

Polymerisation of gluten proteins in developing wheat grain as affected by desiccation

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Abbreviations^a

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^a **Abbreviations used:** ADG, artificially dried grain; DAA, days after anthesis; FDG, freeze-dried grain; GMP, glutenin macropolymer; HMW-GS, high molecular weight-glutenin subunits; LMW-GS, low molecular weight-glutenin subunits; R_{\max} , maximum resistance to extension; RP-HPLC, reversed-phase high-performance liquid chromatography; SDS, sodium dodecyl sulphate; SE-FPLC, size-exclusion fast performance liquid chromatography; TFA, trifluoroacetic acid; UPP, SDS-unextractable polymeric proteins; %UPP, the proportion of SDS-unextractable polymeric protein in total polymeric proteins; YR, yellow ripeness

Abstract

The breadmaking quality of wheat is affected by the composition of gluten proteins and the polymerisation of subunits that are synthesised and accumulated in developing wheat grain. The biological mechanisms and time course of these events during grain development are documented, but not widely confirmed. Therefore, the aim of this study was to monitor the accumulation of gluten protein subunits and the size distribution of protein aggregates during grain development. The effect of desiccation on the polymerisation of gluten proteins and the functional properties of gluten were also studied. The results showed that the size of glutenin polymers remained consistently low until yellow ripeness (YR), while it increased during grain desiccation after YR. Hence, this polymerisation process was presumed to be initiated by desiccation. A similar polymerisation event was also observed when premature grains were dried artificially. The composition of gluten proteins, the ratios of glutenin to gliadin and high molecular weight-glutenin subunits to low molecular weight-glutenin subunits, in premature grain after artificial desiccation showed close association with the size of glutenin polymers in artificially dried grain. Functional properties of gluten in these samples were also associated with polymer size after artificial desiccation.

1. Introduction

Wheat gluten has the unique ability to form a viscoelastic network that gives wheat dough the capability to retain gas produced by yeast and to provide leavened bread with porous crumb structure after baking. The physical properties of the gluten network are primarily determined by the composition of gluten proteins that are synthesized in the endosperm cells during grain development. Gliadins (mainly monomeric proteins) and glutenins (polymeric proteins) are two major types of gluten proteins that account for about 60-70% and 30-40% of total gluten proteins, respectively (Wieser et al., 2004; Wieser and Seilmeier, 1998). Gliadins contribute to the viscosity, while glutenin polymers contribute to the elasticity of wheat dough. Tosi et al. (2009) observed gluten protein bodies in developing grain as early as 8 days after anthesis (DAA). Shewry et al. (2009) reported that low molecular weight-glutenin subunits (LMW-GS) and gliadins were synthesised and accumulated most rapidly between 12 DAA and 35 DAA, while high molecular weight-glutenin subunits (HMW-GS) accumulated more slowly but for a longer period during grain filling. Glutenin subunits are linked by intermolecular disulphide bonds and form large glutenin polymers with molecular weights ranging from about 500,000 to more than 10 million (Shewry and Tatham, 1997; Wahlund et al., 1996). The proportion between glutenins and gliadins, as well as the allelic variations of both HMW-GS and LMW-GS in glutenins, have been shown to influence the technological properties of gluten, in particular breadmaking quality (Flaete and Uhlen, 2003; Gupta et al., 1994; Payne et al., 1979; 1983; Uthayakumaran et al., 1999). Moreover, the proportion of the largest glutenin polymers, known as sodium dodecylsulphate- (SDS) unextractable polymeric proteins (UPP) or glutenin macropolymer (GMP), are associated with the strength and the elasticity of dough (Don et al., 2003; Gupta et al., 1993). The size of these glutenin polymers is controlled to a large extent by genotype (the composition of HMW-GS as well as LMW-GS) but also by environmental effects (Gupta and MacRitchie, 1994; Moldestad et al., 2014)

The initial assembly of glutenin subunits into glutenin polymers occurs shortly after synthesis, while the rapid increase in the molecular weight of glutenin polymers occurs during the desiccation/maturation phase at the end of grain development (Carceller and Aussenac, 1999, 2001; Daniel and Triboi, 2002; Shewry et al., 2009). Carceller and Aussenac (2001) demonstrated the close relationship between the composition of glutenin polymers, particularly the ratio of HMW-GS to LMW-GS, and the polymerisation index (SDS-insoluble polymers/total polymers), when premature grains were desiccated during the cell enlargement phase. In their study, the amount of GMP in desiccated grains increased from 15 DAA to 32 DAA during grain development, and plateaued from 32 DAA to maturity (53 DAA) (Carceller and Aussenac, 1999). Daniel and Triboi (2002) evaluated the effects of environmental factors, particularly temperature and drought, on the aggregation of gluten proteins and showed that drought caused the early onset of the rapid polymerisation of gluten proteins leading to poor solubility. From these results, Shewry et al. (2009) suggested that grain desiccation at the end of grain development drives protein polymerisation, which leads to an increase in the molecular weight of glutenin polymers. However, the number of publications reporting the time course of the accumulation of gluten proteins as well as the polymerisation of glutenin subunits during grain development and desiccation, is still limited.

The aim of the present work was to study the accumulation of gluten proteins and the polymerisation of glutenin subunits in developing wheat grain during maturation / desiccation and to demonstrate the effect on the functional properties. For this purpose, we monitored the synthesis, the accumulation and the polymerisation of proteins in both freeze-dried and artificially dried wheat grain harvested from 10 DAA to 40 DAA during grain development.

The effects that occurred on the molecular level were verified by analysing the functional properties of gluten from artificially dried premature grains by means of rheological methods.

2. Material and Methods

2.1 Wheat material

The Norwegian spring wheat cultivar, Bjarne (HMW-GS; 1Ax2*, 1Bx6+1By8, 1Dx5+1Dy10) was grown in field at Vollebekk experimental farm, Norwegian University of Life Science, Ås, Norway in the 2009 and 2010 seasons. The experiments followed a block design with two replicates, and time-point of harvest during grain development was the experimental factor. One whole plot was harvested randomly at each harvest within each block. Size of the harvested plot was 1.5 x 5 m. The first harvest was performed at 10 DAA, and carried out with five days intervals until 35 DAA, which was morphological YR in 2009, while an extra harvesting was performed after YR at 40 DAA in 2010. Additionally, one plot was harvested with a combiner at maturity. A supplemental field experiment was carried out with the same experimental design in the 2011 season. The harvest regime was the same as in the 2010 season, while harvesting continued after 40 DAA with two days intervals until 50 DAA to follow the polymerisation of glutenin polymers during whole grain desiccation period. Nitrogen was applied at optimal level (120 kgN/ha) as NPK (Yara 22-2-10) at sowing. The date of anthesis was recorded on plot basis, when anther extrusion was seen in 50% of the spikes. In addition, 20-25 ears were randomly selected and tagged with the exact date of anthesis for each plot. At each harvest, 10 of the tagged spikes were taken and grains were collected from well-developed spikelets in the middle of the spikes, which resulted in about 13 grains per spike. Harvested grains were immediately frozen in liquid N₂ and freeze-dried (freeze-dried grain - FDG). The rest of the plot was hand-harvested with a sickle and the spikes were dried in a drying cabinet at 25 °C until the moisture content was below 15% and threshed (artificially dried grain - ADG). This

method was chosen to obtain the amounts of grain necessary for milling and rheological measurements.

2.2 Milling

ADG and mature grains were cleaned and milled into wholemeal flour on a Falling number 3100 hammer mill with 0.8 mm sieve. Due to the small sample size, the FDG samples were milled into wholemeal flour on a Retsch mill with a 0.5 mm sieve.

2.3 Protein content

Protein content was determined in the artificially dried grains with the combustion method according to Dumas using a Leco CHN 1000 Elemental Analyzser (Leco corporation, MI, USA). The factor of 5.7 was applied for protein calculation from the nitrogen content.

2.4 Large deformation rheology

The rheological analysis was performed on artificially dried grain harvested in 2009 and 2010 with the SMS/Kieffer Dough and Gluten Extensibility Rig (Kieffer et al., 1998). Gluten was prepared in a Glutomatic 2200 (Perten AB, Huddinge, Sweden) from wholemeal flour. A 2% (w/v) NaCl solution was used for mixing the dough and washing out starch, bran particles and the salt soluble components. The dough was mixed for 1 min before 10 min of washing. Two types of filter were used for gluten preparation; the filter was changed from 88 μm to 840 μm after 2 min of washing. The gluten was centrifuged in a special centrifuge mould in a swing-out rotor (Rotor 5.51) at $3000 \times g$ for 10 min at 20 °C, pressed in a standard mould, and rested for 45 min at 30 °C for further analysis. Three pieces of gluten from each gluten preparation were stretched with the Kieffer rig until they disrupted. The maximum resistance to extension (R_{max}) was recorded by a TA.XT *plus* Texture Analyser (Stable Micro Systems, Godalming, UK).

2.5 Quantitation of SDS-unextractable polymeric protein (UPP)

Both FDG and ADG samples were analysed by extraction/size exclusion fast performance liquid chromatography (SE-FPLC) (data from ADG harvested at 35 DAA is missing in 2010). Flour samples of 240 mg were extracted sequentially to obtain two extracts (SDS-extractable and SDS-unextractable) according to the method of Morel et al. (2000) with modifications described by Tronsmo et al. (2002). The sonication was done by a Sonics VC130 (Sonics and Materials, CT, USA) for 3 min with 70% automatic amplitude compensation. 100 µL of each extract were separated on Superose[®] 12HR 10/300 (GE Healthcare, Little Chalfont, UK) connected to an ÄKTA SE-FPLC (GE Healthcare) with 0.1% SDS, 0.08 mol/L NaCl, 0.05 mol/L sodium phosphate elution buffer (pH 6.9) at a flow rate of 0.4 mL/min. The effluent was monitored by UV-absorbance at 210 nm.

The SDS-unextractable fraction gave one main peak, denoted F1*, and contained the largest polymers. The SDS-extractable protein fraction gave four main peaks, with F1 - F2 consisting of polymeric proteins and F3 - F4 consisting of monomeric proteins. The proportion of each fraction was calculated by dividing the area of each peak by the sum of the area of all peaks (F1* and F1-F4), and presented as %. The %UPP corresponded to the relative proportion of UPP in total polymeric proteins, calculated as $[F1*/(F1*+F1)] \times 100$.

2.6 Quantitation of protein fractions and protein types

Grain (FDG and ADG) harvested in the 2009 and 2010 seasons were analyzed for the content of protein fractions and protein types by means of an extraction/reversed-phase high-performance liquid chromatography (RP-HPLC) method according to Wieser et al. (1998). Proteins were extracted sequentially from 100 mg wholemeal flour with 0.4 mol/L NaCl + 0.067 mol/L HKNaPO₄, pH 7.6 (2 x 1.0 mL) for 10 min at ≈ 20 °C (albumins/globulins), with 60%

(v/v) ethanol (3 x 0.5 mL) for 10 min at ≈ 20 °C (gliadins), and with 50% (v/v) 1-propanol containing Tris·HCl (0.05 mol/L, pH 7.5), urea (2 mol/L), and 1% (w/v) dithioerythritol (2 x 1.0 mL) for 20 min at 60 °C under nitrogen (glutenins). Each extraction step was initiated with vortexing for 2 min at ≈ 20 °C and continued with magnetic stirring. The suspensions were then centrifuged for 20 min at 6,000 x g at 20 °C. The corresponding supernatants were combined and diluted to 2.0 mL with the respective extraction solvents. Two or three fractionation experiments per flour were performed.

The RP-HPLC procedure was according to Thanhaeuser et al. (2014). Aliquots (≈ 0.5 mL) of the three extracts were filtered through a 0.45 μ m membrane and 10 μ L of each extract was separated at 60 °C on an Acclaim C₁₈ column (3 μ m, 30 nm, 2.1 x 150 mm (Dionex, Idstein, Germany)) using a Jasco X-LC instrument (Jasco, Gross-Umstadt, Germany). Elution was carried out with linear gradients of two elution solvents, A: 0.1% (v/v) trifluoroacetic acid (TFA) in water and B: 0.1% (v/v) TFA in acetonitrile, with gradients of 0 min 20% B, 20 min 60% B for the albumin/globulin fraction and 0 min 24% B, 30 min 56% B for the gliadin and glutenin fractions at a flow rate of 0.2 mL/min and detection of the UV-absorbance at 210 nm. PWG-gliadin was used as calibration reference (van Eckert et al., 2006). From the results, the ratios of glutenin to gliadin (glutenin:gliadin) and HMW-GS to LMW-GS (HMW-GS:LMW-GS) were calculated.

2.7 Statistical analyses

Analyses of variance (ANOVA) and simple linear regression were performed for each season using R Commander modified at the Norwegian University of Life Sciences (Ås, Norway).

3. Results

3.1 Accumulation of proteins and glutenin polymerisation during grain development and maturation

Figure 1 shows the accumulation of dry matter (A), the moisture content (B) and protein content (C) during grain development in all three seasons. Dry matter showed linear increases until approximately 35-40 DAA, while the moisture content decreased steadily until 35 DAA. A rapid decrease in moisture content was observed from 35 DAA, when morphological YR was reached, to 40 DAA in the 2010 season. The moisture content decreased slower in the 2011 season than 2010 during desiccation. The total protein content (mg protein/grain) increased until 35-40 DAA in all seasons. The protein content was highest both during grain development and at the maturity in the 2009 season, while it was lowest in grain grown in 2010 (12.8% in 2009 vs. 11.3% in 2010).

The accumulation of albumins/globulins, gliadins and glutenins (mg/grain) and their proportions in FDG are shown in Figure 2 (A-D). The proportion of albumins/globulins were higher than gliadins and glutenins at 10 DAA. The amount of albumins/globulins increased with constant accumulation rate until 35 DAA in both seasons, and reached approximately 20% of total grain proteins at YR. The amounts of gliadins and glutenins were very low at 10 DAA, while they accumulated faster than the albumins/globulins. The accumulation rate of gliadins was highest among the three fractions, particularly after 15 DAA in both seasons and their proportion accounted for 60% and 48% in 2009 and in 2010, respectively, at YR. On the other hand, the proportion of glutenins accounted for about 20% and 27% in 2009 and 2010, respectively at YR. The glutenin:gliadin ratio was highest at the beginning of grain filling and gradually decreased, while the HMW-GS:LMW-GS ratio increased during grain development in both seasons (Figure 3).

Size exclusion chromatography was performed to study changes in the molecular size distribution of gluten proteins as well as in the polymerisation of glutenin polymers during grain development (Table 1 and Figure 3). The %UPP of the FDG stayed at low level until 35 DAA for all seasons. The results from the 2009 season showed that %UPP at 35 DAA was as low as at 25-30 DAA, and that the formation of UPP had not yet taken place at YR. Therefore, an extra harvesting was performed at 40 DAA in 2010 (moisture content at harvest corresponding to 24.5%); moreover, grains were harvested more frequently from 35 until 50 DAA in the 2011 season. Increase in %UPP was observed after 35 DAA in both seasons (Figure 4(B) and (C)). In the 2010 season, sharp increases in both %F1*, %F1, and %UPP were observed from 35 DAA to 40 DAA and concurrent decreases in %F2 and %F3 (Table 1 and Figure 3(B)). Separation of these FPLC fractions (F1*, F1-F4) by SDS-PAGE showed that HMW-GS and LMW-GS were present in F1-F2 and F1-F3 fractions, respectively, in grain harvested at 35 DAA, while most HMW-GS were found in F1* and F1, and the majority of LMW-GS were in F1* and F1- F2 in grain harvested at 40 DAA (Supplemental Figure A). The changes in the molecular size distribution of gluten proteins from 35 DAA to 40DAA were coincident with the rapid decrease in moisture content observed at the same period in the 2010 season (Figure 1B). The polymerisation of UPP occurred slower in the 2011 season than the 2010 season (Figure 4(C)).

3.2 Effects of desiccation on formation of large glutenin polymers

The ADG from each harvest was also analysed by RP-HPLC (Figure 2 (E-H), Figure 3 and Supplemental Figure C) and SE-FPLC (Figure 4). The albumin/globulin fraction was a major protein fraction at 10 DAA (Figure 2 (B) and (D)), while the level of the gliadin fraction was higher than or as high as of the albumin/globulin fraction at 10 DAA, when grains were dried

artificially (Figure 2(F) and (H)). The accumulation of all three fractions in ADG showed to a large extent a similar pattern to that in FDG. However, the ratios of glutenin:gliadin and HMW-GS:LMW-GS in ADG during grain development differed from these ratios observed in FDG. The glutenin:gliadin ratio was lower in ADG than in FDG at the beginning of grain filling, while it increased gradually. A similar ratio was observed between ADG and FDG after 25 DAA. A strong decrease in the glutenin:gliadin ratio was observed from 10 DAA to 15 DAA in the 2010 season. The HMW-GS:LMW-GS ratio in ADG was constant throughout grain development, and consistently higher than that in FDG until 25 DAA and 30 DAA in 2009 and in 2010, respectively, and an equivalent ratio was observed in ADG and in FDG after these time-points.

Large differences were found in %UPP between the FDG and ADG (Figure 4). Artificial desiccation of premature grains caused substantial increase in %UPP of the grains ($P < 0.001$) in both seasons. Although, the time course of %UPP was slightly different from 2009 to 2010, %UPP, in general, increased during grain development in ADG, except grain harvested at 10 DAA that showed a large increase in %UPP after artificial drying, particularly in the 2010 season. The value of %UPP reached a maximum in ADG harvested at 25 DAA and 30 DAA in 2009 and in 2010, respectively.

The viscoelastic behaviour of gluten during large deformation rheology was analysed for ADG and R_{\max} is shown in Figure 5. The R_{\max} values were similar throughout grain development in the 2009 season. On the other hand, significant differences in R_{\max} were observed from grains harvested at different time-points during grain development in the 2010 season. A higher R_{\max} value was obtained from grains harvested at 10 DAA compared to those harvested between 15 and 30 DAA in this season (Figure 5 (B)). The value decreased from 10 to 15 DAA and

increased again during grain development, particularly from 15 to 20 DAA and from 30 to 35 DAA. Within both seasons, a weak but significant correlation was observed between %UPP and R_{\max} of ADG ($r=0.61$ and $r=0.68$ in 2009 and 2010 samples, respectively, $P<0.05$).

4. Discussion

The amount and the size of glutenin polymers are amongst the most important factors that influence the breadmaking quality of wheat. This study was carried out to increase our knowledge of both the accumulation of gluten proteins and the polymerisation of glutenin polymers during grain development.

Similar increases in dry matter and decreases in grain moisture content were observed during grain development from the three seasons. The results showed that grain development occurred in a similar manner for the three seasons. Both dry matter and total protein content peaked at 35 DAA and 40 DAA in the 2010 season and in the 2011 season, respectively, which demonstrated that accumulation of starch and proteins was completed at 35-40 DAA, when the plants reached YR. The moisture content of the grain declined rapidly after YR in the 2010 season, while it decreased slower in the 2011 season. Weather conditions, particularly precipitation, probably influenced the rate of grain desiccation during this period. In fact, frequent precipitation at the end of grain maturation was observed in 2011 (Supplemental figure D). Although grain dry matter was similar, the protein content varied between the three seasons. This primarily reflects differences in growth conditions. Albumins and globulins predominated the grain protein at the early stage of grain development and accumulated throughout grain development. However, their proportion decreased rapidly from 10 to 15 DAA, when the synthesis of gluten proteins started, and they accounted for about 20% of total grain proteins at the end of the protein accumulation phase. As according to Tosi et al. (2009), synthesis of gluten proteins starts at about 8 DAA, it was expected that grain harvested at 10 DAA would have a

very low content of gluten proteins, and their proportion at this stage was about 12-15% of total grain proteins in our study. Both gliadins and glutenins were accumulated rapidly until 35 DAA with gliadins being accumulated faster than glutenins. Consequently, the glutenin:gliadin ratio decreased during grain development. Gliadins predominated the grain protein, while the proportion of glutenins was similar to the proportion of albumins/globulins at YR. It has been reported that increasing N content in grain leads to a higher proportion of gliadins (Wieser and Seilmeier, 1998). Our results also showed that higher protein content in the 2009 season was followed by higher proportion of gliadins. We were able to detect glutenins already at 10 DAA by RP-HPLC and the results showed that their accumulation continued until YR. Although LMW-GS are the predominant protein subunits in glutenins, the HMW-GS:LMW-GS ratio increased during grain development. Both the synthesis and the accumulation of glutenins as well as the HMW-GS:LMW-GS ratio increased during grain development, however, the size of glutenin polymers remained small until YR. Most HMW-GS and LMW-GS were found in smaller polymers that were extractable in SDS buffer at 35 DAA. According to Tronsmo et al. (2002), the molecular weight of these polymers (F1 and F2) was roughly 670,000 and 200,000 - 300,000, respectively. A rapid increase in the molecular size of glutenin polymers was first observed after YR. The majority of HMW-GS and LMW-GS were found in the F1* and F1 fractions at 40 DAA, which revealed that an increase in the molecular weight of glutenin polymers occurred between 35 and 40 DAA in the 2010 season. The increase in %F1* as well as %UPP indicated that both the size and the amount of large glutenin polymers increased from 35 to 40 DAA. Moreover, these changes coincided with rapid grain desiccation observed between 35 and 40 DAA in the 2010 season. The formation of UPP also started after 35 DAA in the 2011 season, but it took longer to reach the same level as in the 2010 season. Frequent precipitations led to slower desiccation of grain at the end of grain maturation in the 2011 season, and the formation of UPP followed the delayed desiccation pattern. Our results

concurrent with the results of Carceller and Aussenac (2001) which demonstrated a close relationship between the polymerisation rate of UPP and the grain desiccation rate. Our results demonstrated that glutenins exist as smaller polymers until YR, and these smaller polymers were assembled to large polymers during grain desiccation after YR. This is in accordance with previous reports (Carceller and Aussenac, 1999; Gupta et al., 1996; Shewry et al., 2009). A similar polymerisation pattern was observed during the desiccation of premature grain harvested at 10 and 25 DAA in the 2011 season (Supplemental figure B (A), (B), (D), and (E)). Moisture content in grains harvested at 10 DAA remained high (60-70%) during the first eight days of artificial drying and no change in %UPP was observed during this period. However, both rapid increase in %UPP and grain desiccation occurred simultaneously after this period. Moisture content in grains harvested at 25 DAA was already below 60% at the harvest and the formation of large glutenin polymers followed desiccation.

To investigate whether desiccation initiates formation of large glutenin polymers, both freeze-dried and artificially dried (at 25 °C) premature grains were analysed with RP-HPLC and SE-FPLC. The content of the three protein fractions albumins/globulins, gliadins, and glutenins increased linearly from 10 DAA to 35 DAA in both FDG and ADG, however, the amount of proteins was higher in FDG than in ADG. This difference is probably caused by differences in the sample preparation methods. The freeze-dried sample consisted of selected well-developed grains from 10 spikes, thus representing grains from a particular and uniform stage. On the other hand, the artificially dried sample consisted of grains from whole spikes from one whole plot giving more variations in the developmental stage within ADG samples but they represented the average of grains at that developmental stage. In fact, the dry matter of ADG was consistently lower than that of FDG throughout grain development ($P < 0.001$). The results indicate that on average, grain development of ADG was retarded as compared to FDG.

Predominant differences were seen in the proportions of protein fractions between ADG and FDG, particularly in grains harvested before 25 DAA. Grains harvested at early stages had high moisture content (i.e., about 73% at 10 DAA), and artificial drying in cabinets at 25 °C took long time (about two weeks). Hence, it was assumed that the synthesis of gluten proteins in premature grain, especially those that were harvested at an early stage, continued at the beginning of the drying process and led to changes in the proportion of protein fractions. The supplemental study in 2011 showed that the protein content increased during the first 4 days of artificial drying in grain harvested at 10 DAA, while this was not the case for the grain harvested at 25 DAA (Supplemental Figure B (C)) and (F)). Our results showed that gliadins accumulated more rapidly than glutenins, and HMW-GS accumulated more rapidly among glutenin subunits in grain harvested at 10 DAA under artificial desiccation.

The %UPP of ADG, in general, increased with grain development. Our observations are comparable to the results of Carceller and Aussenac (2001) who demonstrated that the polymerisation index increased from 15 DAA and reached maximum at around 30 DAA. They also showed that the polymerisation index after artificial drying depended on the composition of glutenin polymers (the HMW-GS:LMW-GS ratio) in grain before drying. Our results showed a similar relationship as both the HMW-GS:LMW-GS ratio in FDG and the %UPP in ADG increased during grain development in both seasons. However, the HMW-GS:LMW-GS ratio already reached its maximum level in grains harvested at 10 DAA after artificial drying. Therefore, no clear relationship between the HMW-GS:LMW-GS ratio and %UPP in ADG was observed. On the other hand, our results showed a significant correlation between the glutenin:gliadin ratio and the %UPP in ADG in both seasons ($r=0.86$ and $r=0.91$ in 2009 and 2010 samples, respectively, $P<0.001$). The HMW-GS:LMW-GS ratio in FDG may influence the degree of polymerisation of glutenin polymers in ADG, however, it is more reasonable to

assume that changes in the composition of gluten proteins (both the ratios of glutenin:gliadin and HMW-GS:LMW-GS) after artificial desiccation directly influence the degree of polymerisation, especially in the grain harvested at earlier stages of grain development. Because the proportion of glutenins was considerably lower than that of gliadins in grain harvested at the early stages of grain development, a higher HMW-GS:LMW-GS ratio has probably little effect on the formation of large glutenin polymers in these samples. The maximum level of %UPP was found in ADG harvested around 25-30 DAA, which was concomitant with the ratios of glutenin:gliadin and HMW-GS:LMW-GS in ADG that reached the same level as those in FDG.

Artificially dried grain also had higher %UPP compared to FDG throughout grain development. Grain lost water by both desiccation methods, while the biological status of grain differed between the two methods. All the biological activities were ceased in FDG, since grains were frozen in liquid N₂. On the other hand, biological activities were supposed to continue in ADG dried at 25 °C, as long as the moisture content was high enough for activities. Our results showed that the accumulation of gluten proteins was completed, while %UPP differed largely between FDG and ADG at YR. The results indicated that polymerisation of large glutenin polymers was not caused by desiccation itself, but desiccation obviously initiated the polymerisation process, which was also suggested previously (Carceller and Aussenac, 1999; Gupta et al., 1996; Shewry et al., 2009). The desiccation process probably activates enzyme activities that are involved in folding and polymerisation of glutenin polymers as Osipova et al. (2012) described in their review article.

It has been reported that the rheological properties of dough are influenced by the size distribution of glutenin polymers (Gupta et al., 1993). As our results and also those of Carceller

and Aussenac (1999, 2001) showed that %UPP increased in artificially dried premature grains, it was of great interest to investigate the functionality of gluten proteins in premature grains that were dried artificially. Therefore, the rheological analysis was carried out on ADG harvested during grain development to establish a relationship between %UPP and R_{\max} . The results showed significant correlations between %UPP and R_{\max} in both the 2009 and 2010 seasons. Although, the synthesis and the accumulation of gluten proteins were not completed in premature grains, particularly in those that were harvested at early stages, these processes continued during the beginning of grain desiccation and the formation of large polymers occurred at the end of desiccation. These events probably improve the elastic properties of gluten. Both %UPP and R_{\max} values in the ADG were high in grain harvested at 10 DAA, particularly in the 2010 season, and the values from this season were similar to the values obtained from grain harvested at 35 DAA. The reason for these high values in grain harvested at 10 DAA is unclear, however, the results showed the association with high glutenin:gliadin and HMW-GS:LMW-GS ratios after artificial desiccation of these samples.

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References

- Carceller, J.L., Aussenac, T., 1999. Accumulation and changes in molecular size distribution of polymeric proteins in developing grains of hexaploid wheats: role of the desiccation phase. *Australian Journal of Plant Physiology* 26, 301-310.
- Carceller, J.L., Aussenac, T., 2001. SDS-insoluble glutenin polymer formation in developing grains of hexaploid wheat: the role of the ratio of high to low molecular weight glutenin subunits and drying rate during ripening. *Australian Journal of Plant Physiology* 28, 193-201.
- Daniel, C., Triboi, E., 2002. Changes in wheat protein aggregation during grain development: effects of temperatures and water stress. *European Journal of Agronomy* 16, 1-12.
- Don, C., Lichtendonk, W., Plijter, J.J., Hamer, R.J., 2003. Glutenin macropolymer: a gel formed by glutenin particles. *Journal of Cereal Science* 37, 1-7.
- Flaete, N.E.S., Uhlen, A.K., 2003. Association between allelic variation at the combined *Gli-1*, *Glu-3* loci and protein quality in common wheat (*Triticum aestivum* L.). *Journal of Cereal Science* 37, 129-137.
- Gupta, R.B., Khan, K., MacRitchie, F., 1993. Biochemical basis of flour properties in bread wheats. I. Effects of variation in the quantity and size distribution of polymeric protein. *Journal of Cereal Science* 18, 23-41.
- Gupta, R.B., MacRitchie, F., 1994. Allelic variation at glutenin subunit and gliadin loci, *Glu-1*, *Glu-3* and *Gli-1* of common wheats. II. Biochemical basis of the allelic effects on dough properties. *Journal of Cereal Science* 19, 19-29.
- Gupta, R.B., Masci, S., Lafiandra, D., Bariana, H.S., MacRitchie, F., 1996. Accumulation of protein subunits and their polymers in developing grains of hexaploid wheats. *Journal of Experimental Botany* 47, 1377-1385.
- Gupta, R.B., Paul, J.G., Cornish, G.B., Palmer, G.A., Bekes, F., Rathjen, A.J., 1994. Allelic variation at glutenin subunit and gliadin loci, *Glu-1*, *Glu-3* and *Gli-1*, of common wheats. I. Its additive and interaction effects on dough properties. *Journal of Cereal Science* 19, 9-17.
- Kieffer, R., Wieser, H., Henderson, M.H., Graveland, A., 1998. Correlations of the breadmaking performance of wheat flour with rheological measurements on a micro-scale. *Journal of Cereal Science* 27, 53-60.
- Moldestad, A., Hoel, B., Böcker, U., Koga, S., Mosleth, E.F., Uhlen, A.K., 2014. Temperature variations during grain filling obtained in growth tunnel experiments and its influence on protein content, polymer build-up and gluten viscoelastic properties in wheat. *Journal of Cereal Science* 60, 406-413.
- Morel, M.H., Dehlon, P., Autran, J.C., Leygue, J.P., Bar-L'Helgouac'h, C., 2000. Effects of temperature, sonication time, and power settings on size distribution and extractability of total wheat flour proteins as determined by size-exclusion high-performance liquid chromatography. *Cereal Chemistry* 77, 685-691.
- Osipova, S.V., Permyakova, M.D., Permyakov, A.V., 2012. Role of Non-prolamin Proteins and Low Molecular Weight Redox Agents in Protein Folding and Polymerization in Wheat Grains

and Influence on Baking Quality Parameters. *Journal of Agricultural and Food Chemistry* 60, 12065-12073.

Payne, P.I., Corfield, K.G., Blackman, J.A., 1979. Identification of a high-molecular-weight subunit of glutenin whose presence correlates with bread-making quality in wheats of related pedigree. *Theoretical and Applied Genetics* 55, 153-159.

Payne, P.I., Holt, L.M., Lawrence, G.J., 1983. Detection of a novel high molecular-weight subunit of glutenin in some Japanese hexaploid wheats. *Journal of Cereal Science* 1, 3-8.

Shewry, P.R., Tatham, A.S., 1997. Disulphide bonds in wheat gluten proteins. *Journal of Cereal Science* 25, 207-227.

Shewry, P.R., Underwood, C., Wan, Y.F., Lovegrove, A., Bhandari, D., Toole, G., Mills, E.N.C., Denyer, K., Mitchell, R.A.C., 2009. Storage product synthesis and accumulation in developing grains of wheat. *Journal of Cereal Science* 50, 106-112.

Thanhaeuser, S.M., Wieser, H., Koehler, P., 2014. Correlation of Quality Parameters with the Baking Performance of Wheat Flours. *Cereal Chemistry* 91, 333-341.

Tosi, P., Parker, M., Gritsch, C.S., Carzaniga, R., Martin, B., Shewry, P.R., 2009. Trafficking of storage proteins in developing grain of wheat. *Journal of Experimental Botany* 60, 979-991.

Tronsmo, K.M., Faergestad, E.M., Longva, A., Schofield, J.D., Magnus, E.M., 2002. A study of how size distribution of gluten proteins, surface properties of gluten and dough mixing properties relate to baking properties of wheat flours. *Journal of Cereal Science* 35, 201-214.

Uthayakumaran, S., Gras, P.W., Stoddard, F.L., Bekes, F., 1999. Effect of varying protein content and glutenin-to-gliadin ratio on the functional properties of wheat dough. *Cereal Chemistry* 76, 389-394.

van Eckert, R., Berghofer, E., Ciclitira, P.J., Chirido, F., Denery-Papini, S., Ellis, H.J., Ferranti, P., Goodwin, P., Immer, U., Mamone, G., Mendez, E., Mothes, T., Novalin, S., Osman, A., Rumbo, M., Stern, M., Thorell, L., Whim, A., Wieser, H., 2006. Towards a new gliadin reference material-isolation and characterisation. *Journal of Cereal Science* 43, 331-341.

Wahlund, K.G., Gustavsson, M., MacRitchie, F., Nylander, T., Wannerberger, L., 1996. Size characterisation of wheat proteins, particularly glutenin, by asymmetrical flow field flow fractionation. *Journal of Cereal Science* 23, 113-119.

Wieser, H., Antes, S., Seilmeier, W., 1998. Quantitative determination of gluten protein types in wheat flour by reversed-phase high-performance liquid chromatography. *Cereal Chemistry* 75, 644-650.

Wieser, H., Gutser, R., von Tucher, S., 2004. Influence of sulphur fertilisation on quantities and proportions of gluten protein types in wheat flour. *Journal of Cereal Science* 40, 239-244.

Wieser, H., Seilmeier, W., 1998. The influence of nitrogen fertilisation on quantities and proportions of different protein types in wheat flour. *Journal of the Science of Food and Agriculture* 76, 49-55.

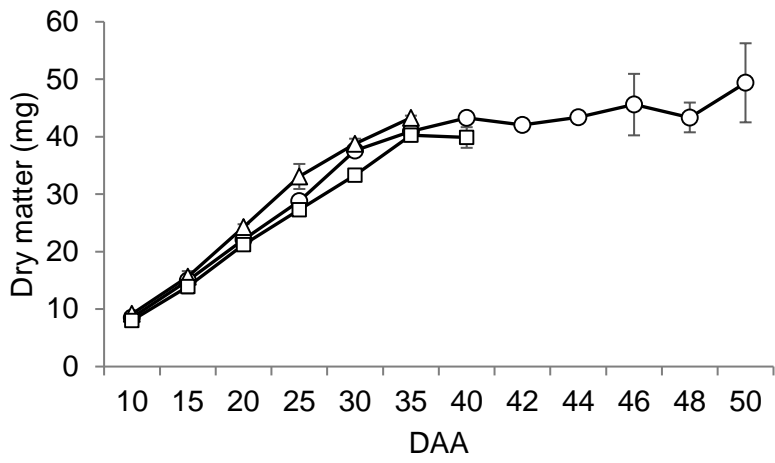
Table 1. Proportion of SE-FPLC fractions (%F1*, %F1-%F4) and %UPP of freeze-dried grain harvested during grain development in 2009 and 2010.

2009 (DAA)	SE-FPLC fraction					
	%F1*	%F1	%F2	%F3	%F4	%UPP
10	3.1	14.8	16.3	35.2	24.1	16.9
15	3.0	16.4	17.4	35.7	22.1	15.4
20	2.6	14.4	18.3	36.9	22.1	15.4
25	1.7	15.7	18.9	34.6	25.5	9.7
30	1.7	17.0	17.4	33.6	26.9	9.2
35	1.6	13.9	18.7	33.1	28.7	10.2
2010 (DAA)	SE-FPLC fraction					
	%F1*	%F1	%F2	%F3	%F4	%UPP
10	2.8	14.4	18.0	34.0	26.5	16.4
15	2.6	15.8	19.2	29.8	27.6	14.3
20	2.7	14.0	20.1	31.1	27.6	16.0
25	3.5	17.7	18.3	29.4	26.0	16.4
30	1.8	10.7	21.0	33.6	28.5	14.3
35	1.7	10.9	20.1	30.6	30.5	13.4
40	10.0	22.2	14.6	20.0	29.2	31.1

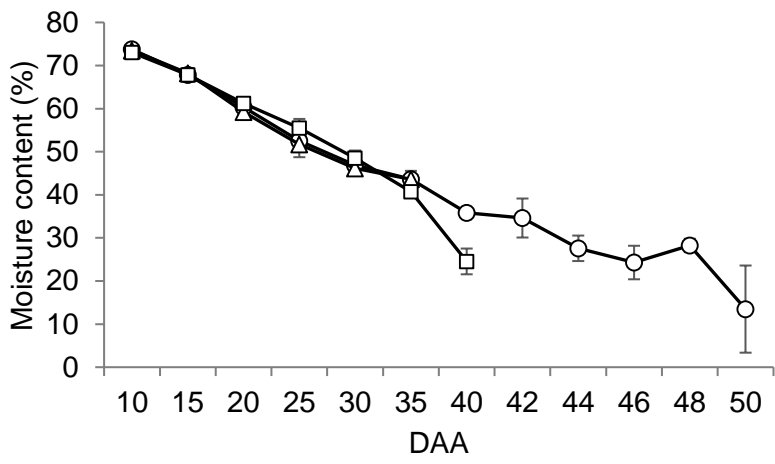
F1* = SDS-unextractable polymeric proteins, F1 and F2 = SDS-extractable polymeric proteins,

F3 and F4 = SDS-extractable monomeric proteins. %UPP = $[F1^* / (F1^* + F1)] \times 100$

(A)



(B)



(C)

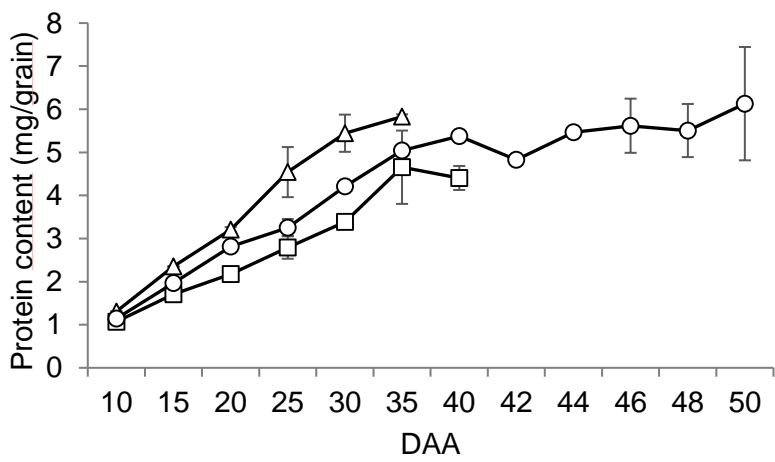
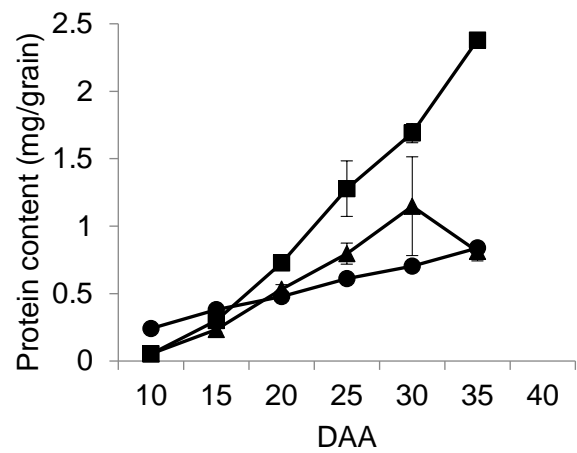
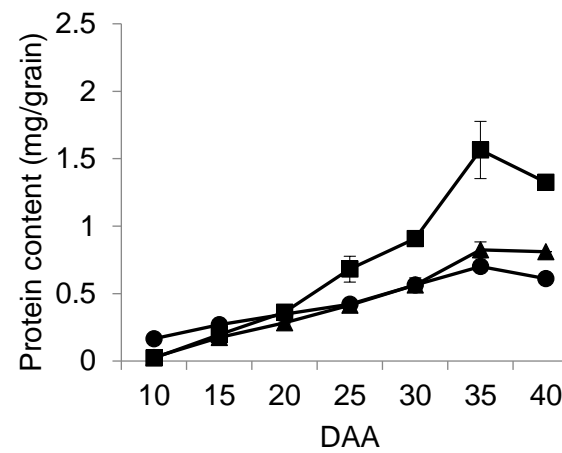
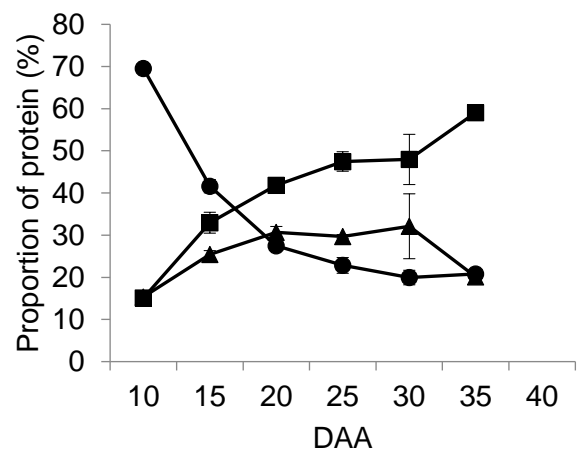
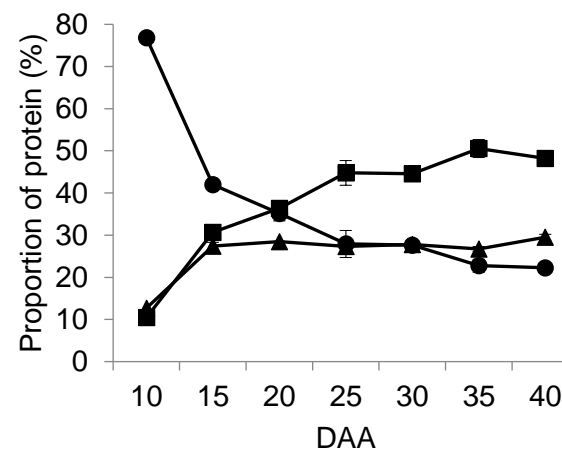


Figure 1

Changes in (A) dry matter (mg), (B) moisture content (%) and (C) protein content (mg/grain) in wheat cv. Bjarne harvested during grain development in 2009 (Δ), 2010 (\square), and 2011 (\circ). The results were based on FDG samples. The vertical bars represent the standard deviation (n=2).

A)**C)****B)****D)**

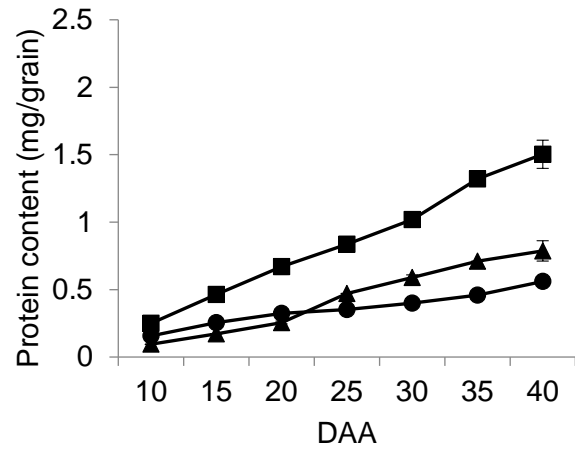
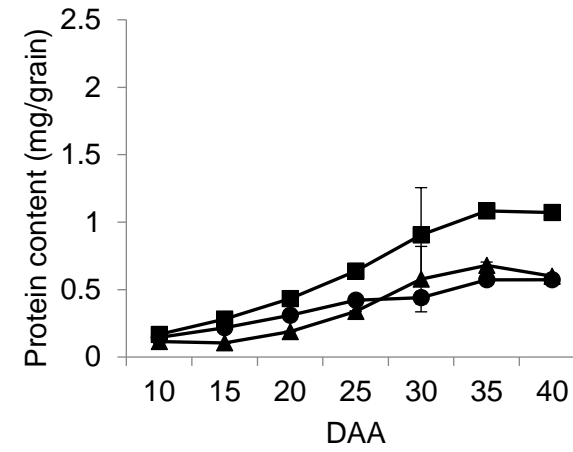
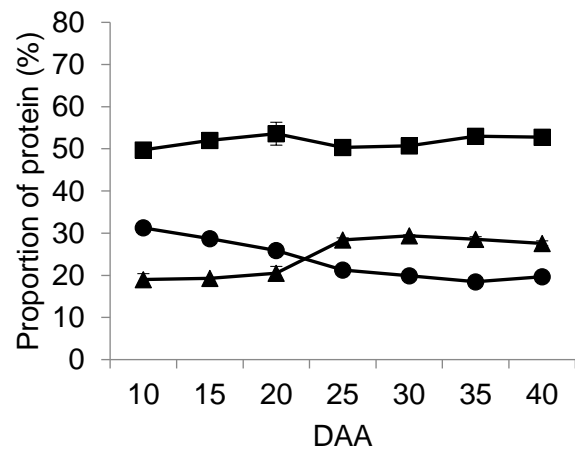
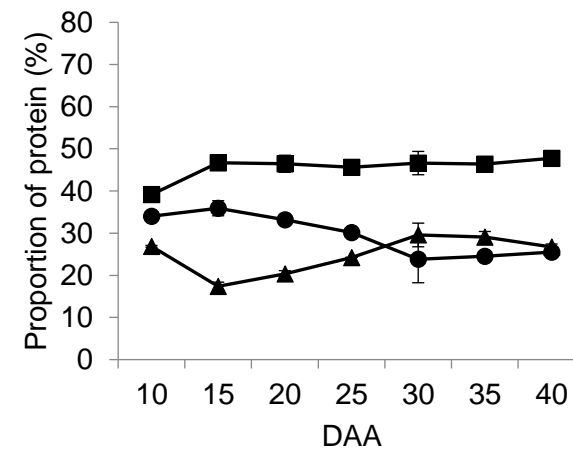
E)**G)****F)****H)**

Figure 2

Accumulation (mg/grain) and proportion of different protein fractions during grain development in freeze-dried grain (A-D) and artificially dried grain (E-H) from 2009 (A, B, E and F) and 2010 (C, D, G and H). ●: albumins/globulins, ■: gliadins and ▲: glutenins. The vertical bars represent the standard deviation (n=2).

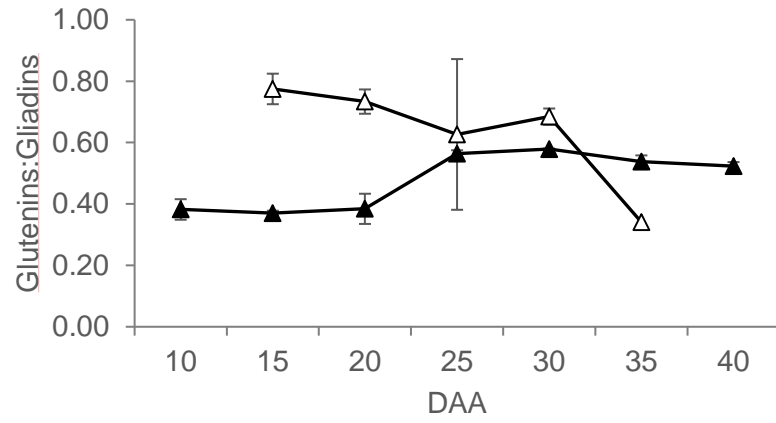
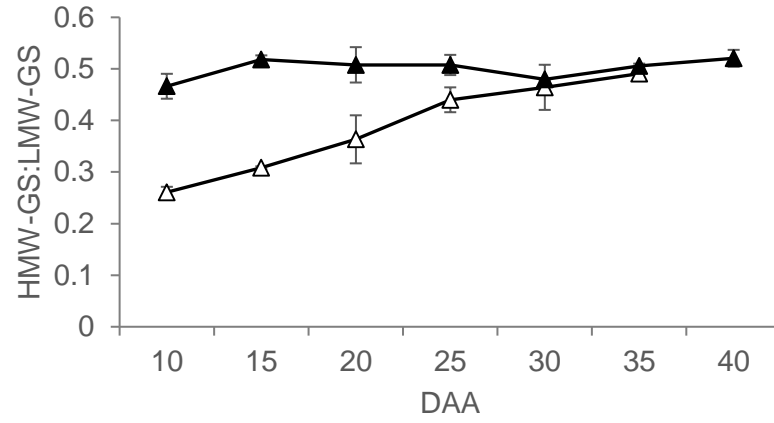
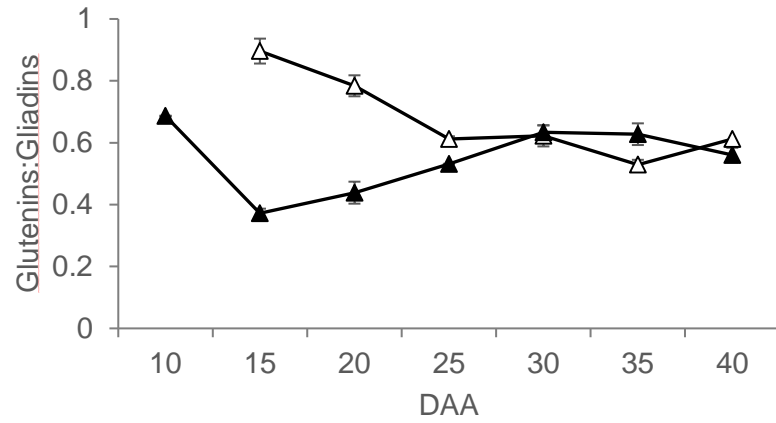
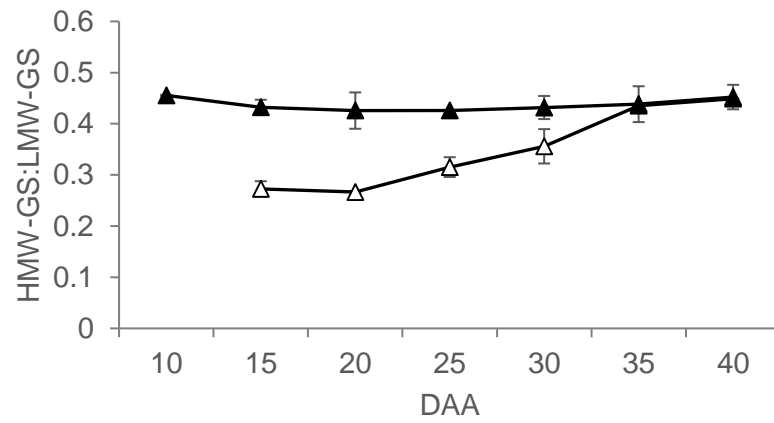
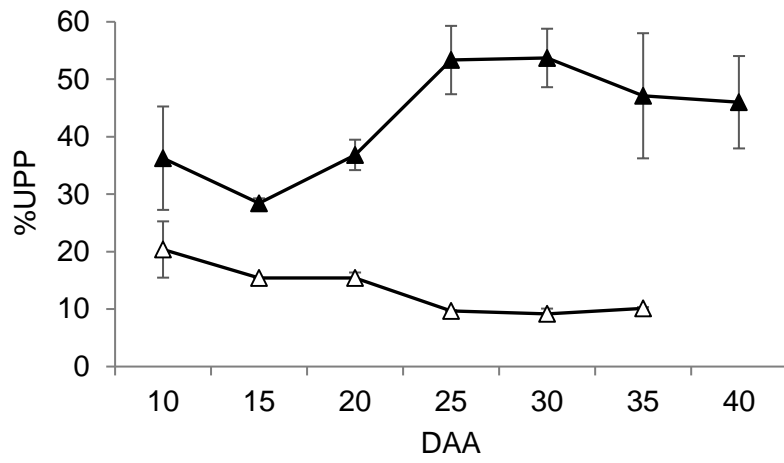
(A)**(C)****(B)****(D)**

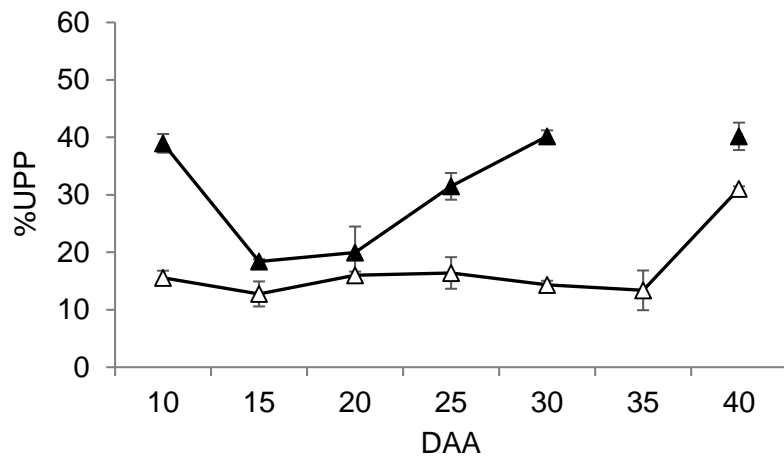
Figure 3

The ratio of glutenins:gliadins (A and B) and HMW-GS:LMW-GS (C and D) in freeze-dried grain (\triangle , open symbol) and in artificially dried grain (\blacktriangle , closed symbol) harvested during grain development in 2009 (A and C) and in 2010 (B and D). The vertical bars represent the standard deviation (n=2).

(A)



(B)



(C)

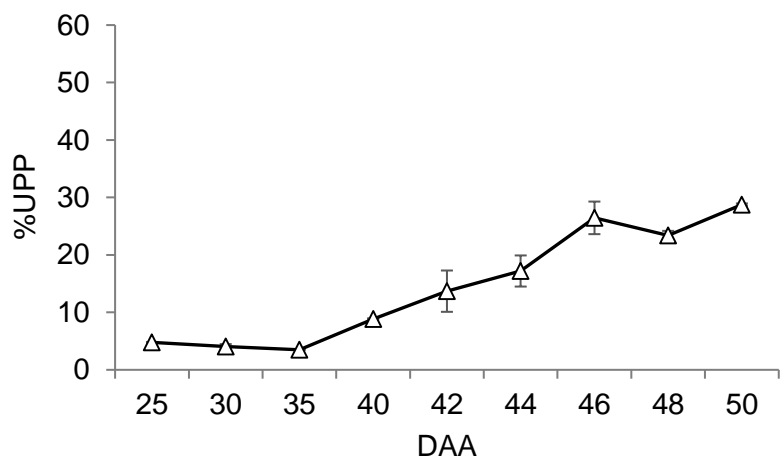


Figure 4

The proportion of SDS-unextractable polymeric proteins in total polymeric proteins (%UPP) in freeze-dried grain (Δ , open symbol) and in artificially dried grain (\blacktriangle , closed symbol) harvested during grain development in (A) 2009, (B) 2010, and (C) 2011. The vertical bars represent the standard deviation (n=2). The %UPP data of ADG harvested at 35 DAA in the 2010 season was missing.

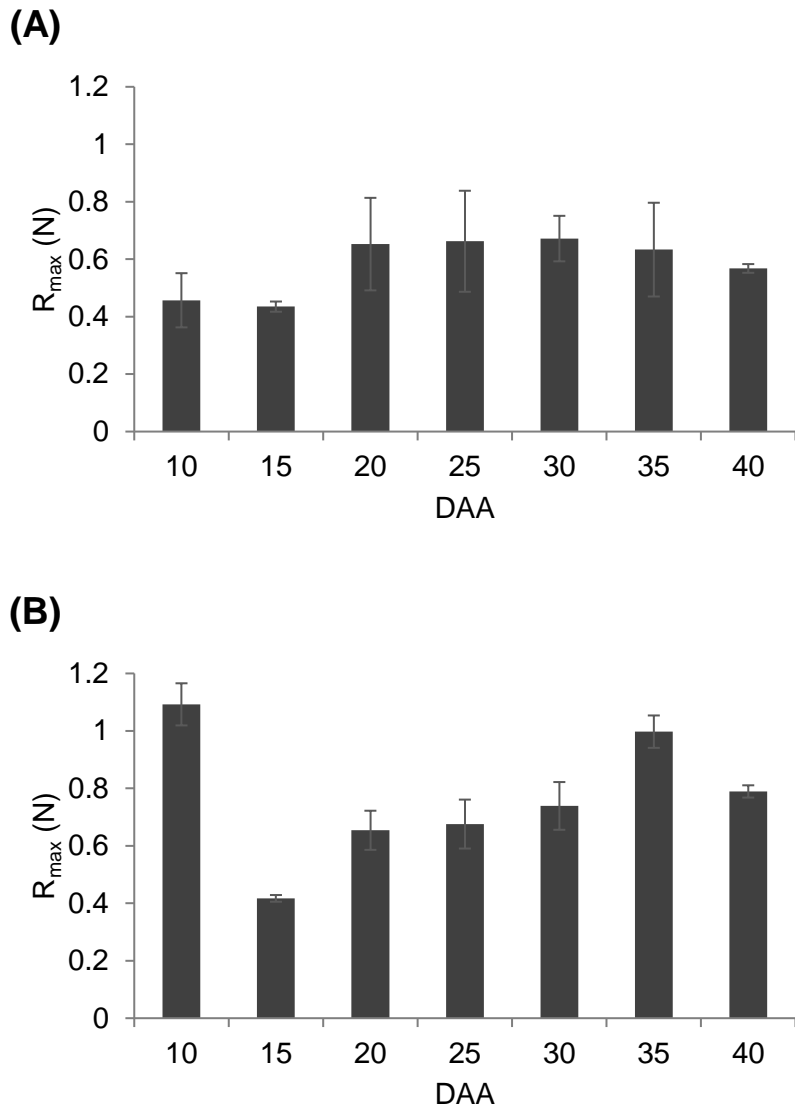
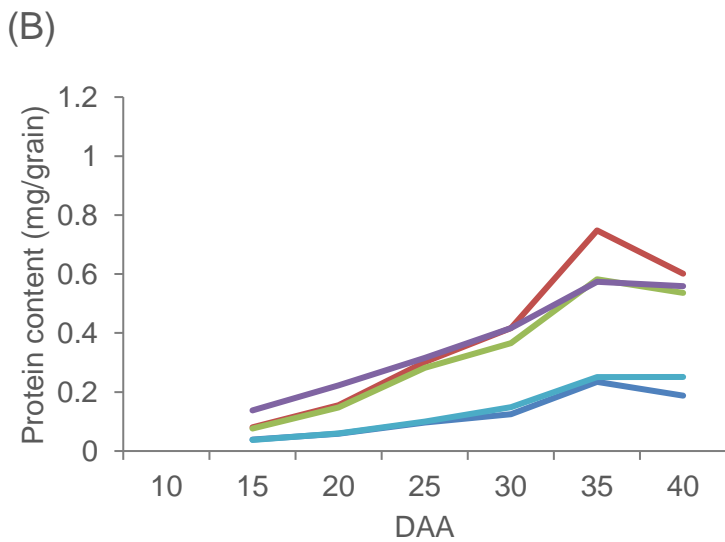
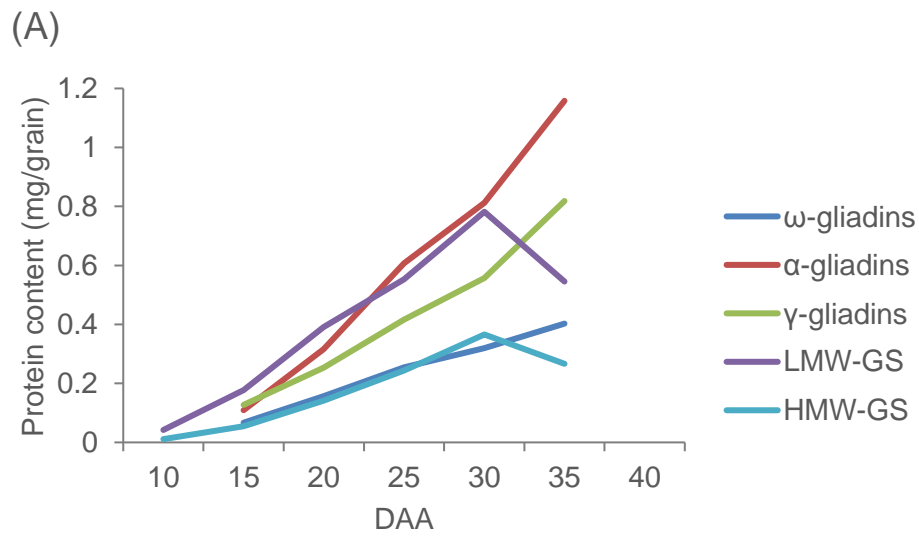


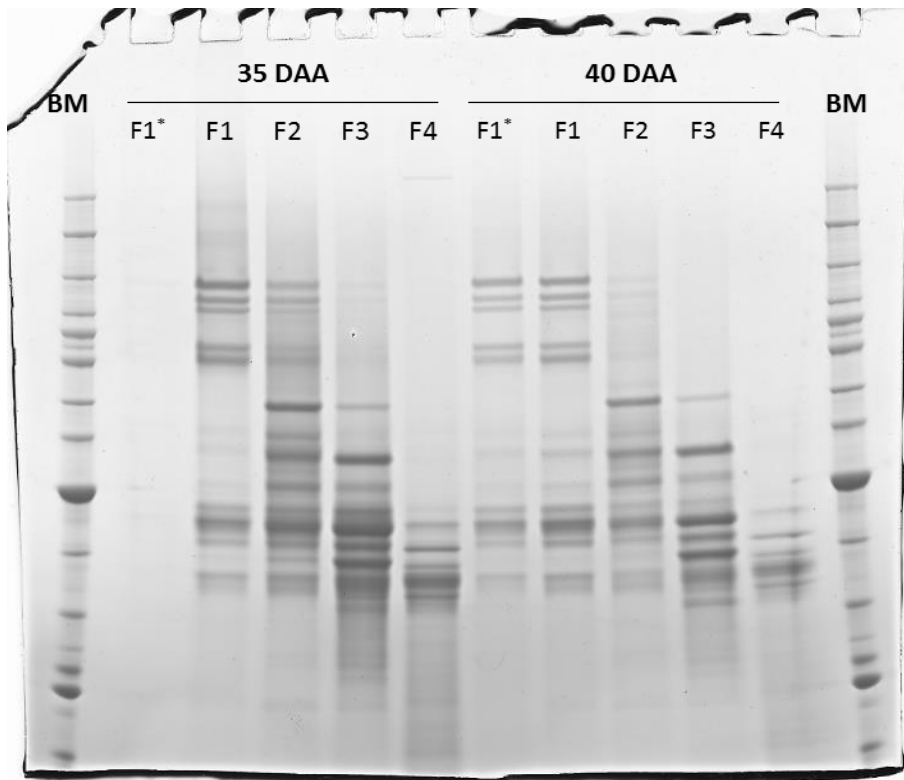
Figure 5

Maximum resistance to extension (R_{max}) of gluten prepared from artificially dried grain harvested during grain development in (A) 2009 and (B) 2010. The vertical bars represent the standard deviation (n=2).



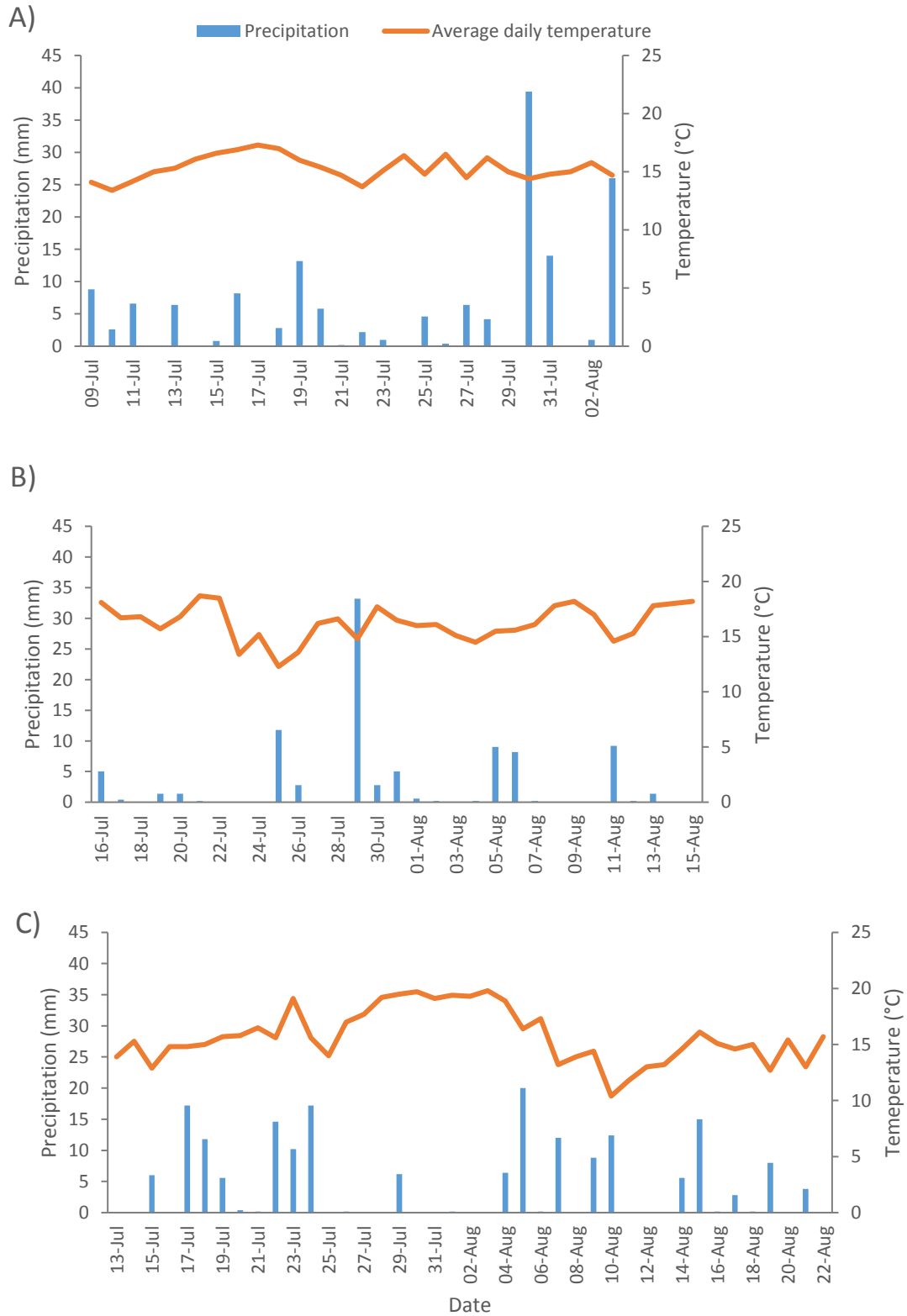
Supplemental Figure A

Content of gluten protein types (mg/grain) in premature grain during development based on freeze-dried grain (A) from 2009 and (B) from 2010.



Supplemental Figure B

Composition of gluten proteins in size exclusion chromatography fractions. SDS-unextractable proteins (F1*) and SDS-extractable proteins (F1-F4) were extracted from freeze-dried grain harvested at 35 DAA and 40 DAA and separated by SE-FPLC. Each fraction was then separated on SDS-PAGE gel. BM: bench marker

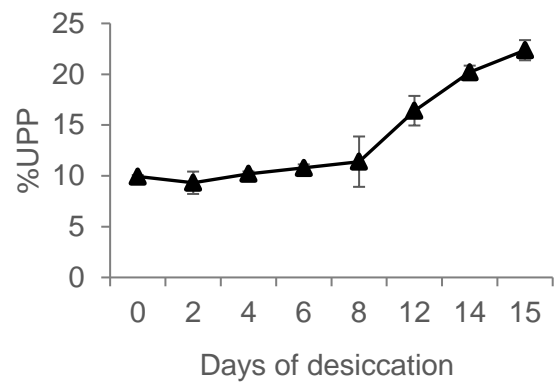


Supplemental figure C

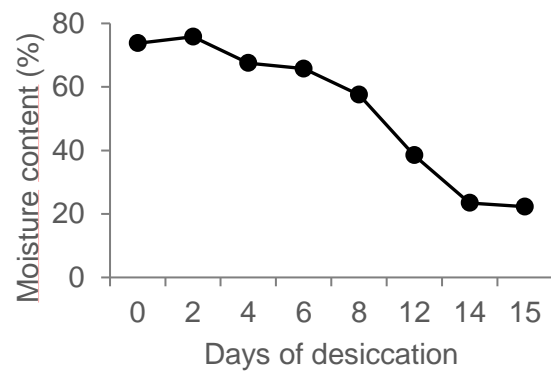
Average daily temperature and precipitation in 2009 (A), in 2010 (B) and in 2011 (C). The first date in each figure corresponds to the first harvest at 10 DAA in each season, and the last date

corresponds to the last harvest (35 DAA, 40 DAA and 50 DAA in 2009, 2010 and 2011, respectively).

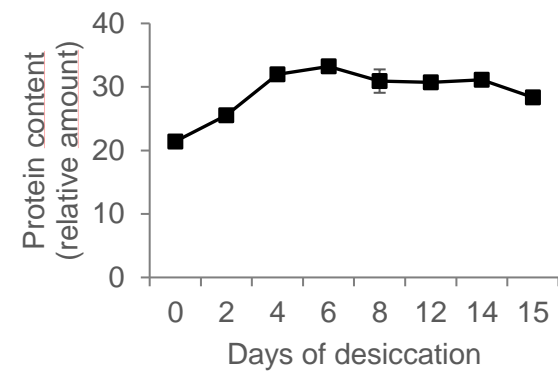
(A)



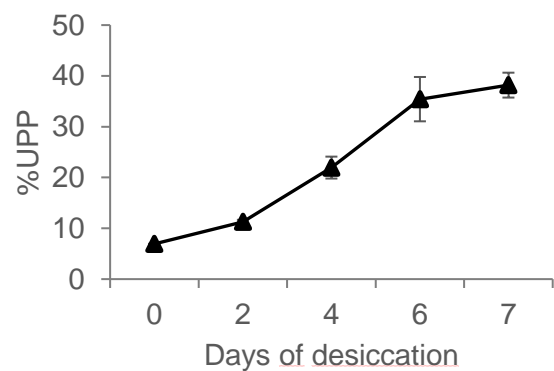
(B)



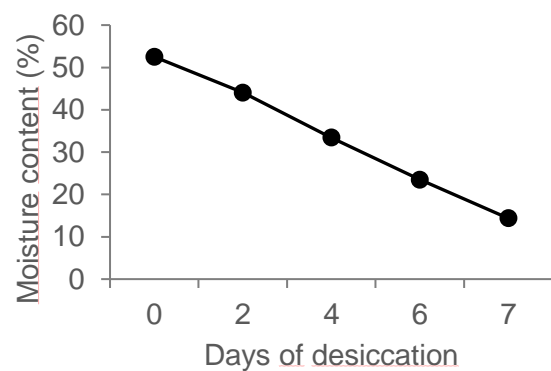
(C)



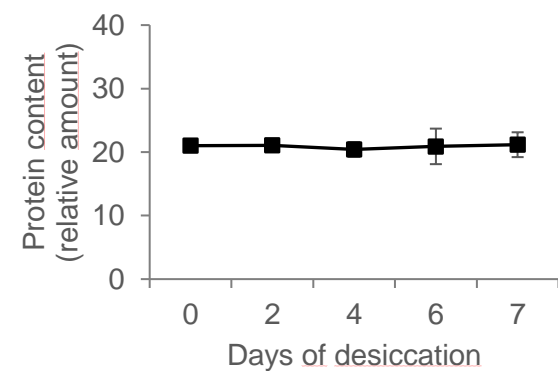
(D)



(E)



(F)



Supplemental Figure D

Changes in %UPP (▲, A and D), moisture content (●, B and E) and protein content (relative amount; ■, C and F) in wheat cv. Bjarne during artificial drying. Grains were harvested at 10 DAA (A-C) and 25 DAA (D-F) during grain development in 2011. The protein content is shown as relative amount measured by SE-FPLC. The vertical bars represent the standard deviation (n=2).