

**Aus dem Institut für Pflanzenernährung und Bodenkunde  
der Christian-Albrechts-Universität zu Kiel**

**Impact of late nitrogen fertilization on protein and baking  
quality of winter wheat**

**Dissertation  
zur Erlangung des Doktorgrades  
der Agrar- und Ernährungswissenschaftlichen Fakultät  
der Christian-Albrechts-Universität zu Kiel**

**vorgelegt von  
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Kiel, 2015**

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**Tag der mündlichen Prüfung: 4. November 2015**

**Gedruckt mit der Genehmigung**  
**der Agrar- und Ernährungswissenschaftlichen Fakultät**  
**der Christian-Albrechts-Universität zu Kiel**

*The roots of education are bitter, but the fruit is sweet.*

Aristotle

(384-332 BC)

## **Acknowledgement**

I never felt more disappointed with my ability in English writing when it comes to this part. It has been 11 years since I left my hometown and went to college. Along the whole journey, feelings are far beyond words. Especially during these four years abroad, I have learned and grown a lot. Hereby I want to express my deepest acknowledgements to my supervisor, my colleagues, my friends and my family, all your help and support will be remembered in my heart.

First of all, I would like to express my sincere thanks to **Prof. Dr. Karl H. Mühling** for providing me the chance to study in your group as a Ph.D. student. Time really flies. It was like yesterday when I just arrived in Germany. The scene when I met him the first time is still vivid in my head. I directly went to his office without any appointment and he was surprised but so kindly introduced the whole group to me and communicated warmly with me for several hours. Afterwards, occasionally I noticed that he was so fully scheduled every day and then realized that I must totally disturb his schedule at our first meeting. Even now I thought of this scene, I still felt funny but embarrassed. In terms of my Ph.D. work, he spent a lot of time with me discussing about the selection of topic and experimental design. Without his continued supervision, advice and valuable help, it was impossible for me to set up the first pot experiment within two months since I arrived in Kiel. Besides, he warmly discussed with us on the latest results we achieved and tried to solve problems we encountered during research, which ensured the smooth progress in our research. Especially for me, without a driver's license, conducting field experiments are almost impossible on my own. However, he established cooperation with another group where field experiments were conducted with relatively similar experimental set up with our pot experiment. Therefore, it was possible to verify our results from pot experiments in the field by collecting and analyzing samples from the field according to our aims. Moreover, for some analyses (e.g. baking test and protein identification) which need special equipment but not available in our group or in Kiel University, he was so helpful and guaranteed the successful analyses through cooperation with other groups. During the process of writing my thesis, he gave me many important advices and spent much time in the revision of the manuscripts and my thesis. I really appreciate all these helps and supervisions from him. In addition, not only in research work, he is also very concerned about me in life. When he heard that I found a very good opportunity and would go back to China for a job interview, he was so supportive and willing to offer me a recommendation letter which I did not even ask for. I felt really moved by his care and encouragement. It was thanks to his continued supervision, kind advice and valuable help made me have the chance to finish my Ph.D. today. Hereby, I would like to express my deepest gratitude to Prof. Dr. Karl H. Mühling.

My deepest appreciation also goes to **PD. Dr. Gunda Schulte auf'm Erley**, for her valuable help and guidance through my research and the revision of the manuscripts of thesis. Although she was not very familiar with my topic in the beginning, every time when I have questions or

encountered problems she was so kind and patient helping and solving problems with me. When I needed to take samples from the field, she not only drove me there but also helped me with sample collection. Especially when I was writing my thesis, she spent much time with me in discussing about the structure and contents in details, and revising the manuscripts and thesis. I really appreciate for all the patient and kind help as well as the valuable encouragement from her.

I also want to thank **Dr. Christoph-Martin Geilfus**. He was so patiently and nicely showed me how to conduct gel electrophoresis and shared his valuable insights in research. My gratitude is also extended to **Dr. Britta Pitann**, who taught me how to use the software Delta 2D for 2D gel analysis. I also thank **Ramona Schuster** and **Sulamith Rücker** for their excellent conduction of baking test in Freising. My warm and sincere acknowledgement also goes to our lab technicians **Stephanie thor Straten**, **Stefan Becker-Fazekas**, **Bärbel Biegler**, **Martina Bach** and **Thoja Heimbeck** for their technical assistance and help during my four-year study.

I want to give my special thanks to my dear officemate **Marcus Rohwer**. He really helped me a lot during these four years, and every time he was so warm-hearted and kind to offer me the help. There were so many times that he translated information in German for me. I also learnt a lot of German traditions and cultures from him. He also helped me a lot with living in Kiel. For example, when I was sick he helped me search for doctor information and make appointments. Things like that were countless. Although they might be small things for him, they were really tough for me without his help.

My special thanks also go to my Chinese colleagues **Li Wang** who helped me with setting up of pot experiments and taking samples in the field as well as **Jiawen Wu** for her valuable support and encouragement during the research work. I also acknowledge my dear friends **Wenjuan Li**, **Liwei Ma**, **Xiaohui Hu**, **Chanjuan Ren**, **Yanjun Ren**, **Jie Ding**, **Hao Chang**, **Kaihuai Liao**, **Uta Lungershausen**, **Gerrit Lungershausen** and **Katinka Seeger**. It is their company making my living abroad no longer lonely but delightful and colourful.

My deepest appreciation also goes to Chinese scholarship council for providing me financial support to work and stay as a Ph.D. student in Germany.

Last and most importantly, I must acknowledge with tremendous to my parents, my wife and my parents-in-law for their love, support and sacrifices. They have been central to my completion of this study as they have given me confidence and motivated me in so many ways. Being so far away from them, I can deeply feel the care from them and I also miss them a lot. Special thanks to my dear wife **Yang Wang**. In December 2014, the most wonderful thing happened in our life that our lovely and cute baby was born in China. My wife, herself is a Ph.D. student in China. She is very busy taking care of our lovely baby and, in the meantime, working hard on her doctoral dissertation. However, being so far away from them, I could not take the responsibility of taking care of the baby as a father, and I could not accompany my wife to help and support her as a husband. Even so, she had never complained about me. Whenever thinking

of this, I deeply feel remorse. Words cannot express my feelings, thank you with all my heart and soul.

Cheng Xue  
Kiel University, Germany  
September 2015

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# **Chapter 1**

## **General introduction**

# **1. General Introduction**

## **1.1 Wheat production, end-use and genetic background**

Wheat is one of the dominant food crops in the world, with over 600 million tonnes being harvested annually. According to the end-use, about 65% of the wheat crop is used for food, 17% for animal feed and 12% in industrial applications (FAO, 2013). Germany, the second important country for wheat production in Europe, produced 24.1 million tonnes of wheat in 2010, which accounted for 22.8% and 3.7% of the wheat production in Europe and in the world, respectively (FAO, 2012).

The success of wheat depends not only on its adaptability and high yield potential but also on the unique properties of its dough can be processed into bread, pasta, noodles and many other food products. These properties mainly result from the structures and interactions of the grain storage proteins (gluten proteins). In the bread-making processes, wheat flour is mixed with water, yeast, salt and other functional ingredients (such as fat and sugar) to form the dough, which is fermented and baked. The presence of the unique gluten complex provides dough the capacity to hold carbon dioxide gas produced during fermentation, which is preferred for the leavened bread (Delcour et al., 2012).

The genetic background of today's wheat cultivars are built by einkorn and emmer. Einkorn wheat is a simple species containing two sets of chromosomes (AA) and is referred to as diploid wheat. Emmer wheat is tetraploid wheat having four sets of chromosomes (AABB). Durum wheat, being cultivated mainly in the Mediterranean region, is the most important tetraploid wheat species suitable for pasta production. Afterwards, hybridization of cultivated emmer with *Triticum tauschii* (DD) formed today's bread wheat (common wheat or *Triticum aestivum*). Bread wheat has six sets of chromosomes (AABBDD) and is thus hexaploid wheat (Cornell, 2012; Feldman, 2001). Due to its major end-use, about 95% of the current wheat production in the world is hexaploid bread wheat, with tetraploid durum wheat taking up the majority of the remaining 5% (Shewry, 2009).

## **1.2 Composition of wheat proteins and their roles in bread-making quality**

Wheat grain protein is recognized playing a crucial role in affecting end-use quality of wheat products. Based on grain protein concentration, wheats are classified into hard (about 15% protein) and soft (near 10% protein) wheats. Wheat grain proteins are an extremely diverse group of compounds ranging from low-molecular-weight (LMW) to very high-molecular-weight (HMW) proteins due to their differences in amino acid composition. All proteins contain particular sequences of amino acids, referred to as the 'building blocks' of proteins. The structural organization of protein molecules determines their properties and the storage proteins have unique properties in affecting bread-making quality of wheat. The major proteins in the wheat grains can be classified into four groups and separated stepwise based on their solubility in different solvents according to the "Osborne fractionation" procedure (Osborne, 1924): albumins (water soluble), globulins (soluble in dilute saline), gliadins (soluble in aqueous alcohols) and glutenins (soluble in dilute acids or alkalis, chaotropic agents or detergents). These proteins are very rich in glutamine (30%) and proline (10%). Comparing to gliadins and glutenins, albumins have lower glutamine/glutamic acid (23%) and proline (9%) while higher cysteine (6%) contents. Globulins have even lower glutamine/glutamic acid (15%) while also higher cysteine (5%) contents (Cornell, 2012; Wieser et al., 1994). The gliadins and glutenins together form the gluten protein. The differences in solubility of these four groups of proteins result not from fundamental differences in sequence or structure but from their polymerization behaviour. Gliadins are monomeric proteins that are classified into three sub-types based on their electrophoretic mobility at low pH and amino acid compositions: the  $\omega$ -gliadins, which lack cysteine residues and thus cannot form disulphide bonds; the  $\alpha/\beta$ -type and  $\gamma$ -type gliadins, which form only intrachain disulphide bonds. Glutenins comprise polymers stabilized by interchain disulphide bonds which may have molecular weights of up to 10 million. After reduction, the glutenin subunits are readily soluble in alcohol-water mixtures like the gliadins and can be separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) into HMW-glutenin subunits (HMW-GS) and LMW-GS. The LMW-GS are further divided into a major group of B-type subunits and minor groups of subunits related to  $\alpha$ -type/ $\gamma$ -type gliadins (C-type) and  $\omega$ -gliadins (D-type). Due to the availability of complete amino acid sequences of representatives of the gluten proteins, three

broad groups are classified: sulphur (S)-rich, S-poor and HMW proteins. The types and characteristics of wheat grain gluten proteins are summarized in Fig. 1 according to Shewry and Halford (2002) and Shewry (2011).

It has been known for a long time that the wheat baking quality within one cultivar is positively correlated to the total grain protein concentration (Stein et al., 1992). As a consequence, grain protein concentration is applied as the most widespread criterion in evaluating wheat baking quality, thus determining the price of wheat. Higher grain protein concentration is usually associated with increased kernel hardness, gluten strength and loaf volume. Although wheat grain protein takes up only 8-14% of the grain dry weight, about 80% of these proteins in white flour is composed of gluten proteins in European wheats (Shewry, 2009). These gluten proteins play key roles in determining bread-making quality of wheat flour, as glutenin polymers are considered to form the ‘backbone’ of the gluten network and to confer elasticity (strength) to gluten and dough, while the gliadins interact with the glutenin polymers and other gliadins by strong noncovalent forces (notably hydrogen bonds) and confer viscosity and extensibility (Shewry, 2011). To achieve the desired soft bread with a large loaf volume, an optimal balance between dough extensibility and elasticity is needed. Therefore, not only quantity but also the compositions (quality) of proteins, such as gliadin/glutenin ratio and HMW-GS/LMW-GS ratio, are also important for bread-making quality as shown in Fig. 2 adapted according to Veraverbeke and Delcour (2002). In terms of glutenin quality, the HMW-GS are regarded affecting bread-making quality to a greater extent, while the LMW-GS mainly affects pasta-making quality of durum wheats (Masci et al., 2000). The HMW-GS affect bread-making quality through both quantitative effects which are related to differences in gene expression as affected by environmental factors and qualitative effects which relate to differences in the structures and properties of allelic subunits determined by genotype. Cultivars of bread wheat express three to five HMW-GS genes. Although the encoded HMW-GS account for only about 12% of the total grain protein, 45-70% of the variation in baking quality was supposed to be determined by these HMW-GS (Halford et al., 1992; Shewry et al., 2001).

### **1.3 Factors affecting bread-making quality**

Many factors influence the bread-making quality of wheat through effects on grain protein concentration and composition as mentioned above. These factors are genotypes, environmental conditions mainly temperature regimens and water availability and nutrient supply. Analysis of grain protein accumulation kinetics showed that albumins-globulins accumulate from anthesis to approximately 20 days after anthesis (daa), while gluten proteins accumulate from around 6 daa to the end of grain filling, being gliadins accumulate earlier than glutenins (Gupta et al., 1996; Panozzo et al., 2001; Stone and Nicolas, 1996). Heat stress during the grain filling period affects the quantity and quality of grain protein. Blumenthal et al. (1995) studied the heat stress ( $> 35^{\circ}\text{C}$ ) on grain quality using 45 wheat genotypes and found that the high temperature increased the gliadin/glutenin ratio and decreased the proportion of large polymers in flour. Moreover, heat stress effects varied among genotypes. Grain protein composition of genotypes having alleles for the HMW-GS pair 1Dx5 and 1Dy10 generally were more stable in response to high temperature than that having 1Dx2 and 1Dy12. Daniel and Triboï (2002) demonstrated that post-anthesis drought had no effect on glutenin accumulation rate, but shortened the period of grain-filling before the start of polymer insolubilization. Triboï et al. (2003) reported that the accumulation rates and durations of both gliadins and glutenins expressed in thermal time were not affected by moderately high temperature ( $< 35^{\circ}\text{C}$ ). Besides, post-anthesis drought stress had no significant effect on the accumulation rates of different protein fractions, but significantly reduced the durations of accumulation. Therefore, heat and drought stresses mainly affected the quantity and quality of grain protein through the shortening of grain-filling period while the process of N partitioning in grain protein synthesis is neither significantly influenced by post-anthesis temperature nor drought. In contrast to these environmental factors described above, the factor of nutrient supply can be more easily adjusted by appropriate fertilization management with essential nutrients. Mineral nutrition may influence grain protein synthesis from two aspects. On one hand, the minerals mainly nitrogen (N) and S contribute to the structure of proteins due to their roles in amino acid structure. Therefore, variations in the availability of these minerals may affect the amount and/or composition of grain proteins. On the other hand, the availability of substrates or the uptake,

transport, or biosynthesis of other components that compete for substrates may be affected by minerals in an indirect way (Shewry, 2011).

#### **1.4 Nitrogen fertilization effects in wheat production and quality**

Nitrogen is an important nutrient not only due to its high demand by plant but also to its importance in almost all the biological processes. Nitrogen supply plays a crucial role in storage protein synthesis, determining the total protein content and the protein composition. Since most of N present in the grain is in the form of proteins (with small amounts of free amino acids and peptides), a standard factor of 5.7 is used to convert grain N% to protein% in wheat grain (Shewry, 2011). Although the main focus here is the N effect on grain protein, the consideration of N influences on grain yield cannot be avoided due to the high correlations between grain yield and protein. Increasing grain yield from correcting nutrient shortages without further providing additional N usually reduce grain protein concentration because the N required for optimal storage protein synthesis may exceed that required to maximize yield. The grain N is derived from two sources. Wheat accumulates and stores the majority of its N before anthesis in vegetative organs and this N is remobilized later to the grain for protein synthesis, while the rest grain N (5-40%) comes from the lately absorbed N after anthesis (Triboi and Triboi-Blondel, 2002; Yoneyama, 1983). Therefore, in order to increase grain protein concentration, both N sources need to be enhanced. It is well known that the N uptake rate and accumulation highly depend on the wheat growth stages. During vegetative growth, the N accumulation in wheat follows a sigmoidal pattern. Wheat takes up and accumulates N slowly during early stages until tillering, while the N uptake proceeds rapidly until heading, and slows down again after flowering until grain filling (McGuire et al., 1998). The N accumulation during the early and mid-vegetative growth stages is mainly used for enhancing grain yield, while N uptake during and after heading primarily contribute to the grain protein synthesis. Therefore, effective N fertilization management throughout the wheat growth stages is essential for assuring both adequate yield production and acceptable grain protein concentration.

In wheat production, maximizing economic benefits is the ultimate goal for farmers. To pursue such aim, a rational balance should be achieved between grain yield and protein concentration

rather than highly increasing one parameter at the expense of the other. Besides, the process of wheat production should be environmentally friendly by minimizing detrimental effects on environment such as N leaching and gaseous losses. At present, normally three applications of N fertilizer during early and mid-vegetative growth stages are conducted to increase grain yield and usually a late N fertilizer (the fourth N application - quality fertilization) is supplemented to enhance grain protein content in wheat production. The first N application is conducted at sowing or at the beginning of tillering (EC21) (Lancashire et al., 1991) to enhance tillering and ear number, which are typically the greatest components affecting grain yield; the second N application is given at the beginning of stem elongation (EC30) in order to ensure the number of tillers and ears; the third N dose is applied at flag leaf stage (EC39) to increase grain number per ear and thousand kernel weight; and the fourth N application is conducted at the beginning of heading (EC51) or even later to increase grain protein content.

Considering the total N availability, under most conditions the grain protein concentration is increased by increasing N fertilizer input (Fuertes-Mendizábal et al., 2010; Garrido-Lestache et al., 2005; López-Bellido et al., 1998; Wieser and Seilmeier, 1998). Benizian and Lane (1981) summarized data of wheat experiments in Southern England from 20 years and found that in most experiments linear or convex responses exist between grain protein content and fertilizer up to 175 kg N ha<sup>-1</sup>. Moreover, N fertilization changes not only quantity but also quality (composition) of grain proteins. With the enhancement in grain protein concentration, the concentrations of both the gliadins and glutenins increase, being gliadin fraction increases proportionally faster than that of glutenin, thus resulting in higher gliadin to glutenin ratio (Martre et al., 2006). Besides, it is speculated that since the  $\omega$ -gliadins and HMW-GS are mainly composed of glutamine and proline which are the metabolically inexpensive amino acids, they may be good sinks for N when there is surplus N or (relatively) insufficient S (Dupont and Altenbach, 2003). One of the representative studies is from Wieser and Seilmeier (1998), who conducted a detailed quantitative study of N fertilization on gliadin and glutenin components within 13 hexaploid wheat varieties. With increasing N fertilization rate, the protein concentration (mg g<sup>-1</sup> flour) enhanced 44-68%. The amount of  $\omega$ -gliadins and HMW-GS increased more than two-fold and 56-101%, respectively. The proportional changes



of  $\alpha$ - and  $\gamma$ -gliadins and LMW-GS were relatively small. Besides, the amount of albumin and globulin was unaffected by N fertilization rate.

Ideally, the application of a late season N fertilizer either split from the total N rate or as additional N amendment, should have positive effect in enhancing grain protein concentration since this portion of N is supposed to be predominantly used for protein synthesis over yield formation as described above. However, the effects of this late season N application vary considerably between different years and locations, and depend on many factors including total N application rate, timing, N fertilizer type, yield potential and the plant N status at flowering (Ayoub et al., 1994; Fuertes-Mendizábal et al., 2010, 2013; Garrido-Lestache et al., 2004, 2005). Therefore, the effects of late season N application on grain protein concentration are inconsistent and uncertain. Besides, by summarizing results from many studies, it has been reported that large differences existed in the proportion of the variation in loaf volume that can be explained by protein concentration, especially between different cultivars (Weegels et al., 1996). Therefore, even this effect of late season N application does exist, whether it will influence the baking quality of the resulting wheat flour still remains a question, particularly for cultivars whose baking quality are relatively stable within a certain range of protein concentration. However, the practical problems and extra costs (energy, fertilizer and manpower) of applying this late season N are undisputed. Furthermore, without cooperation with appropriate precipitation, the late season N fertilizer may cause potential ecological problems such as N leaching and gaseous losses as mentioned above. Therefore, it is needed to investigate the effects of late season N application on wheat grain protein and bread-making quality more comprehensively and thus give evidences on whether this late N application can be removed or combined with early N application without decreasing wheat yield and quality, which will benefit greatly to wheat producers.

### **1.5 Aims of the study**

From literatures supporting the positive effects of late season N application (either split from the same total N rate or with additional N amendments) on grain protein concentration and composition (Blandino et al., 2015; Fuertes-Mendizábal et al., 2010; Islas-Rubio et al., 2011;

Wieser and Seilmeier, 1998; Wuest and Cassman, 1992), it seems the late season N application has similar effects as caused by increasing N fertilization rate. As fertilizer N use efficiency can be increased by skilful handling of N fertilization such as split N application (Ercoli et al., 2013; Hamid, 1972), the plant N uptake would be increased by the late season N application in contrast to applying all N fertilizers at early stages of wheat growth. Therefore, to clarify the late season N application effect and distinguish it from the N rate effect, the plant N uptake and recovery of the late fertilizer N should be considered under controlled conditions. Unfortunately, limited studies aimed at this issue are available.

Therefore, it is hypothesized that late season N application had no significant effect in affecting N partitioning in the grain, whereas its influence on grain protein quantity and quality actually results from increased plant N uptake due to the enhanced N use efficiency. If this is true, the late N fertilizer can be applied earlier and reduce the overall N application times.

Based on this main hypothesis, two pot experiments with two winter wheat cultivars belonging to different quality groups (having similar baking quality while different grain protein concentrations) were conducted under natural conditions with supplemental irrigation. Nitrogen fertilization treatments were set up by applying increasing N rates and splitting them into two or three amendments plus N form management to investigate the effect of split N application and late N fertilization on wheat grain protein concentration and composition and thus baking quality (Chapter 2).

Baking quality of wheat flour is highly affected not only by fertilization but also by many other environmental factors (e.g. precipitation, soil types, and locations). Since environmental conditions could be well controlled (e.g. soil moisture) and plant N uptake could be assured by pot experiments, thus the intrinsic effects of late N fertilization on wheat baking quality could be better revealed using pot experiments, avoiding interactions from other environmental factors. However, whether these results achieved from pot experiments could be applied to wheat production, the effects of late N fertilization need to be verified also from field studies. Therefore, a field study was performed as well at two locations in two consecutive years using the same cultivars as used in pot experiments (Chapter 3).

Based on the results from both pot and field experiments (Chapter 2 and 3), two most interesting treatments (splitting of the same total N fertilization rate into two and three doses with the third N application at late boot stage) from the first pot experiment were selected to further investigate the individual protein changes caused by the third N application (Chapter 4).

Taken together, the present study was conducted with the following objectives:

- (i) To determine the effect of late N fertilization on the quantity and composition of wheat grain protein and baking quality.
- (ii) To distinguish whether the late N effect resulted from increased total N fertilization rate or from N splitting.
- (iii) To reveal how N fertilizer types influence the protein and baking quality when applied as late N.
- (iv) To evaluate how cultivars belonging to different quality groups respond to late N fertilization.

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## **Figures**

### **Figure Captions**

**Figure 1** The classification and nomenclature of wheat gluten proteins separated by electrophoresis at low pH (gliadins) and SDS-PAGE (glutenins) according to Shewry (2011) and Shewry and Halford (2002).

**Figure 2** Factors determining dough rheological properties and bread-making quality adapted according to Veraverbeke and Delcour (2002).



Figure 1

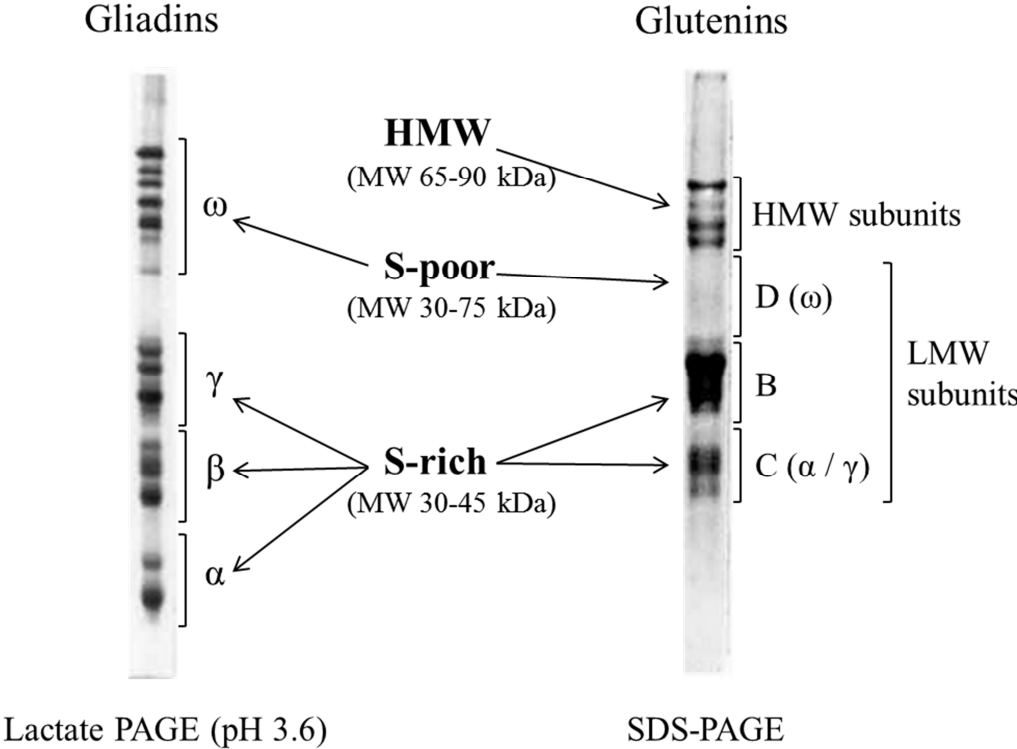
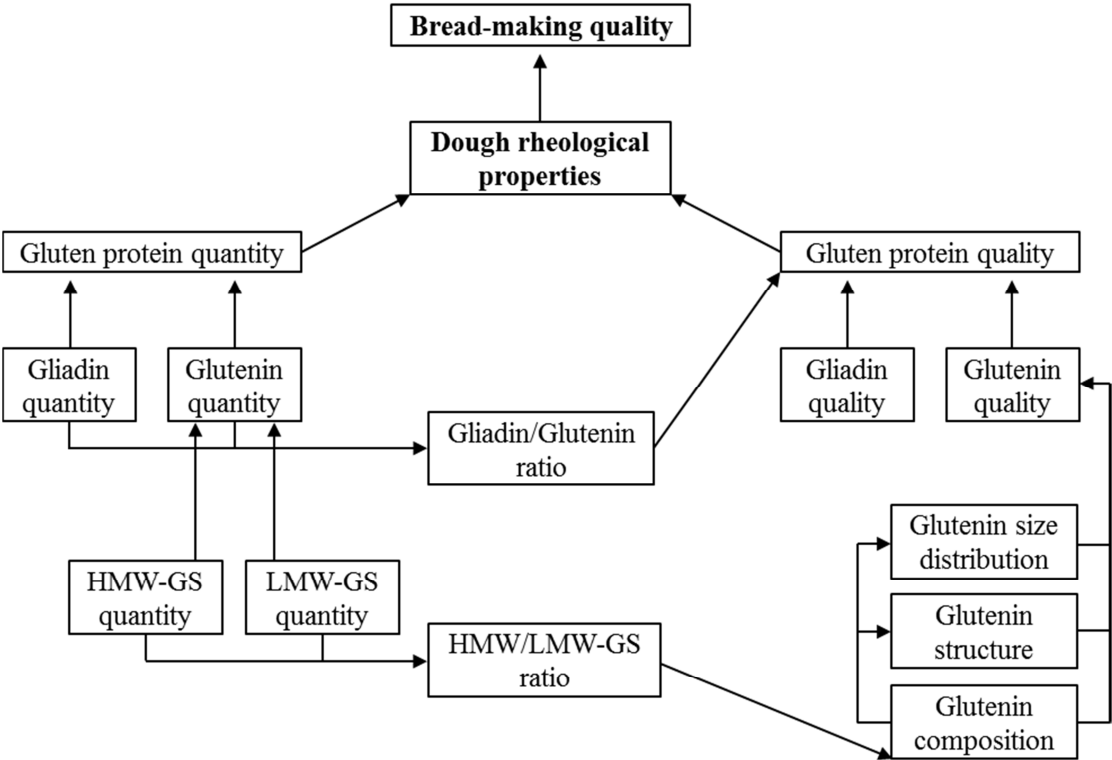


Figure 2



## **Chapter 2**

**Split nitrogen application improves wheat baking quality by  
influencing protein composition rather than concentration**

Journal of the Science of Food and Agriculture (submitted)

## **2. Split nitrogen application improves wheat baking quality by influencing protein composition rather than concentration**

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Key words: Late nitrogen application, gliadin, glutenin, loaf volume

## **Abstract**

Two pot experiments were conducted to evaluate how late nitrogen (N) fertilization influences protein and baking quality of two wheat cultivars, as well as to distinguish if this effect resulted from increased N rate or N splitting. Late N was applied either as additional N or split from the basal N in the form of nitrate-N or urea. Results showed that late N fertilization improved loaf volume of wheat flour by increasing grain protein concentration and altering its composition. Increasing N rate mainly increased protein and gluten concentrations and gliadin/glutenin ratio. However, split N application enhanced not only the quantities but also the proportions of gliadins and glutenins as well as certain high molecular weight glutenin subunits (HMW-GS), which led to an improved baking quality of wheat flour. The late N effects were greater when applied as nitrate-N than urea. The late N effect was more remarkable in wheat cultivars whose baking quality was more dependent on protein concentration. The proportions of glutenin and x-type HMW-GS were more important than the overall protein concentration in determining baking quality. However, when the total N rate was further increased to higher levels, application of late N fertilization was less effective.

## 2.1 Introduction

Wheat, one of the main food crops, is unique for the viscoelastic properties of its dough which could be processed mainly into bread for hard wheat. Two crucial determinants of bread-making quality are the grain protein concentration and protein composition, including the amounts and ratios of gluten protein fractions as well as their subunits (Weegels et al., 1996; Zhang et al., 2009). Gluten consists of gliadin (mainly monomeric proteins soluble in aqueous alcohols, heterogeneous mixture of different gliadin types) and glutenin fractions (polymeric proteins forming large aggregates, subunits soluble in aqueous alcohols after reduction). Gluten plays an important role for the bread-making quality of wheat flour as gliadins mainly contribute to dough viscosity and extensibility, while glutenins to dough strength and elasticity (Wieser, 2007).

Wheat bread-making quality is highly affected by both genetic and environmental factors. Within environmental factors, fertilization, especially nitrogen (N) fertilization plays an important role and can be easily adjusted comparing to climatic and irrigation conditions. Numerous studies demonstrated that wheat bread-making quality was improved with higher N fertilization rate mainly through the increase in grain protein concentration, as well as gliadin to glutenin and high molecular weight glutenin subunits (HMW-GS) to low molecular weight glutenin subunits (LMW-GS) ratios (Fuertes-Mendizábal et al., 2010; Garrido-Lestache et al., 2004; Jia et al., 1996; Wieser and Seilmeier, 1998; Zörb et al., 2010).

Therefore, late N fertilization (application of additional N at heading or anthesis) is widely recognized as useful for enhancing grain protein concentration and is practically conducted. Many studies have reported improved bread-making quality of wheat flour after late N fertilization (Blandino et al., 2015; Wieser and Seilmeier, 1998). This effect may result not only from increased total N fertilization rate but also from split N application. The split N application (splitting of the same N rate distributed in several applications at different growth stages) also influences the wheat flour quality since the delayed N application predominantly benefits protein build-up over starch in the grain and prolongs the duration of grain-filling (Sowers et al., 1994). However, the results of split N application on wheat quality varied among

studies (Fuertes-Mendizábal et al., 2010; Garrido-Lestache et al., 2004, 2005; Schulz et al., 2015). The positive effects may be attributed mainly to the N rate effect since the fertilizer N use efficiency can be enhanced by split N application under appropriate environmental conditions, which resulted in higher plant N uptake (Hamid, 1972). On the contrary, the results showing no such effect from split N application probably resulted from reduced availability of N from the soil caused by the different field conditions, thus the delayed application of fertilizer N is not taken up efficiently by plants. Similarly, it was also shown that the effects of late N fertilization varied between different environmental conditions such as locations and total N fertilization rate (Jia et al., 1996). Therefore, it is hypothesized that the effects of late N fertilization on wheat grain protein concentration and composition were actually due to N fertilization rate and not to split N application. If this is true, the late N fertilizer could be combined with early applications, thus simplifying the N fertilization management. Besides, taking into account the practical problems and extra costs of applying a late N dose, further investigations are needed on whether a late N application is necessary. To clarify the split N application effect and to distinguish it from the N rate effect, the plant N uptake and recovery of the late fertilizer N should be considered under controlled conditions. Unfortunately, limited studies aimed at this issue are available.

In order to avoid N losses and contamination of underground water and air through leaching and gaseous losses, the accumulation of large amount of soil-nitrate should be avoided. Unlike nitrate-N, which can be stored in vacuole when extensive supply of N as nitrate-N was taken up by plants, ammonium-N cannot be stored in plant cells after uptake by plants. Therefore, it is hypothesized that the process of amino acids synthesis from ammonium-fed wheat would be enhanced, thus promoting protein accumulation in the final grain. Fuertes-Mendizábal et al. (2013) reported that ammonium as sole N source combined with split N application improved grain quality in wheat compared to nitrate-N source. Nevertheless, the differences between N forms as late N application on wheat grain quality still need further investigation.

Bread-making quality of cultivars differing in protein concentration or quality (such as HMW-GS composition) may respond differently to variations in protein concentration

(Weegels et al., 1996). For cultivars exhibiting high baking quality with relatively low protein concentration, measures to increase protein concentration might be dispensable. Therefore, to investigate the effect of late N fertilization more comprehensively, cultivars belonging to different quality groups should be considered. Moreover, the correlation between protein concentration and bread-making quality varies widely over years and cultivars (Koppel and Ingver, 2010). Therefore, a suitable baking test is needed to avoid incorrect evaluation of the bread-making quality of wheat cultivars. Unfortunately, very limited studies have used the baking test to evaluate wheat flour quality due to a lack of material or equipment.

The main objectives of this study were (i) to evaluate the effect of late N fertilization on the quantity and composition of gluten proteins and thus baking quality (loaf volume), (ii) to distinguish whether this effect resulted from higher N rate or split N application, (iii) to determine how different N forms affect the protein and baking quality when applied as late N, (iv) to determine how cultivars belonging to different quality groups respond to late N fertilization, (v) to check if protein concentration is a reliable parameter in evaluating baking quality under such conditions.

## **2.2 Materials and Methods**

### Experimental design

Two winter wheat cultivars Tobak and JB Asano, belonging to different baking quality classes according to the German Federal Office of Plant Varieties, were used in this study. Tobak (baking quality class B) had similar loaf volume but lower raw protein concentration comparing to JB Asano (baking quality class A). Besides, according to ‘Glu-1 quality score’ (Payne et al., 1987) based on HMW-GS, the ‘Glu-1 quality score’ for Tobak is five, whereas for JB Asano it is seven. The N fertilization experiment was conducted in the year 2011-2012, and was repeated in the following year 2012-2013 with some modifications in total N fertilization rate and timing of late N application.

The first experiment (2011-2012) was performed in Mitscherlich pots containing 6 kg of soil with supplemental irrigation under natural conditions, i.e. outdoor, except during strong frost and exceptionally high rainfall. The fertilization treatments were designed as follows: N1 (low



N rate treatment), an N rate of 1.5 g N pot<sup>-1</sup> in two doses with 1 g N pot<sup>-1</sup> applied before seeding and 0.5 g N pot<sup>-1</sup> at EC30 (beginning of stem elongation) (Lancashire et al., 1991); N2 (higher N rate treatment), 2 g N pot<sup>-1</sup> in two doses with 1 g N pot<sup>-1</sup> applied before seeding and 1 g N pot<sup>-1</sup> at EC30; N3 (late nitrate treatment), 2 g N pot<sup>-1</sup> applied in three doses with 1 g N pot<sup>-1</sup> applied before seeding, 0.5 N pot<sup>-1</sup> at EC30 and 0.5 g N pot<sup>-1</sup> in the form of calcium nitrate (<sup>15</sup>N labelled) applied as a late N application at EC45 (late boot stage); N4 (late urea treatment), 2 g N pot<sup>-1</sup> applied in three doses with 0.5 g N pot<sup>-1</sup> in the form of urea (<sup>15</sup>N labelled) with nitrification inhibitor dicyandiamide (DCD) applied as a late N application at EC45 comparing to N3. Thus, the effects of N fertilization rate (N2 vs N1), split N application (N3 and N4 vs N2), late N fertilization (N3 and N4 vs N1) and N forms (N4 vs N3) could be determined in the present study. The N fertilizers applied before seeding and at EC30 for all treatments were in the form of ammonium nitrate. Other nutrients including P (0.6 g pot<sup>-1</sup>), K (2.3 g pot<sup>-1</sup>), S (0.5 g pot<sup>-1</sup>), Mg (0.33 g pot<sup>-1</sup>), Ca (1.19 g pot<sup>-1</sup>) and the minor elements Cu (10 mg pot<sup>-1</sup>), Zn (15 mg pot<sup>-1</sup>) and Mn (30 mg pot<sup>-1</sup>) were applied before winter wheat seeding. Each treatment was replicated five times. Winter wheat was sown on 30 November 2011 and harvested on 6 August 2012. Nitrogen fertilization was conducted on 25 November 2011, 2 April 2012 and 30 May 2012, respectively. Fungi disease and insects were well controlled by spraying fungicide Capalo three times and insecticide Biscaya once during winter wheat growth.

The second experiment (2012-2013) was conducted as a replicate of the first experiment with some modifications. For each treatment (N5, N6, N7 and N8 comparing to N1, N2, N3 and N4, respectively), 0.5 g more N pot<sup>-1</sup> was given at EC30 and the application of late N fertilizer was postponed from EC45 to EC51 (beginning of heading). Winter wheat was sown on 30 October 2012 and harvested on 23 July 2013 for JB Asano and 2 August 2013 for Tobak. Nitrogen fertilization was conducted on 26 October 2012, 25 March 2013 for both cultivars and on 24 May 2013 for JB Asano and 29 May 2013 for Tobak. Fungi disease and insects were well controlled by spraying fungicide Capalo two times and insecticide Biscaya once during winter wheat growth. The soil used for both pot experiments was classified as sandy loam, pH 6.6, and the nutrient concentrations were 250 mg P<sub>2</sub>O<sub>5</sub> kg<sup>-1</sup>, 180 mg K<sub>2</sub>O kg<sup>-1</sup>, 3.3 mg S kg<sup>-1</sup> and 1300 mg N kg<sup>-1</sup> and organic matter of 2.3%.

### Yield, nitrogen and protein concentration

After harvest, grains, stems (incl. leaves except flag leaf), flag leaves and glumes were separated, dried and the dry weight of each fraction was determined for each pot. Grains were milled in a Titan laminated mill using a 500 µm sieve (Retsch, Haan, Germany). Nitrogen concentration of each fraction was determined by a CNS elemental analyser (Flash EA 1112 NCS, Thermo Fisher Scientific, Waltham, MA, USA). Crude protein concentration of wheat flour was calculated by multiplying the N concentration by 5.7.

### Elemental analysis isotope ratio mass spectrometry

Grain flour was analysed by elemental analyser - stable isotope ratio mass spectrometry (EA-SIRMS) using a DELTA V PLUS Isotope Ratio MS (Thermo Electron Corporation) interfaced with a Flash 2000 Organic Elemental Analyser (Thermo Fisher Scientific, Waltham, MA, USA) to determine N isotope ratios.

### Chlorophyll meter measurement

The chlorophyll content was determined by reading the absorbance on a chlorophyll meter (SPAD-502, Minolta, Langenhagen, Germany) five times at one, 11, 24, 31 and 43 days after the late N application, respectively, on flag leaf, the first, second and third leaf below flag leaf for both cultivars. At each time, measurements were made on five fixed leaves of each leaf fraction in each pot (measurements on 20 leaves pot<sup>-1</sup> in total), 10 measurements were done for each leaf, and the average was used as SPAD reading for each leaf.

### Extraction and quantification of storage protein fractions

Extraction of flour storage protein fractions was performed stepwise according to Wieser and Seilmeier (1998) and Zörb et al. (2010) with some modifications. First albumins and globulins were extracted from 100 mg wholemeal flour with 1 mL extraction buffer (0.4 mol L<sup>-1</sup> NaCl, 67 mmol L<sup>-1</sup> Na<sub>2</sub>HPO<sub>4</sub>, 7.7 mmol L<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, pH 7.6) using a 2 mL tube. After vortexing shortly the samples were stirred for 20 min at 4 °C and centrifuged (16,000 × g, 20 min, 4 °C). The supernatant was collected as albumin-globulin fraction. This procedure was repeated once. The pellet was then extracted with 500 µL 60% ethanol to obtain the gliadin fraction using the same

procedure as described above and repeated twice. The three supernatants were combined as one gliadin fraction. The pellet was then washed with 1 mL of deionized water, stirred for 5 min, and centrifuged ( $16,000 \times g$ , 15 min,  $4^\circ\text{C}$ ). The supernatant was discarded. Afterwards, the glutenin fraction was extracted by adding 1 mL extraction buffer [50% 1-propanol (v/v),  $2 \text{ mol L}^{-1}$  urea, 1% dithiothreitol (DTT),  $0.05 \text{ mol L}^{-1}$  Tris-HCl, pH 7.5] to the pellet. Extraction was done by vortexing for one min and stirring for 25 min at  $60^\circ\text{C}$  followed by centrifugation ( $16,000 \times g$ , 15 min,  $20^\circ\text{C}$ ). The supernatant was collected and the extraction repeated once. In the end, all gluten fractions were centrifuged ( $16,000 \times g$ , 25 min,  $4^\circ\text{C}$  for gliadin and  $20^\circ\text{C}$  for glutenin) and each resulting supernatant was transferred into a new 2 mL tube and stored at  $-20^\circ\text{C}$ . A subsample ( $400 \mu\text{L}$ ) of each protein fraction was transferred into a new 2 mL tube and the protein was precipitated by adding  $600 \mu\text{L}$  DTT/acetone solution ( $50 \text{ mmol L}^{-1}$  DTT) and stored at  $-20^\circ\text{C}$  overnight. After centrifugation ( $16,000 \times g$ , 25 min,  $4^\circ\text{C}$ ), the supernatant was discarded and the pellet was dissolved by dissolving buffer { $8 \text{ mol L}^{-1}$  urea (serdolit washed),  $2 \text{ mol L}^{-1}$  thiourea (serdolit washed), 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS),  $30 \text{ mmol L}^{-1}$  DTT,  $20 \text{ mmol L}^{-1}$  Tris base} and stored at  $-20^\circ\text{C}$ . The concentration of each protein fraction was determined using 2D Quant-kit (GE-Healthcare, Munich, Germany).

#### Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Gliadin and glutenin compositions were determined by SDS-PAGE performed according to the method of Laemmli (1970) with some modifications. Medium sized ( $18 \text{ cm} \times 16 \text{ cm}$  glass plates and 1.5 mm spacers in thickness) gels (12.5% separating gels and 6.3% stacking gels) were casted one day before use. Separating gels were prepared by mixing 24 mL deionized water, 18 mL separating gel buffer ( $1.5 \text{ mol L}^{-1}$  Tris base, 0.4% SDS, pH 8.8), 30 mL acrylamide / bis-acrylamide (37.5/1, v/v; Rotiphorese Gel 30),  $599.4 \mu\text{L}$  10% ammonium persulfate (freshly made) and  $59.4 \mu\text{L}$  tetramethylethylenediamine (TEMED) in a beaker and then pipetted between the plates leaving 2 cm space to the top and covered by 2-propanol. After 1 h, 2-propanol was removed and replaced by stacking gel [prepared by mixing 15 mL deionized water, 7.5 mL stacking gel buffer ( $0.5 \text{ mol L}^{-1}$  Tris base, 0.4% SDS, pH 6.8)], 6 mL

polyacrylamide, 120  $\mu\text{L}$  10% ammonium persulfate and 120  $\mu\text{L}$  TEMED in a beaker). A comb was then placed. Protein extracts were mixed with the same volume of sample buffer (10% glycerol, 2.3% SDS, 5% 2-mercaptoethanol, 0.0025% bromophenol blue, 63  $\text{mmol L}^{-1}$  Tris-base, pH 6.8) and shaken for 5 min at 95  $^{\circ}\text{C}$  and cooled at room temperature. Each well was loaded with sample containing 20  $\mu\text{g}$  protein. A molecular weight standard 'Rotimark 10-150' (RotiMark, Roth, Crailsheim, Germany) was loaded on one lane of each gel. Two gels were run simultaneously in the same vertical electrophoretic unit (SE600, Hoefer) at 50 mA and 17  $^{\circ}\text{C}$  for around 6 h. The gels were then fixed (40% ethanol, 10% acetic acid), stained (1 tablet 'PhastGel<sup>TM</sup> Blue R' in 1.6 L 10% acetic acid) and destained (10% acetic acid). Gels were digitized by scanning on an image scanner (Epson PerfectionV700) at 300 dpi and 16 bits per pixel.

#### Image analysis

Gel electrophoresis images were analysed using the Quantity One software (Bio-Rad, Hercules, CA, USA). Bands on each gel were determined and matched with molecular weight markers to calculate the molecular weight of each protein band. The average band intensity and relative quantity of each band were then determined.

#### Micro-baking test (MBT)

The MBT was performed according to Thanhaeuser et al. (2014) with the modification that wholemeal flour was used. After grinding, wheat flours were stored for at least 14 days before being used for baking. The moisture of each wholemeal flour sample was measured using an infrared moisture analyser (MA35, Sartorius). Then, for each sample the water absorption and dough development time were determined using a modification of the ICC-Standard method 114/1 by mixing 10 g flour, 0.2 g NaCl, and water at 22  $^{\circ}\text{C}$  for 20 min in a micro-farinograph (Brabender, Duisburg, Germany) according to Thanhaeuser et al. (2014). Afterwards, the MBT was performed. Briefly, 10 g flour, 0.7 g fresh yeast (Wieninger, Passau, Germany), 0.2 g NaCl, 0.2 g sucrose, 0.1 g coconut fat, 0.3 mL L-ascorbic acid solution (0.67  $\text{g L}^{-1}$ ) and water were mixed in the micro-farinograph for 4-6 min according to the dough development time. Then the dough was weighed and placed in a proofer for 20 min (30  $^{\circ}\text{C}$ , water-saturated atmosphere).

After rounding and weighing again, the dough was fermented 35 min on the baking line at 29 °C in a water-saturated atmosphere. The dough was then baked for 10 min (three temperature stages: 120 °C, 180 °C and 250 °C).

The bread was weighed immediately and again at 1 h after baking. The bread volume was measured using Volscan Profiler 600 (Stable Micro Systems, Godalming, UK).

### Statistical analysis

Data for each parameter was presented as the mean value of five replicates analysed. The software SPSS 13.0 (Chicago, IL, USA) was used. Significance of treatments within each cultivar was determined at  $P < 0.05$  level by analysis of variance (One-way ANOVA) followed by Tukey's least significant test. Before, a two-way ANOVA was conducted testing treatment and cultivar effects and their interactions in order to get an overview over parameter changes.

## 2.3 Results

### 2.3.1 Grain yield and N uptake

Winter wheat grain yield and plant N uptake were increased by late N fertilization for both cultivars and in both experiments through increased N fertilization rate, while split N application had no such effects (Table 1). Around 63 - 68% of the total N uptake was translocated to the kernels. However, N harvest index was mainly enhanced by late nitrate fertilization, except that there was no effect on Tobak in the second experiment, and was also increased with higher N rate on JB Asano in the second experiment (Table 1). The recovery of late N fertilizer was higher when applied as nitrate-N compared to urea, except for Tobak in the second experiment (Table 1). Comparing to N fertilizer applied at early growth stage of wheat, late N fertilizer was more efficiently used in the kernels, resulting in much higher late N harvest index (80 - 86%) than N harvest index (63 - 68%) (Table 1).

### 2.3.2 Protein concentration and composition

Protein concentration was mainly increased by higher N fertilization rate and further increased by split N application in nitrate-N in JB Asano when total N rate was medium (first experiment) (Table 2). However, protein concentration was only increased by late N fertilization in JB

Asano when total N rate was further increased (second experiment). Protein yield showed the same trend as grain yield. Furthermore, due to the much higher grain yield in the second experiment and similar protein concentration between both experiments, protein yield in the second experiment was much higher than in the first experiment.

The effects of late N fertilization on the concentrations and proportions of gluten fractions varied between experiments (Table 2). In the first experiment, gliadin concentration, gliadin proportion and gluten concentration were increased by higher N fertilization rate and further increased by split N application. Glutenin concentration was increased by higher N rate and split N application in nitrate-N (Tobak) or just by split N application (JB Asano), whereas glutenin proportion was increased by late N fertilization and split N application in nitrate-N. Although the response of gliadin and glutenin concentration varied among fertilization treatments and cultivars, gliadin to glutenin ratio was only increased by late N fertilization through higher N fertilization rate for both cultivars. Furthermore, nitrate-N had greater effects in increasing concentrations of gluten fractions than urea when applied as late N. Comparing to JB Asano, Tobak had higher glutenin concentration and similar gliadin concentration, thus the gliadin/glutenin ratio was lower in Tobak than in JB Asano.

However, in the second experiment, only glutenin and gluten concentration were increased by late N fertilization through higher N fertilization rate in Tobak (Table 2). Late N fertilization had no significant effects on gliadin concentration, glutenin concentration (JB Asano), gliadin and glutenin proportions and their ratio. Besides, there was no difference between N forms for all these parameters.

### 2.3.3 Gluten subunits pattern

Gliadin and glutenin protein subunits were separated by SDS-PAGE, and the protein band patterns of both cultivars were shown in Fig. 1A and 1B. For the gliadin fraction, 14 protein bands were detected and their relative quantities were determined for Tobak and JB Asano, respectively. In the first experiment (Table 3), the relative quantities of two  $\gamma$ -gliadin subunits in Tobak (band 6, MW 40.1 kDa and band 7, MW 38.7 kDa) and one  $\gamma$ -gliadin subunit in JB Asano (band 8, MW 39 kDa) were decreased by late N fertilization through the combined effect

of higher N rate and split N application, respectively. Besides, one  $\omega$ -gliadin subunit in JB Asano (band 2, MW 58.4 kDa) was increased with late nitrate fertilization through split N application. However, no change of band relative quantity was found in both cultivars in the second experiment (data not shown).

For the glutenin fraction, 11 and 14 protein bands were determined for Tobak and JB Asano, respectively. In the first experiment (Table 4), the relative quantity of four and two glutenin bands in Tobak (band 2, 3, 5 and 6) and JB Asano (band 2 and 6), respectively, were increased by late N fertilization, while one band in Tobak (band 11) and one band in JB Asano (band 14) were decreased by late N fertilization, respectively. Particularly, the relative quantity of the same HMW-GS (subunit 7), band 2 in both Tobak (at  $P < 0.05$  level) and JB Asano (at  $P < 0.1$  level), was only increased with late N fertilization through split N application. In the second experiment, the relative quantities of two LMW-GS bands were decreased with late N fertilization through higher N rate in JB Asano, while no change was observed in Tobak (data not shown). In terms of HMW-GS to LMW-GS ratio (Table 4), JB Asano had higher ratio than Tobak. However, no difference was found between treatments in both cultivars and in both years (data from the second experiment not shown).

#### 2.3.4 Baking quality

Micro-baking tests were performed only for the first experiment since differences in gluten concentration and composition were mainly observed in the first experiment. For both wheat cultivars, baking quality of wheat flour expressed as bread volume was improved by late N fertilization through split N application (Fig. 2). Besides, nitrate-N was better than urea in improving flour baking quality when applied as late N, since bread volume of Tobak was only increased by late nitrate fertilization. Furthermore, the positive effect of late N fertilization on JB Asano was greater than on Tobak. JB Asano had a lower bread volume than Tobak when N fertilizer was only applied in two instead of three doses, however, this difference no longer existed after a late N application since bread volume of JB Asano increased more (from 29.5 to 32.3 mL, increased by 9.4%) by late N fertilization than Tobak (from 31.3 to 32.6 mL, increased by 3.9%).

## 2.4 Discussion

### 2.4.1 Protein and baking quality of wheat flour as influenced by late N fertilization

In our study, late N fertilization increased bread volume of wheat flour through the enhanced grain protein concentration, gliadin and glutenin concentrations as well as their proportions, and relative quantity of certain HMW-GS in the first experiment when the total N fertilization rate was low to medium (from 1.5 to 2 g N pot<sup>-1</sup>). However, the effects of late N fertilization were less obvious under higher N fertilization conditions in the second experiment (from 2 to 2.5 g N pot<sup>-1</sup>). Since the total N fertilization rate was not high in the first experiment, it could be speculated that the N fertilizer applied at early stages was all taken up by the plant before anthesis, while there was still plant N uptake in the split N application treatment. Therefore, this lately uptake of N was more directly used for grain protein synthesis, affecting not only protein quantity but also the N partitioning in the final grain (further discussed below). However, although 2.5 g N pot<sup>-1</sup> was given at early growth stages in the second experiment (treatment N6), it might not be taken up completely before anthesis. Therefore there might still have been plant N uptake in both treatments N6 and late N fertilization treatments after anthesis. Consequently, the effect of split N application was not observed in the second experiment. Based on these results, the discussion of the present study was mainly focused on the first experiment at the N fertilization rate between 1.5 and 2 g N pot<sup>-1</sup>.

In agreement with other studies (Blandino et al., 2015; Wieser and Seilmeier, 1998), our results confirmed that the late N fertilization was an effective measure in increasing grain protein concentration (Table 2). The results also clearly indicated that this effect was mainly due to the increased N fertilization rate rather than split N application since the plant N uptake (Table 1) was only increased by higher N fertilization rate, thus more N was available for grain protein synthesis. The fact that grain protein concentration was increased by higher N fertilization rate was already described in many studies (Garrido-Lestache et al., 2005; Wieser and Seilmeier, 1998). Therefore, with respect to the inconsistent results describing the effect of split N application on grain protein concentration (Fuertes-Mendizábal et al., 2010; Garrido-Lestache et al., 2004, 2005; Schulz et al., 2015), it might be explained by variations in plant N uptake.



Since different climatic conditions varying in total precipitation or its distribution would have affected the fertilizer N availability in the soil and thus plant N uptake, which led to the same variations as caused by different N fertilization rate.

With the increase in protein concentration, the gliadin and glutenin concentrations as well as their ratio were also increased (Table 2). This was in agreement with other studies (Martre et al., 2006; Triboï et al., 2003; Wieser and Seilmeier, 1998). Besides, in agreement with Jia et al. (1996), increasing N supply led to an increase of gliadin proportion, while that of glutenins was not changed. However, split N application further increased gliadin and glutenin concentrations and proportions at the same N fertilization rate (Table 2). In contrast to literatures describing that the process of N partitioning in the grain was not significantly altered by environmental conditions and depended mostly on the grain protein content (Triboï et al., 2003), our results showed that split N application affected N partitioning in the grain, increasing mainly gliadin as well as glutenin proportions (Table 2). One possible explanation for this split N application effect may due to the two sources of amino acids exported to the phloem for protein synthesis in the grain. One export pool that supplies amino acids to the phloem is fed by the recently reduced nitrate-N and depends on the N uptake by the plant. A second storage pool is where amino acids accumulate when N is not limiting, and are slowly released after the export pool is depleted (Barneix, 2007). The amino acids more readily available for export to the phloem are those that have been synthesized from the reduction of the recently absorbed nitrate-N (Yoneyama and Takeba, 1984). It can be speculated from our results that the corresponding quantitative reduction of non-storage protein fractions at the expense of increased gliadins and glutenins existed, although some studies described that the quantity of structural/metabolic proteins were scarcely influenced by N nutrition (Triboï et al., 2003). Nevertheless, the proportional changes of gliadin and glutenin fractions by split N application in our study were relatively small compared to the broad ranges that have been reported and used for modelling (Martre et al., 2006). However, it had a decisive effect on baking quality (Fig. 2), and therefore needs to be acknowledged.

Bread wheat cultivars express between three and five HMW-GS. Although HMW-GS account for only up to 12% of entire grain protein in wheat, 45-70% of the variation in baking quality was supposed to be determined by these glutenin subunits within European wheats (Shewry et al., 2001a). Both increases (Wieser and Seilmeier, 1998) and decreases (Pechanek et al., 1997) in the proportions of HMW-GS have been reported in response to N application. However, in contrast to literatures describing that the relative proportions of each HMW-GS remained constant regardless of the increment of N fertilization rate or its splitting (Fuertes-Mendizábal et al., 2010), our results showed that the relative quantities of certain HMW-GS were increased by late N fertilization (Table 4). Particularly, the relative quantity of the same HMW-GS (subunit 7) in both cultivars was increased by late N fertilization only through the effect of split N application. Generally, the responses of protein subunits to N fertilization are related to the proportion of the sulphur (S)-containing amino acids in the different gluten protein classes. The proportions of low S-proteins ( $\omega$ -gliadins) and low to medium S-proteins (such as HMW-GS) increased and those high S-proteins (such as LMW-GS) remained the same or decreased with N fertilization (Daniel and Triboi, 2000; Shewry et al., 2001b). Therefore, it can be speculated that split N application might promote the relative availability of N compared to S, resulting in the proportional increases of certain HMW-GS.

Based on our results from both experiments, it is suggested that the late N fertilization, especially split N application can be crucial in improving baking quality of wheat flours. Although these effects might be highly affected or even hidden by environmental factors such as total N fertilization rate and variations from years and locations, it should not be easily considered to abandon the late N application in order to produce high quality wheat.

#### 2.4.2 Protein and baking quality of wheat flour as affected by N forms

In the present study, the late application of N was taken up in nitrate-N form in treatment N3 and ammonium-N form in treatment N4. It was hypothesized that the process of amino acid synthesis from ammonium-fed wheat would be enhanced, thus promoting protein accumulation in the final grain. However, results from our study showed that nitrate-N had greater effects than ammonium-N in improving grain protein quality such as gliadin and glutenin

concentrations and proportions (Table 2) and baking quality (Fig. 2). The differences between nitrate-N and ammonium-N on grain protein composition and baking quality mainly resulted from the uptake of late N fertilizer, since the late N recovery from late nitrate application was significantly higher than that from late urea application (Table 1). The SPAD values from the second experiment also showed that the late N fertilizer in nitrate form had slightly higher SPAD values in the flag leaves than that of urea during grain filling (data not shown). However, our results were in contrast to a recent study by Fuertes-Mendizábal et al. (2013) who reported that ammonium-N as sole N source improved wheat grain quality compared to a nitrate nutrition when split into two and especially three doses. In that study, the N source ( $\text{NO}_3^-$  or  $\text{NH}_4^+$ ) was varied during the whole growth period of wheat. The different results to our study might therefore arise from the fact that no N storage in vacuoles occurred in ammonium-fed plants, therefore the second and third application of ammonium-N was more efficiently used for plant growth and protein synthesis than in nitrate-fed plants, as we originally expected in our hypothesis. Another possible explanation was that the plant N uptake in nitrate-fed plants might be lower than in ammonium-fed plants due to N loss such as nitrate-N leaching. However, the plant N uptake was not determined in that study.

#### 2.4.3 Cultivar differences in baking quality as affected by late N fertilization

In terms of cultivar responses to late N fertilization, the improvement of loaf volume in JB Asano (from 29.5 to 32.3 mL) was greater than that in Tobak (from 31.3 to 32.6 mL) (Fig. 2). Based on the quality parameters according to the German Federal Office of Plant Varieties, JB Asano is a cultivar that usually contains higher protein concentrations at optimum baking quality as compared to Tobak. It can be assumed that JB Asano might be more dependent on grain protein concentration to achieve a certain baking quality. Therefore, the bread volume of JB Asano was eventually increased to a higher extent with the improvement in protein concentration and composition compared to Tobak. These results indicated that measures for enhancing grain protein concentration and composition might be less necessary for cultivars such as Tobak in order to get optimum baking quality. A possible reason for this might be the mostly higher glutenin proportions in the flour of Tobak compared to JB Asano (Table 2).

#### 2.4.4 Meaningful parameters in evaluating wheat flour baking quality

Proteins (mainly protein concentration) are generally recognized to be positively correlated to the bread-making quality within one cultivar (Delcour et al., 2012). However, Johansson and Svensson (1998) investigated two spring and two winter wheat cultivars during 21 years and found that although a positive relationship existed between grain protein concentration and bread volume, the coefficient of correlation was lower than 0.57, and grain protein concentration could only explain 19% of the variation during the investigated years. In the present study, we also demonstrated that protein concentration was not an appropriate parameter in evaluating baking quality of wheat flour since higher N rate increased protein concentration in both wheat cultivars while did not lead to higher bread volume (Table 2 and Fig. 2). It has already been reported that although HMW-GS are minor components of gluten in quantitative terms, they are key factors that determine the gluten strength to a great extent and improve baking quality (Shewry et al., 2001a). Besides, it was demonstrated that among HMW-GS, x-type subunits had higher contributions to bread-making quality than y-type subunits (Wieser and Kieffer, 2001). In the present study, x-type HMW-GS subunit 7 present in both wheat cultivars was the most abundant HMW-GS in Tobak (accounting for 36% of the HMW-GS) and second abundant in JB Asano (accounting for 36% of the HMW-GS) (Table 4). Consequently, the relative change of this subunit may exert great influences on baking quality, as was observed in our study.

In this study, grain protein concentration alone was not suitable in evaluating the effect of split N application on baking quality (loaf volume) of wheat flour. However, the proportion of the glutenins and especially the abundance HMW-GS 7 were assumed to play more important roles than other parameters in determining wheat flour baking quality. Therefore, just using grain protein concentration to predict baking quality of the resulting wheat flour from fertilization management in scientific studies might be inappropriate, especially for split N treatments. For industrial purposes, grain protein concentration might still be a reasonable parameter to evaluate the baking quality of wheat flours because the quantitation of glutenin and HMW-GS is inconvenient and time-consuming.

## **2.5 Conclusions**

Late N fertilization has a significant effect in improving baking quality (loaf volume) of wheat flour. This quality improvement is only due to the effect of split N application. Split N application can change the N partitioning in wheat grain by increasing gliadin and glutenin proportions, which is decisive for baking quality. Therefore, although this effect of split N application might be blurred by total N rate effect or hidden by other environmental factors, the practice of split N application should not be abandoned without considering these factors. Grain protein concentration alone is insufficient for evaluating late N effects on baking quality of wheat flour. The proportions of glutenin and HMW-GS seem to be better correlated with baking quality in these cases.

## **Acknowledgements**

We appreciate the help and excellent technical assistance from Stephanie thor Straten, Stefan Becker-Fazekas, Bärbel Biegler, Dr. Reiner Krähmer, and Katharina Schiesser. Cheng Xue gratefully acknowledges the financial support provided by the China Scholarship Council.

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## Tables and figures

**Table 1** Grain yield, plant N uptake, N and late N harvest index and late N recovery as affected by N treatments. Different letters in the same column within the same experiment represent significant differences of mean values at  $P < 0.05$ .

Treatment <sup>a</sup>	Grain yield (g pot <sup>-1</sup> )		Plant N uptake (g N pot <sup>-1</sup> )		N harvest index (%)		Late N harvest index (%)		Late N recovery (%)		
	Tobak	JB Asano	Tobak	JB Asano	Tobak	JB Asano	Tobak	JB Asano	Tobak	JB Asano	
<b>Exp. 1</b>	<b>N1 (1+0.5+0)</b>	64.4 b	64.3 b	1.45 b	1.46 b	63.6 b	64.0 b				
	<b>N2 (1+1+0)</b>	74.5 a	79.5 a	1.81 a	1.84 a	63.7 b	66.0 ab				
	<b>N3 (1+0.5+0.5N)</b>	75.5 a	75.0 a	1.80 a	1.84 a	67.6 a	68.0 a	85.2 a	86.0 a	95.6 a	98.1 a
	<b>N4 (1+0.5+0.5U)</b>	72.7 a	77.6 a	1.73 a	1.85 a	65.7 ab	66.4 ab	84.5 a	84.2 a	81.6 b	79.6 b
<b>Exp. 2</b>	<b>N5 (1+1+0)</b>	89.0 b	92.2 b	2.03 b	2.00 b	64.4	62.9 c				
	<b>N6 (1+1.5+0)</b>	106.1 a	112.6 a	2.47 a	2.52 a	67.1	65.4 b				
	<b>N7 (1+1+0.5N)</b>	111.1 a	113.9 a	2.54 a	2.62 a	67.8	67.6 a	83.8 a	80.8 a	89.4 a	95.2 a
	<b>N8 (1+1+0.5U)</b>	101.5 a	108.0 a	2.45 a	2.48 a	67.1	67.2 ab	83.3 a	80.1 a	88.1 a	83.6 b

<sup>a</sup> In the first experiment, N1 (1+0.5+0) represents treatment N1, with N fertilization rate and timing at 1 g N pot<sup>-1</sup> before sowing, 0.5 g N pot<sup>-1</sup> at EC30 (stem elongation), and 0 g N pot<sup>-1</sup> at EC45 (late boot stage). The N fertilization rate and timing in treatment N2, N3 and N4 were done in the same way as N1. In the second experiment, N5 (1+1+0) represents treatment N5, with N fertilization rate and timing at 1 g N pot<sup>-1</sup> before sowing, 1 g N pot<sup>-1</sup> at EC30, and 0 g N pot<sup>-1</sup> at EC51 (beginning of heading). The N fertilization rate and timing in treatment N6, N7 and N8 were done in the same way as N5. The expression ‘0.5N’ means that 0.5 g N pot<sup>-1</sup> was given as calcium nitrate, ‘0.5U’ means that 0.5 g N pot<sup>-1</sup> was given as urea plus nitrification inhibitor DCD.

**Table 2** Protein concentration and composition as affected by N treatments. Different letters in the same column within the same experiment represent significant differences of mean values at  $P < 0.05$ . Treatments N1 to N8 represent the corresponding treatments as described in Table 1.

Treatment	Protein concentration (mg g <sup>-1</sup> flour)		Protein yield (g pot <sup>-1</sup> )		Gliadin (mg g <sup>-1</sup> flour)		Glutenin (mg g <sup>-1</sup> flour)		Gliadin proportion (%) <sup>a</sup>		Glutenin proportion (%) <sup>a</sup>		Gliadin/Glutenin		Gluten (mg g <sup>-1</sup> flour)		
	Tobak	JB Asano	Tobak	JB Asano	Tobak	JB Asano	Tobak	JB Asano	Tobak	JB Asano	Tobak	JB Asano	Tobak	JB Asano	Tobak	JB Asano	
Exp. 1	N1	81.7 b	82.7 c	5.3 b	5.3 b	12.7 c	12.5 d	19.4 c	18.1 c	15.5 c	15.1 c	23.7 c	21.9 c	0.65 b	0.69 b	32.0 d	30.6 d
	N2	88.4 a	87.0 b	6.6 a	6.9 a	15.8 b	15.5 c	22.7 b	19.8 c	17.9 b	17.8 b	25.7 bc	22.7 bc	0.70 a	0.78 a	38.5 c	35.2 c
	N3	91.9 a	94.9 a	6.9 a	7.1 a	19.4 a	20.4 a	27.3 a	25.7 a	21.1 a	21.5 a	29.8 a	27.1 a	0.71 a	0.79 a	46.7 a	46.2 a
	N4	89.1 a	90.3 b	6.5 a	7.0 a	17.7 a	18.0 b	24.5 b	22.0 b	20.0 a	19.9 a	27.5 ab	24.3 b	0.72 a	0.82 a	42.3 b	39.9 b
Exp. 2	N5	84.0 a	78.0 b	7.5 b	7.2 b	16.8 a	17.6 a	27.4 b	27.8 a	20.1 a	22.5 a	32.6 a	35.6 a	0.62 a	0.64 a	44.2 b	45.3 a
	N6	89.2 a	83.6 a	9.5 a	9.4 a	18.3 a	18.0 a	31.7 a	29.3 a	20.5 a	21.6 a	35.6 a	35.1 a	0.58 a	0.62 a	50.0 a	47.4 a
	N7	88.5 a	88.6 a	9.8 a	10.1 a	18.6 a	17.1 a	32.0 a	30.0 a	21.1 a	19.3 a	36.3 a	33.9 a	0.58 a	0.58 a	50.6 a	47.1 a
	N8	92.6 a	88.2 a	9.4 a	9.5 a	19.3 a	18.2 a	31.3 a	29.1 a	20.8 a	20.6 a	33.8 a	33.0 a	0.61 a	0.62 a	50.6 a	47.3 a

<sup>a</sup> Gliadin proportion (%) refers to the percentage of gliadins to total grain protein, and was calculated as gliadin (mg g<sup>-1</sup> flour) / total grain protein (mg g<sup>-1</sup> flour) × 100. Glutenin proportion (%) refers to the percentage of glutenins to total grain protein, and was calculated as glutenin (mg g<sup>-1</sup> flour) / total grain protein (mg g<sup>-1</sup> flour) × 100.

**Table 3** Gliadin subunits pattern and their relative quantities (%) of two winter wheat cultivars as affected by N treatments in the first experiment. Different letters in the same row within the same wheat cultivar represent significant differences of mean values at  $P < 0.05$ . Treatments N1 to N4 represent the corresponding treatments as described in Table 1.

Tobak						JB Asano					
Band	MW (kDa)	N1	N2	N3	N4	Band	MW (kDa)	N1	N2	N3	N4
1	60.1	3.6	4.4	4.9	5.1	1	59.9	3.2	3.7	3.8	4.3
2	56.4	1.1	1.3	1.4	1.5	2	58.4	1.2 b	1.2 b	1.9 a	1.3 ab
3	52.9	1.0	1.5	1.5	1.4	3	56.2	1.3	1.7	2.0	2.0
4	49.1	3.0	3.3	3.6	3.7	4	52.8	1.6	1.9	2.2	2.3
5	45.5	1.1	1.1	1.3	1.1	5	48.9	3.1	3.3	3.4	3.6
6	40.1	13.3 a	11.7 ab	11.2 b	11.6 b	6	45.4	1.0	1.2	1.1	1.2
7	38.7	32.8 a	30.3 ab	29.2 b	28.4 b	7	41.8	0.5	0.4	0.5	0.7
8	36.4	1.8	2.3	2.5	2.5	8	39.0	37.3 a	35.3 ab	34.0 b	34.2 b
9	33.1	19.8	19.8	19.6	19.3	9	37.7	6.1	6.6	6.9	6.4
10	30.7	8.9	9.6	9.9	10.0	10	33.5	11.3	11.5	11.6	11.3
11	29.8	5.3	5.9	6.3	7.0	11	32.6	9.8	10.1	10.5	10.2
12	28.5	4.3	4.5	4.1	3.8	12	29.7	11.7	11.4	11.3	11.4
13	27.4	0.3	0.3	0.4	0.4	13	28.1	11.2	10.8	10.3	10.4
14	26.1	3.6	4.0	4.1	4.0	14	25.3	0.7	0.8	0.5	0.7

**Table 4** Glutenin subunits pattern and their relative quantities (%) of two winter wheat cultivars as affected by N treatments in the first experiment. Different letters in the same row within the same cultivar represent significant differences of mean values at  $P < 0.05$ . For band 2 in JB Asano, different letters in the same row represent significant differences of mean values at  $P < 0.1$ . Treatments N1 to N4 represent the corresponding treatments as described in Table 1.

<b>Tobak</b>						<b>JB Asano</b>					
<b>Band</b>	<b>MW (kDa)</b>	<b>N1</b>	<b>N2</b>	<b>N3</b>	<b>N4</b>	<b>Band</b>	<b>MW (kDa)</b>	<b>N1</b>	<b>N2</b>	<b>N3</b>	<b>N4</b>
1	114.5	8.0	7.9	8.5	8.5	1	112.9	9.8	9.9	10.3	10.3
2	96.1	11.9 b	12.0 b	13.1 a	13.0 a	2	96.9	13.5 b	13.5 b	14.3 a	14.2 a
3	79.1	2.9 b	3.1 ab	3.1 ab	3.3 a	3	77.7	14.2	14.6	14.5	14.4
4	76.2	10.6	11.0	11.2	11.5	4	58.6	3.7	3.9	3.8	3.7
5	55.8	3.1 b	3.9 a	3.8 a	3.8 a	5	55.7	5.4	5.8	5.6	5.1
6	46.2	2.4 b	2.7 ab	2.9 a	2.9 a	6	46.3	2.9 b	3.4 ab	3.7 a	3.6 a
7	40.5	24.2	23.6	24.2	24.4	7	43.1	0.6	0.4	0.5	0.5
8	38.8	21.7	21.0	21.4	20.6	8	40.5	18.5	18.2	19.0	19.4
9	33.6	6.3	6.3	5.3	5.7	9	39.1	10.6	10.8	11.3	11.5
10	31.4	1.8	1.9	2.0	2.2	10	38.2	2.2	2.7	2.8	2.6
11	29.2	7.1 a	6.6 a	4.6 b	4.2 b	11	33.7	8.9	8.6	7.6	7.9
						12	31.2	2.1	2.2	2.2	2.2
						13	29.9	3.0	2.7	2.2	2.5
						14	28.6	4.6 a	3.2 ab	2.2 b	2.1 b
<b>HMW-GS / LMW-GS</b>		0.50	0.52	0.56	0.57	<b>HMW-GS / LMW-GS</b>		0.60	0.61	0.64	0.64

### **Figure captions**

**Figure 1** Effects of N treatments on protein subunits patterns of gliadin (A) and glutenin (B) fractions separated by SDS-PAGE for the two winter wheat cultivars Tobak and JB Asano. Treatments N1 to N4 represent the corresponding treatments as described in Table 1. The Right Arrow (→) and the Left Arrow (←) indicate the bands that showed relative changes after different N treatments in Tobak and JB Asano, respectively.

**Figure 2** Bread volume of two winter wheat cultivars as affected by N treatments in the first experiment. Error bars represent  $\pm$  standard errors of five independent pot replicates. Statistical significance ( $P < 0.05$ ) is indicated by small letters for Tobak and capitals for JB Asano. Treatments N1 to N4 represent the corresponding treatments as described in Table 1.

Figure 1

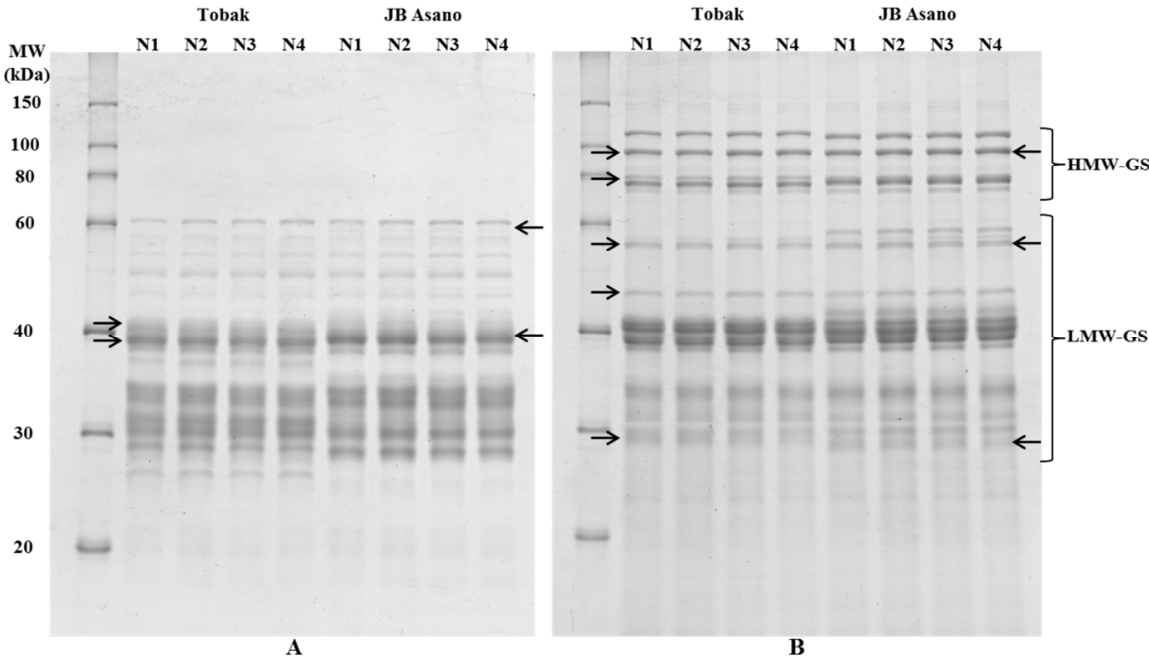
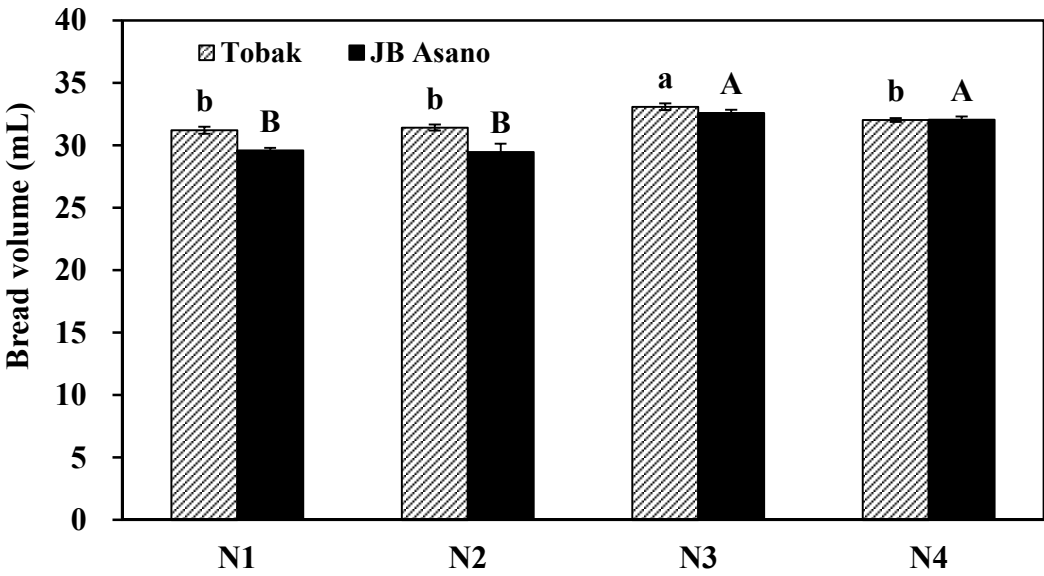


Figure 2



## **Chapter 3**

**Late nitrogen application increases protein concentration but not  
baking quality of wheat**

(In preparation)

### **3. Late nitrogen application increases protein concentration but not baking quality of wheat**

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Key words: Split N application, late N fertilization, gliadin, glutenin, HMW-GS, baking quality



## **Abstract**

Applying late nitrogen (N) fertilizers at heading or anthesis is usually performed to produce wheat with high bread-making quality. However, increasing energy costs and ecological problems due to N losses call for efficient and simplified N fertilization strategies. This study aimed to investigate the effect of late N fertilization on grain protein quality and thus baking quality and to evaluate if similar wheat quality can be maintained without late N application. Field experiments with two winter wheat cultivars differing in quality groups were conducted. The fertilization treatments comprised a rate of 220 kg N ha<sup>-1</sup> applied in two doses or three doses (split N application), and 260 kg N ha<sup>-1</sup> applied in four doses (late N fertilization) with N fertilizer type management. Results showed that although split N application had no effect on grain protein concentration (GPC), it affected N partitioning in the grain, increasing mainly the concentration and proportion of the glutenin fraction. As a result, baking quality was improved by split N application. Late N fertilization enhanced GPC and the relative abundance of certain HMW-GS, however, it had no effect on N partitioning in the grain and did not further improve baking quality. No obvious differences were found between N fertilizer types on grain yield and quality. The N fertilization effect was more remarkable on the wheat cultivar whose baking quality was more dependent on protein concentration. In evaluating baking quality of wheat flour, gliadin and glutenin proportions were better correlated with loaf volume than the overall protein concentration.

### **3.1 Introduction**

Baking quality of wheat is mainly determined by grain protein concentration (GPC) and protein composition. In fact, the quality of wheat is ranked according to the GPC, with increasing GPC resulting in higher water absorption capacity, more extensible and less tenacious dough and eventually larger loaf volumes (Osman et al., 2012). Gluten proteins are considered crucial in determining dough property with gliadins being responsible for viscosity and extensibility, while glutenins provide strength and elasticity (Wieser, 2007). The GPC, quantities and proportions of gliadins and glutenins and their subunits are all important determinants of bread-making quality and highly influenced by genotype, environment, fertilization and their interactions in both direct and indirect ways (Delcour et al., 2012; Osman et al., 2012; Shewry and Halford, 2002). In contrast to climatic conditions, fertilization, particularly nitrogen (N) fertilization can be more easily handled in quality wheat production.

In wheat production, N fertilizer application is usually split into three doses during early and mid-vegetative growth stages to ensure mainly grain yield. This normal practice is defined as split N application. Moreover, in order to increase GPC and achieve optimal baking quality of wheat, the late N fertilization (applying additional N at heading or even anthesis on top of the split N application) is widely considered as useful and is practically conducted in wheat production as well (Olson and Kurtz, 1982). It was reported that N fertilizer applied at late stages comparing to early stages favours protein build-up in the grain over an increased yield (Sowers et al., 1994). However, it was also shown that the effects of late N fertilization varied between different environmental conditions such as locations and total N fertilization rate (Jia et al., 1996). Therefore, it can be speculated that the late N fertilization might be less effective when soil available N was sufficient or under dry conditions where late N might not be taken up efficiently by plants. Besides, taking into account the practical problems and extra costs of applying N fertilizers at these late stages, further investigations are needed on whether the application of a late N is necessary. Comparing to late N fertilization, less N fertilizer input can be used by split N application, therefore increasing N fertilizer use efficiency and minimizing risks of N leaching and gaseous losses. However, the effects of split N application on wheat

quality varied among studies (Ayoub et al., 1994; Fuertes-Mendizábal et al., 2010; Garrido-Lestache et al., 2004, 2005). These inconsistent results were probably mainly attributed to the N rate effect since different climatic conditions resulted in variations in plant N uptake. Therefore, it is hypothesized that the effects of split N application and late N fertilization on grain protein concentration and composition were actually due to N fertilization rate, and not to N splitting. If this is true, the late N fertilizer could be applied earlier under conditions with enough precipitation, thus reducing the overall N application times. However, to evaluate the effects of split N application or late N fertilization, plant N uptake should be considered. Unfortunately, limited results about this issue are available.

Urea and (calcium) ammonium nitrate (CAN or AN) are often used as N fertilizers in wheat production. It was reported that urea was less effective comparing to CAN on grain yield and protein synthesis especially when precipitation was scarce (Gately, 1994). Ammonia volatilization and ammonium immobilization after urea hydrolysis were regarded as the possible causes for urea inefficiency. However, the problem of N leaching and gaseous losses during denitrification were also often observed from nitrate-N fertilizers when soil water content was in excess. Besides, when plants take up N mainly in the form of ammonium instead of the usually dominating nitrate, the phytohormone production, assimilates distribution and intensity of different organs growth could be affected (Sommer and Scherer, 2009). According to the study from Fuertes-Mendizábal et al. (2013), the application of ammonium as sole N source improved bread-making quality of wheat comparing to nitrate-N. However, Schulz et al. (2015) observed no differences between CAN and urea as N fertilizer on wheat flour quality in terms of GPC. Nevertheless, N fertilizer types should be considered when investigating N fertilization on wheat quality under natural conditions.

Bread-making quality of different cultivars may respond differently to protein concentration variation through N management (Weegels et al., 1996). For cultivars which can reach high baking quality with relatively low GPC, measures to increase GPC might be dispensable. Therefore, to investigate the effect of split N application and late N fertilization more comprehensively, cultivars having similar baking quality while differing in GPC should be

considered. Furthermore, the widespread assumption between GPC enhancement and baking quality improvement was questioned due to the high variations between these two parameters, especially when modern cultivars were included into the study (Koppel and Ingver, 2010; Timms et al., 1981). Therefore, to avoid evaluating baking quality of wheat flour incorrectly, loaf volume should be determined by baking test. Unfortunately, very limited studies have conducted a baking test for wheat flour due to the lack of materials or equipment.

Previously, two pot experiments regarding late N fertilization influences on protein and quality of two wheat cultivars were conducted (Chapter 2). Results showed that late N application at late boot stage improved loaf volume of wheat flour mainly through the effect of N splitting on the proportions of gliadins and glutenins (% total protein) and the relative abundance of certain HMW-GS. The quantitative increases in grain protein and its fractions were mainly through the effect of increased N fertilization rate, which did not lead to improvement in loaf volume.

To verify whether our results from pot experiments are applicable to field conditions, field experiments with the same cultivars and a similar experimental design were conducted in the present study with the main following objectives: (i) to evaluate the effects of split N application and late N fertilization on the quantity and composition of gluten protein and thus bread-making quality (loaf volume), (ii) to determine how different N fertilizer types affect the protein and bread-making quality, (iii) to determine how cultivars belonging to different quality groups respond to split N application and late N fertilization, (iv) to check if GPC is a reliable parameter in evaluating baking quality under such conditions.

### **3.2 Materials and Methods**

#### **Study sites**

The investigation was performed at two locations, Futterkamp (2011-2012 and 2012-2013) and Sönke-Nissen-Koog (SNK) (2011-2012), Northern Germany. At Futterkamp (54°17' N, 10°38' E, and 4 m above sea level), the soil type was sandy loam with the annual precipitation of 706 mm and average temperature of 9 °C. At SNK (54°37' N, 8°52' E, 0 m in altitude), the soil type was clay, the annual precipitation and average temperature were 833 mm and 8.7 °C, respectively.

## Weather conditions

The monthly precipitations and temperatures during experimental years and the mean values during the last three decades (1981-2010) at Futterkamp and SNK were shown in Fig. 1. The annual precipitation for Futterkamp was 662 mm and 705 mm in the year of 2011-2012 and 2012-2013, respectively (Fig. 1A). Comparing to the 30-year average (706 mm), both experimental years at Futterkamp could be regarded as normal years. However, the precipitations during the key period (May, June and July) of wheat growth were 230 mm and 252 mm in 2011-2012 and 2012-2013, respectively, which were higher than that in the 30-year average (188 mm). At SNK (Fig. 1B), the annual precipitation in 2011-2012 was 684 mm, which was much lower than the 30-year average (833 mm). Therefore, the experimental year at SNK was a dry year. However, the precipitation during May to July was 262 mm, which was much higher than that in the 30-year average (199 mm).

The annual mean temperature at Futterkamp (Fig. 1A) was 8.7 °C in 2011-2012, which was similar to the 30-year average (9.0 °C). In terms of monthly mean temperature, the temperatures in June and August were slightly lower (around 1.5 °C lower) than the corresponding period over the 30-year average. The annual mean temperature in 2012-2013 was 1 °C lower than the 30-year average, which was mainly due to the cold winter during December 2012 to March 2013. At SNK (Fig. 1B), the annual mean temperature in 2011-2012 (8.6 °C) was almost the same comparing to that in the 30-year average (8.7 °C). The monthly mean temperatures in 2011-2012 were comparable to that in the 30-year average as well, except in February 2012, the temperature was 3 °C lower than that in the 30-year average.

## Experimental design

Four N fertilization treatments including split N application, late N fertilization and N type were designed and conducted at both locations as follows: N1 (Simplified fertilization), an N rate of 220 kg N ha<sup>-1</sup> split into two doses at EC21 (Lancashire et al., 1991) and EC30; N2 (Split N application), an N rate of 220 kg N ha<sup>-1</sup> split into three doses at EC21, EC30 and EC41; N3 (Late N fertilization - CAN), an N rate of 260 kg N ha<sup>-1</sup> split into four doses at EC21, EC30, EC41 and EC51 in the form of calcium ammonium nitrate; N4 (Late N fertilization - urea), as

N3, but in the form of urea. Detailed information on N fertilizer type and N rate for each application was summarized in Table 1. Each treatment was replicated four times in a completely randomized block design, with each plot being 15 m<sup>2</sup> and 10.15 m<sup>2</sup> at Futterkamp and SNK, respectively. At both sites, winter wheat was sown in the end of September or the beginning of October at a seeding rate of 300 and 425 seeds m<sup>-2</sup> in Futterkamp and SNK, respectively, and harvested in the middle of August in the following year. Winter wheat was grown under rain-fed conditions without any supplemental irrigation.

Two winter wheat cultivars Tobak and JB Asano were used in this study. Both were chosen because these cultivars belong to different baking quality classes according to the German Federal Office of Plant Varieties based on the GPC level. Tobak (class B) had lower raw protein concentration while similar loaf volume comparing to JB Asano (class A). Besides, according to ‘Glu-1 quality score’ (Payne et al., 1987) based on HMW-GS, the ‘Glu-1 quality score’ is five and seven for Tobak and JB Asano, respectively.

#### Harvests, yield and nitrogen determination

At Futterkamp, a hand-harvest was performed shortly before machine-harvest of the plots. Wheat plants were cut just above soil-level from a representative area (4 rows, 0.5 m along each row) in each plot. Each plant was separated into flag leaves, rest straw, glumes and grains. Samples were oven-dried at 60 °C, milled and analysed for N by a CNS elemental analyser (Flash EA 1112 NCS, Thermo Fisher Scientific, Waltham, MA, USA). Raw protein concentration of wheat flour was calculated by multiplying the N concentration by 5.7. A sub-sample of the grains was air-dried and used for further protein analysis in 2013. At both locations and years, plots were also harvested by a plot-thresher. After harvest, wheat grains from the four replicates of each treatment and cultivar were well mixed and one average sample was taken from the well mixed materials for protein and baking quality analyses. Grains were air-dried and milled in a Titan laminated mill using a 500 µm sieve (Retsch, Haan, Germany) prior to analyses.

#### Extraction and quantification of storage protein fractions

Extraction of wholemeal flour storage protein fractions was performed stepwise according to Wieser and Seilmeier (1998) and Zörb et al. (2010) with some modifications (Chapter 2). First, albumins and globulins were extracted from 100 mg wholemeal flour with extraction buffer (0.4 mol L<sup>-1</sup> NaCl, 67 mmol L<sup>-1</sup> Na<sub>2</sub>HPO<sub>4</sub>, 7.7 mmol L<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, pH 7.6). The pellet was then extracted with 60% ethanol to obtain the gliadin fraction. The pellet was washed with 1 mL of deionized water. Afterwards, the glutenin fraction was extracted with extraction buffer [50% 1-propanol (v/v), 2 mol L<sup>-1</sup> urea, 1% DTT, 0.05 mol L<sup>-1</sup> Tris-HCl, pH 7.5]. The protein was precipitated by DTT/Acetone solution (50 mmol L<sup>-1</sup> DTT), dissolved in another buffer [8 mol L<sup>-1</sup> urea (serdolit washed), 2 mol L<sup>-1</sup> thiourea (serdolit washed), 4% CHAPS, 30 mmol L<sup>-1</sup> DTT, 20 mmol L<sup>-1</sup> Tris base] and stored at -20 °C for further use.

The protein concentration of each fraction was determined using 2D Quant-kit (GE-Healthcare, Munich, Germany).

#### Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Gliadin and glutenin subunit compositions were detected by SDS-PAGE performed according to the method of Laemmli (1970) with some modifications (Chapter 2). Medium sized gels (18 cm × 16 cm glass plates and 1.5 mm spacers in thickness, 12.5% separating gels and 6.3% stacking gels) were casted one day before use. Protein extracts were mixed with the same volume of sample buffer (10% glycerol, 2.3% SDS, 5% 2-mercaptoethanol, 0.0025% bromophenol blue, 63 mmol L<sup>-1</sup> Tris base, pH 6.8) and a sample containing 20 µg protein was loaded per well. A molecular weight standard 'Rotimark 10-150' (RotiMark, Roth, Crailsheim, Germany) was loaded on one lane of each gel. Two gels were run simultaneously in the same vertical electrophoretic unit (SE600, Hoefer). The gels were then fixed (40% ethanol, 10% acetic acid), stained (1 tablet 'PhastGel<sup>TM</sup> Blue R' in 1.6 L 10% acetic acid) and destained (10% acetic acid). Gels were digitized by scanning on an image scanner (Epson Perfection V700) at 300 dpi and 16 bits per pixel.

#### Image analysis

Gel electrophoresis images of gliadin and glutenin were analysed using the Quantity One software (Bio-Rad, Hercules, CA, USA). Bands on each gel were determined and matched with

molecular weight markers to calculate the molecular weight of each protein band. The average band intensity and relative quantity of each band were then determined.

### Baking test

The baking test was performed according to Thanhaeuser et al. (2014) with some modifications using wholemeal flour. After grinding, wheat flours were rested for at least 14 days before being used for baking. The moisture of each sample was measured using infrared moisture analyser (MA35, Sartorius). Then, for each sample the water absorption and dough development time were determined by mixing 10 g flour, 0.2 g NaCl and water at 22 °C for 20 min in a micro-farinograph (Brabender, Duisburg, Germany) according to the ICC-Standard method 115/1 with modifications. Afterwards, the baking test was performed. For each sample, 50 g flour, 3.5 g fresh yeast (Wieninger, Passau), 1 g NaCl, 0.5 g sucrose, 0.5 g coconut fat, 1.5 mL L-ascorbic acid solution (0.67 g L<sup>-1</sup>) and water were mixed in the farinograph for 4-12 minutes according to the dough development time. Then the dough was weighed and placed in a proofer for 20 min (30 °C, water-saturated atmosphere). After rounding and weighing again, the dough was fermented 20 min on the baking pan at 30 °C in a water-saturated atmosphere. The dough was then baked for 20 min at 230 °C.

The bread was weighed immediately and again at 1 h after baking. The bread volume was measured using Volscan Profiler 600 (Stable Micro Systems, Godalming, UK).

### Statistical analysis

The statistical software R (2014) was used to evaluate the data. The data evaluation started with the definition of an appropriate statistical mixed model. The data were assumed to be normally distributed and to be homoscedastic. These assumptions were based on a graphical residual analysis. The statistical model included treatment and cultivar, as well as one random factor (year and location). Based on this model, an analysis of variances (ANOVA) was conducted, followed by multiple contrast tests (Tukey's least significant test at  $P < 0.05$  level) in order to compare the several levels of the influence factors, respectively.

## 3.3 Results



Wheat grain yield and protein concentration varied between two experimental sites (data not shown). For both cultivars, wheat grown at Futterkamp had higher grain yield (98-114 dt ha<sup>-1</sup>) than that at SNK (80-96 dt ha<sup>-1</sup>), whereas variations in GPC were lower, ranging from 11-13% at Futterkamp and 10-13% at SNK. Nevertheless, the grain yield, protein concentration and its composition as well as baking quality responded in the same way to different N treatments for both wheat cultivars at both experimental sites.

### 3.3.1 Grain yield, plant N uptake and N use efficiency

The plant N uptake and protein yield of both winter wheat cultivars were mainly enhanced with the increased N fertilization rate from 220 to 260 kg N ha<sup>-1</sup> by late N fertilization (Table 2). Late N fertilization only showed a trend to increased grain yield. Split N application (N2 vs N1) and N fertilizer types (N3 vs N4) had no significant effects on these parameters. Furthermore, the application of urea compared to CAN (N4 vs N3) showed a tendency to lower the grain yield, plant N uptake and protein yield in Tobak, while no such effect was detected in JB Asano. Nitrogen use efficiency (NUE) was decreased with the increase in N fertilization rate in both cultivars. However, N fertilization managements had no influence on nitrogen harvest index (NHI) in both cultivars.

### 3.3.2 Grain protein concentration and composition

Wheat GPC was mainly increased by late N fertilization (Table 3). Split N application (N2 vs N1) and N fertilizer types had no significant influences on GPC. The gliadin concentration was enhanced with the increase in GPC by late N fertilization in Tobak, while was not significantly changed in JB Asano (Table 3). However, the glutenin concentration was increased by split N application in both cultivars to a higher extend (from around 33 to 40 mg g<sup>-1</sup> flour) and was further increased by late N fertilization only in Tobak (from 38 to 41 mg g<sup>-1</sup> flour) (Table 3). The total gluten concentration (gliadin + glutenin) was increased by late N fertilization in Tobak while by split N application in JB Asano. The gliadin, glutenin and gluten concentrations were not affected by N fertilizer types.

In terms of protein composition, gliadin and glutenin proportions were only enhanced by split N application except the gliadin proportion in Tobak, which was not changed (Table 3). However,

late N fertilization did not affect the gliadin and glutenin proportions. The gliadin/glutenin ratio in both cultivars was not affected by N fertilization management. Besides, N fertilizer types had no effect on grain protein composition.

### 3.3.3 Gluten subunit pattern

Gliadin and glutenin fractions were further separated by SDS-PAGE and the protein band patterns of both wheat cultivars were shown, respectively (Fig. 2A and 2B). For gliadin fraction, 14 protein bands were detected and their relative quantities were determined for Tobak [molecular weight (MW) ranging from 26 kDa to 61 kDa] and JB Asano (MW ranging from 25 kDa to 61 kDa), respectively (Table 4). However, no changes of band relative quantity were found in both cultivars as affected by split N application or late N fertilization.

For the glutenin fraction, 11 protein bands were detected in Tobak (MW between 29 kDa and 115 kDa) and 14 protein bands in JB Asano (MW ranging from 28 kDa to 113 kDa), respectively (Table 5). In Tobak, the relative quantities of two HMW-GS and one LMW-GS were changed by N fertilization treatments. Band 2 (MW 96.5 kDa, HMW-GS 7) was relatively enhanced by late N fertilization in CAN (N3), and less so by split N and late N fertilization in urea. Moreover, the relative quantity of band 4 (MW 76.2, HMW-GS 12) was only increased by late N fertilization in CAN comparing to other treatments. However, band 8 (MW 37.2 kDa, LMW-GS) was relatively decreased by late N fertilization. In JB Asano, only one LMW-GS (band 9, MW 37.4 kDa) was changed by N fertilization treatments, being relatively decreased by split N application and further decreased by late N fertilization, while differences were only significant between treatment N3 and N1. In terms of HMW-GS/LMW-GS ratio (Table 5), JB Asano had higher HMW-GS/LMW-GS (ranging from 0.64 to 0.68) than Tobak (between 0.51 and 0.62). However, HMW-GS/LMW-GS was not affected by N fertilization treatments within each cultivar.

### 3.3.4 Baking quality

Baking tests were performed and the most direct parameter loaf volume was used to indicate bread-making quality (Fig. 3). The loaf volume was increased by split N application (N2 vs N1) while late N fertilization had no influence on loaf volume in JB Asano. Moreover, by

comparing fertilizer types, CAN had greater effect than urea in JB Asano. For Tobak, no significant difference in loaf volume was detected by N fertilization treatments.

### **3.4 Discussion**

Wheat bread-making quality is highly complex that depends on both genetic and environmental factors. In the present study, variations caused by years, locations and N fertilization managements existed in grain yield, protein concentration and composition and baking quality of wheat flours. The variations between locations mainly lied in grain yield, being around 20 dt ha<sup>-1</sup> higher at Futterkamp comparing to that at SNK in both cultivars. However, the variations in protein concentration and composition between locations were relatively low. At Futterkamp, the differences in all investigated parameters between experimental years were relatively small as well. Furthermore, considering locations and years, all the investigated parameters responded in the same way to N fertilization management. Therefore, N fertilization was the main factor affecting grain protein concentration and composition as well as baking quality of wheat flour in the present study.

#### **3.4.1 Grain protein and baking quality as influenced by late N fertilization and split N application**

In agreement with our previous study (Chapter 2) and many other studies (Blandino et al., 2015; Wieser and Seilmeier, 1998), our results confirmed that late N fertilization was an effective measure in enhancing GPC comparing to split N application (Table 3). This increase in GPC arose from increased total N fertilization rate since the plant N uptake was also enhanced only by late N fertilization (Table 2), thus more N was available for grain protein synthesis. The fact that higher N fertilizer input increases GPC was already described in many studies (Garrido-Lestache et al., 2005; Wieser and Seilmeier, 1998; Zörb et al., 2010). However, baking quality (loaf volume) was only improved by split N application at the same GPC level compared to simplified fertilization, whereas increases in GPC by late N fertilization did not lead to further improvements in baking quality (Table 3 and Fig. 3). This effect of split N application on baking quality mainly resulted from the increased percentage of gluteins to total protein. It has been reported that the process of N partitioning between storage protein fractions

depended mostly on the GPC (Triboï et al., 2003). However, our previous study with pot experiments already showed that split N application affected the N partitioning in the grain by increasing percentages of both gliadins and glutenins to total protein (Chapter 2) and this effect was verified in the present study (Table 3). It might result from the different N sources for grain protein synthesis, which are mainly the mobilization of previously assimilated N present in vegetative tissues before anthesis and the direct uptake and assimilation of N during grain filling. The amino acids that were synthesized from the recently absorbed N seem more readily available to be exported to the phloem to meet the demand of grain protein synthesis than that slowly released from the storage pool (Yoneyama and Takeba, 1984). Moreover, by comparing the effects of late N fertilization and split N application on the proportions of gliadins and glutenins, it seemed that the timing of N application might be crucial in determining N partitioning in the final grain since no further effect was observed when additional N was applied at heading (Table 3). Similar results were also found in our previous study (Chapter 2) that baking quality was improved when the third N dose was applied at late boot stage but not so in a second experiment when it was applied at heading. Therefore, N fertilizer applied at boot stage seems to predominantly benefit gluten protein synthesis over structural/metabolic proteins in the final grain, which was decisive for wheat flour baking quality. Nevertheless, the mechanism behind N partitioning process in the grain needs further investigation.

In terms of protein subunits, HMW-GS were reported to attribute up to 45-70% of the variation in baking quality within European wheat cultivars (Shewry et al., 2001a). Our results showed that the relative abundance of certain HMW-GS could be altered by N fertilization management (Table 5). Particularly, the relative abundance of x-type HMW-GS 7 (band 2) in Tobak was mainly increased by late N fertilization. These results caused by split N application and late N fertilization might arise from the relatively enhanced availability of N as compared to sulphur (S). It was reported that the percentages of low to medium S-containing proteins (e.g. HMW-GS) increased and those S-rich proteins (e.g. LMW-GS) remained the same or decreased with N fertilization or under S deficiency (Hurkman et al., 2013; Shewry et al., 2001b).

Therefore, split N application can improve baking quality (loaf volume) of wheat flour by increasing the proportions of gliadin and glutenin proteins. In comparison, although late N fertilization increased GPC as well as gliadin and glutenin concentrations, it had no effect on the N partitioning in the grain. Therefore, late N fertilization had no significant effect on baking quality of wheat flour in contrast to split N application. Although a trend of increasing grain yield was shown by late N fertilization, NUE was significantly reduced. Therefore, taking into account the extra cost of fertilizer ( $40 \text{ kg N ha}^{-1}$ ) and the energy cost of applying this N dose, the fourth N application should be abandoned at the present conditions.

#### 3.4.2 Nitrogen fertilizer type effects on grain protein and baking quality

Urea and CAN are often used as N fertilizers in wheat production. However, results regarding different N fertilizer types on wheat grain yield, protein and quality varied among studies (Fuertes-Mendizábal et al., 2013; Gately, 1994; Schulz et al., 2015). In this study, generally no significant differences were found between CAN and urea on grain yield and quality related parameters. However, the effects of CAN were greater compared to urea in Tobak (Table 2, 3 and 5). These effects mainly resulted from differences in plant N uptake since it was  $12 \text{ kg N ha}^{-1}$  less in urea comparing to CAN treatment (Table 2). However, since no such difference was found in JB Asano, this difference in plant N uptake could not be attributed simply to N losses from ammonia volatilization or the immobilization of ammonium in the soil from urea treatment as described by Watson et al. (1990). Therefore, the variations in plant N uptake caused by N fertilizer types might be due to the cultivar differences. It can be speculated that Tobak is a cultivar having high N uptake potential mainly during vegetative growth. Therefore, the plant N uptake achieved around  $260 \text{ kg N ha}^{-1}$  when fertilizer N was given no later than EC41 at a rate of  $220 \text{ kg N ha}^{-1}$  (treatment N1 and N2). However, when additional  $40 \text{ kg N ha}^{-1}$  was applied at EC51 (treatment N3 and N4), the plant N uptake enhancement was  $24 \text{ kg N ha}^{-1}$  when applied as CAN, while was only  $12 \text{ kg N ha}^{-1}$  when applied as urea. This was probably due to the delayed N availability caused by the processes of urea hydrolysis and ammonium nitrification. In association with our previous results showing that the late N recovery was lower when N fertilizer was applied in ammonium-N (urea + nitrification inhibitor) comparing

to nitrate-N (Chapter 2), we hereby demonstrate that different N sources (CAN or urea; nitrate-N or ammonium-N) affected protein and quality of wheat mainly through the variations in plant N uptake. Therefore, the N fertilizer type which can better ensure plant N uptake under the given environmental conditions should be recommended in quality wheat production.

### 3.4.3 Cultivar responses to N fertilization management

Generally, both cultivars Tobak and JB Asano responded similarly to N fertilization management in the present study. Besides that cultivar differences might exist in N uptake ability and timing as discussed above, the main cultivar differences lied in grain yield and the extent of quality parameters responding to N fertilization management. Based on cultivar information from the German Federal Office of Plant Varieties, Tobak is a cultivar which has similar baking quality but a lower raw protein content compared to JB Asano. It can be assumed that Tobak might be less dependent on GPC to achieve a certain baking quality. In the present study, the loaf volume was significantly increased by 9% (from 161 to 176 mL) in JB Asano while no significant changes were found in Tobak with the improvement in grain protein concentration and its composition (Fig. 3). These results indicated that in order to achieve optimum baking quality for cultivars such as Tobak, measures in enhancing grain protein concentration and composition might be less necessary.

### 3.4.4 Parameters in evaluating wheat flour baking quality

The GPC of wheat is one of the most important quality indicators for millers and bakers. Market adjustments for wheat have been established worldwide based on protein content, with premiums commonly paid for protein contents above baseline levels (Woolfolk et al., 2002). The major effect of N fertilization on wheat quality is usually exerted throughout changes in GPC. However, as reported by Johansson and Svensson (1998) who found that the coefficient between GPC and loaf volume was lower than 0.57, and GPC could only explain no more than 20% of the variation during a 21-years investigation, which indicated that GPC might not be a reliable parameter in predicting baking quality of the resulting flour. Previously we already found that the changes in loaf volume were inconsistent with variations in GPC in both cultivars of Tobak and JB Asano (Chapter 2). These results were verified in the present study. Therefore,

we demonstrated that the GPC alone was not suitable in evaluating the effect of split N application on baking quality of wheat flour. Moreover, it was reported that HMW-GS, especially x-type HMW-GS were more crucial in determining baking quality (Shewry et al., 2001a; Wieser and Kieffer, 2001). However, although the relative abundance of x-type HMW-GS 7 was increased by late N fertilization in Tobak (N3 vs N1), baking quality was not changed. Therefore, it seemed no single parameter could perfectly explain the variations in baking quality in our study. In relative terms, the gliadin and glutenin proportions correlated better with baking quality in both cultivars (Table 3 and Fig. 3).

Therefore, in scientific studies, where such effort is justified, it is inappropriate just to use GPC to predict baking quality of the resulting wheat flour from fertilization management, especially from split N application. Besides, in order to better explain variations in baking quality, the protein composition (gliadin and glutenin proportions) must be considered, although the determination of these parameters is inconvenient and time-consuming.

### **3.5 Conclusions**

Based on the results from this study we can conclude that the practice of an additional late-N application can be questioned. Nitrogen splitting as already performed for yield increments is sufficient to achieve optimal baking quality. Studies performed on additional locations are needed to verify these results, before this conclusion can be generalized.

### **Acknowledgements**

The authors appreciate the help from Stefan Becker-Fazekas, Bärbel Biegler and from Dr. Mario Hasler regarding statistical advice. We also thank Katharina Schiesser for excellent technical assistance during baking tests. Cheng Xue gratefully acknowledges the financial support provided by the China Scholarship Council.

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## Tables and figures

**Table 1** Overview over fertilization rates, timing of application and fertilizer types used in the field experiments

Treatments	Fertilization time <sup>a</sup>	Fertilizer type <sup>b</sup>	Fertilizer rate (kg N ha <sup>-1</sup> )	Total fertilization rate (kg N ha <sup>-1</sup> )
N1 (Simplified fertilization)	EC21	ASN/Urea	30/70	220
	EC30	Urea	120	
N2 (Split N application)	EC21	ASN/Urea	30/60	220
	EC30	Urea	80	
	EC41	CAN	50	
N3 (Late N fertilization-CAN)	EC21	ASN/CAN	30/60	260
	EC30	CAN	80	
	EC41	CAN	50	
	EC51	CAN	40	
N4 (Late N fertilization-urea)	EC21	ASN/Urea	30/60	260
	EC30	Urea	80	
	EC41	Urea	50	
	EC51	Urea	40	

<sup>a</sup> EC21: beginning of tillering; EC30: beginning of stem elongation; EC41: early boot stage; EC51: beginning of heading

<sup>b</sup> ASN: ammonium sulphate nitrate; CAN: calcium ammonium nitrate

**Table 2** Grain yield, protein concentration and protein yield as affected by N treatments. Different letters in the same column represent significant differences of mean values at  $P < 0.05$ . Treatments N1 to N4 represent the corresponding treatments as described in Table 1.

Treatment	Grain yield (dt ha <sup>-1</sup> )		Plant N uptake (kg N ha <sup>-1</sup> ) <sup>a</sup>		Protein yield (dt ha <sup>-1</sup> )		NUE (kg kg <sup>-1</sup> ) <sup>b</sup>		NHI (%) <sup>a,b</sup>	
	Tobak	JB Asano	Tobak	JB Asano	Tobak	JB Asano	Tobak	JB Asano	Tobak	JB Asano
N1	101.2 b	92.7 b	258.2 b	233.2 b	11.5 c	10.6 b	46.0 a	42.1 a	69.9 a	69.5 a
N2	102.6 ab	95.6 ab	258.8 b	235.0 b	12.1 bc	10.9 b	46.6 a	43.5 a	71.1 a	70.3 a
N3	106.9 a	99.7 a	282.9 a	262.6 a	14.0 a	12.2 a	41.1 b	38.4 b	70.9 a	69.2 a
N4	103.8 ab	98.7 a	270.7 ab	261.5 a	13.2 ab	12.2 a	39.9 b	37.9 b	71.4 a	69.9 a

<sup>a</sup> Data from Futterkamp only. <sup>b</sup> NUE (kg kg<sup>-1</sup>) = grain yield (kg ha<sup>-1</sup>) / N fertilization rate (kg ha<sup>-1</sup>); NHI (%) = N uptake in the grain (kg N ha<sup>-1</sup>) / plant N uptake (kg N ha<sup>-1</sup>) × 100.

**Table 3** Protein concentration and composition as affected by N treatments. Different letters in the same column represent significant differences of mean values at  $P < 0.05$ . Treatments N1 to N4 represent the corresponding treatments as described in Table 1.

Treatment	Protein concentration (%)		Gliadin (mg g <sup>-1</sup> flour)		Glutenin (mg g <sup>-1</sup> flour)		Gliadin proportion (%) <sup>a</sup>		Glutenin proportion (%) <sup>a</sup>		Gliadin/Glutenin		Gluten (mg g <sup>-1</sup> flour)	
	Tobak	JB Asano	Tobak	JB Asano	Tobak	JB Asano	Tobak	JB Asano	Tobak	JB Asano	Tobak	JB Asano	Tobak	JB Asano
N1	11.3 c	11.3 b	19.4 b	19.6 b	33.8 c	33.7 b	17.2 a	17.4 b	30.1 b	30.0 b	0.58 a	0.58 a	53.2 b	53.3 b
N2	11.9 bc	11.5 b	19.7 b	21.9 ab	38.0 b	38.2 a	16.6 a	19.2 a	31.9 a	33.3 a	0.52 a	0.57 a	57.7 b	60.0 a
N3	13.2 a	12.3 a	24.7 a	24.3 a	41.8 a	39.6 a	18.6 a	19.9 a	31.6 a	32.4 a	0.59 a	0.61 a	66.5 a	63.9 a
N4	12.7 ab	12.3 a	23.4 a	23.7 a	41.1 a	39.5 a	18.3 a	19.3 a	32.3 a	32.2 a	0.57 a	0.60 a	64.4 a	63.1 a

<sup>a</sup> Gliadin proportion (%) = gliadin (mg g<sup>-1</sup> flour) / total grain protein (mg g<sup>-1</sup> flour) × 100; Glutenin proportion (%) = glutenin (mg g<sup>-1</sup> flour) / total grain protein (mg g<sup>-1</sup> flour) × 100.

**Table 4** Gliadin subunits pattern, molecular weights (MW) and relative abundance of individual bands (%) as affected by N treatments. Different letters in the same row within the same wheat cultivar represent significant differences of mean values at  $P < 0.05$ . Treatments N1 to N4 represent the corresponding treatments as described in Table 1.

		<b>Tobak</b>				<b>JB Asano</b>					
<b>Band</b>	<b>MW (kDa)</b>	<b>N1</b>	<b>N2</b>	<b>N3</b>	<b>N4</b>	<b>Band</b>	<b>MW (kDa)</b>	<b>N1</b>	<b>N2</b>	<b>N3</b>	<b>N4</b>
1	60.6	3.7	4.3	3.9	4.2	1	60.6	3.5	3.8	3.7	3.7
2	56.2	1.9	2.2	2.3	2.1	2	58.0	2.6	2.8	2.7	2.5
3	51.9	1.0	1.2	1.0	1.1	3	56.4	1.4	1.8	1.7	1.5
4	48.1	1.9	2.2	2.1	2.1	4	52.5	1.4	1.4	1.5	1.6
5	46.0	0.6	0.6	0.6	0.7	5	49.2	1.8	2.0	2.0	2.0
6	39.9	13.4	10.5	11.8	10.6	6	45.9	0.6	0.6	0.4	0.5
7	38.7	29.4	27.8	27.4	28.3	7	42.1	0.9	0.7	0.7	1.0
8	36.4	1.9	2.2	2.4	2.3	8	39.0	34.7	33.7	35.0	34.2
9	32.5	21.1	21.0	20.8	19.3	9	37.7	7.4	7.7	8.4	8.0
10	31.0	8.7	8.5	8.6	7.3	10	32.7	15.8	14.8	15.5	15.2
11	29.9	6.6	8.3	8.6	9.8	11	31.6	8.9	9.1	8.4	9.0
12	28.5	3.8	4.8	4.5	5.9	12	29.8	9.2	9.2	8.8	8.8
13	27.2	0.8	0.9	0.9	1.0	13	27.7	9.7	10.3	9.1	9.8
14	26.3	5.3	5.3	5.2	5.3	14	24.9	2.2	2.3	2.2	2.3

**Table 5** Glutenin subunits pattern, molecular weights (MW) and relative abundance of individual bands (%) as affected by N treatments. Different letters in the same row within the same cultivar represent significant differences of mean values at  $P < 0.05$ . Treatments N1 to N4 represent the corresponding treatments as described in Table 1.

<b>Tobak</b>						<b>JB Asano</b>					
<b>Band</b>	<b>MW (kDa)</b>	<b>N1</b>	<b>N2</b>	<b>N3</b>	<b>N4</b>	<b>Band</b>	<b>MW (kDa)</b>	<b>N1</b>	<b>N2</b>	<b>N3</b>	<b>N4</b>
1	114.6	8.0	7.9	9.2	8.7	1	113.3	9.9	9.9	10.2	10.2
2	96.5	11.5 b	12.2 ab	12.7 a	12.2 ab	2	97.4	13.5	13.1	13.3	13.8
3	79.1	5.4	5.3	6.0	5.9	3	78.0	16.0	15.9	16.9	16.3
4	76.2	8.9 b	8.5 b	10.2 a	9.0 b	4	57.7	3.0	3.4	3.6	3.2
5	54.4	5.3	5.6	5.6	5.5	5	54.4	6.6	6.7	7.2	7.0
6	44.1	2.1	2.0	2.3	2.1	6	44.3	2.1	2.1	2.0	1.8
7	38.7	23.3	23.2	22.1	22.4	7	41.2	0.4	0.4	0.4	0.4
8	37.2	19.0 a	19.2 a	16.6 b	17.5 b	8	38.9	19.0	18.6	18.1	18.6
9	32.3	10.5	10.1	9.7	10.6	9	37.4	8.9 a	8.6 ab	7.7 b	7.9 ab
10	30.5	2.7	2.8	2.5	3.0	10	36.4	3.1	3.1	3.0	2.8
11	28.8	3.2	3.2	3.2	3.1	11	32.4	12.5	12.8	12.8	12.9
						12	30.4	1.1	1.0	0.9	1.0
						13	29.2	2.7	2.7	2.4	2.6
						14	28.2	1.3	1.5	1.6	1.6
<b>HMW-GS / LMW-GS</b>		0.51	0.51	0.62	0.56	<b>HMW-GS / LMW-GS</b>		0.65	0.64	0.68	0.68

## Figure captions

**Figure 1** Monthly precipitation and average temperature during the experimental years and the mean values from 1981 to 2010 for Futterkamp (A) and Sönke-Nissen-Koog (B). Data source: Landwirtschaftskammer Schleswig-Holstein.

**Figure 2** Effects of N treatments on protein subunits pattern of gliadin (A) and glutenin (B) fractions separated by SDS-PAGE for two winter wheat cultivars. Treatments N1 to N4 represent the corresponding treatments as described in Table 1. The Right Arrow (→) and the Left Arrow (←) indicated the bands which were relatively changed by N treatments in Tobak and JB Asano, respectively.

**Figure 3** Loaf volume as affected by N treatments for two winter wheat cultivars. Error bars represent standard deviations. Different letters represent significant differences ( $P < 0.05$ ) of mean values within cultivars (small letters for Tobak and capital letters for JB Asano). Treatments N1 to N4 represent the corresponding treatments as described in Table 1.



Figure 1 (A)

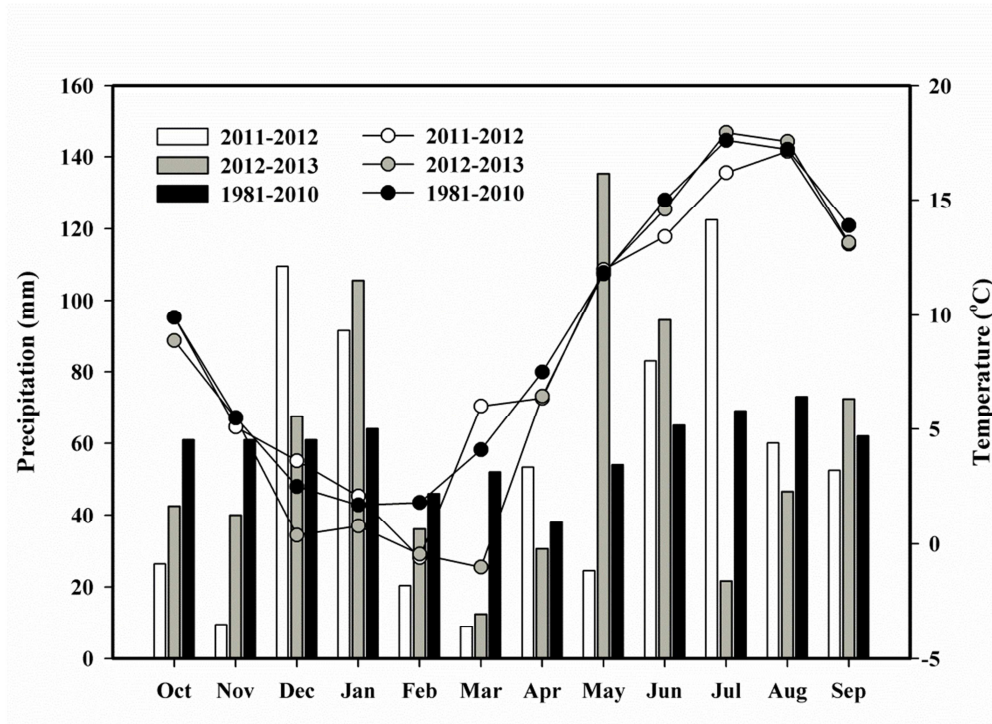
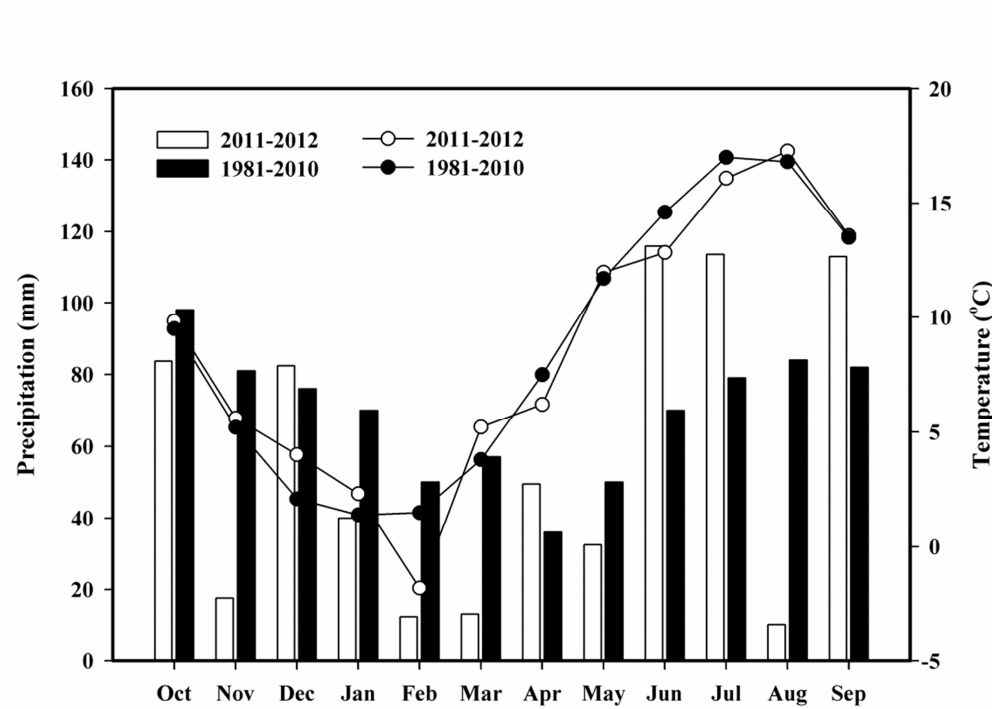
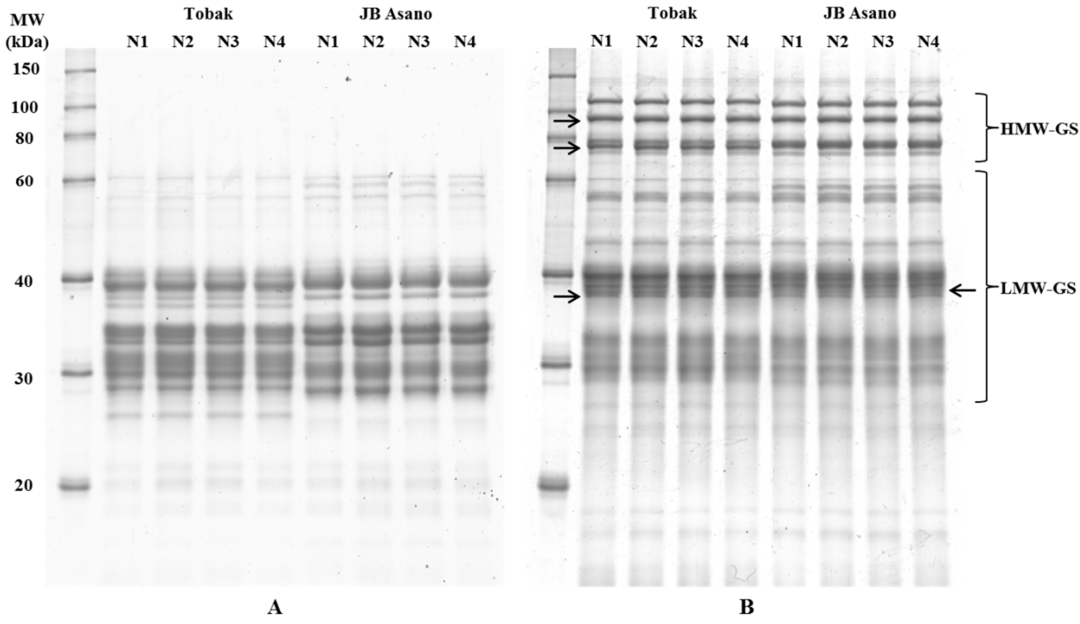


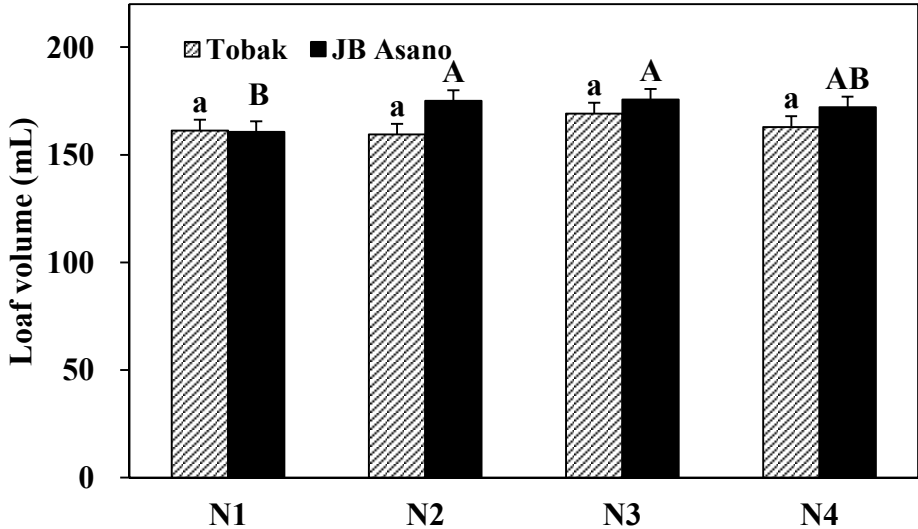
Figure 1 (B)



**Figure 2**



**Figure 3**



## **Chapter 4**

### **Quantitative proteome analysis and baking quality of wheat flour as affected by split N application**

(In preparation)

#### **4. Quantitative proteome analysis and baking quality of wheat flour as affected by split N application**

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Key words: 2D-electrophoresis, gliadins, glutenins, protein composition, loaf volume

## **Abstract**

Bread-making quality of wheat flour is determined by protein concentration and its composition and is highly influenced by environmental factors such as nitrogen (N) fertilization management. This study investigated the effect of split N application on grain protein composition and baking quality of two winter wheat cultivars, Tobak and JB Asano. Loaf volumes in both cultivars were enhanced by split N application. In contrast, grain protein concentration was only significantly increased in JB Asano. Quantitative two-dimensional gel electrophoresis (2-DE) revealed that the relative volumes of 27 and 35 unique protein spots were significantly changed by split N application in Tobak and JB Asano, respectively. In particular, five protein spots were up-regulated while six were down-regulated by more than two-fold in Tobak as affected by split N application. These proteins being relatively increased by more than two-fold may exert greater influences on baking quality of wheat flour.

## 4.1 Introduction

Grain protein concentration is widely used as the main parameter in evaluating bread-making quality of wheat products and a higher price is achieved with high-protein bread wheat. However, the bread-making quality is complex and is determined not only by grain protein concentration but also by its composition (Veraverbeke and Delcour, 2002). Based on the solubility in different solvents, the major flour protein types are classified into albumins, globulins, gliadins and glutenins (Osborne, 1924). Gluten proteins (gliadins and glutenins) play important roles in determining bread-making quality of wheat flour as gliadins mainly contribute to dough viscosity and extensibility, while glutenins to dough strength and elasticity (Wieser, 2007).

One well-known effect of fertilization with nitrogen (N) is the increase in grain protein concentration (Benzian and Lane, 1981). Therefore, in order to produce high quality wheat and to achieve optimum economic benefits, farmers make strategic decisions about the handling of N fertilization such as N rate and N splitting. Studies demonstrated that additional N increased grain protein concentration mainly through the enhanced gliadins and glutenins, as well as increased the proportions of HMW-GS and  $\omega$ -gliadins and decreased the proportions of LMW-GS (Martre et al., 2006; Wieser and Seilmeier, 1998). Apart from total N fertilization rate, split N application (splitting of the same N fertilizer rate distributed in several applications at different growth stages) enhances N use efficiency thus provides more available N for the plant (Ercoli et al., 2013). Moreover, it has been reported that N fertilizer applied at late stages comparing to early stages favours protein build-up in the grain over an increased yield (Sowers et al., 1994). Therefore, split N application is supposed to be an effective way in improving bread-making quality of wheat. However, the results from split N application varied among studies (Fuertes-Mendizábal et al., 2010; Garrido-Lestache et al., 2004, 2005). Our previous study using two wheat cultivars (Chapter 2) showed that split N application significantly improved loaf volume of both wheat cultivars mainly through the proportionally increased gliadin and glutenin fractions as well as certain high-molecular-weight glutenin subunits (HMW-GS) as fractionated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). However, the grain protein concentration was only significantly increased in one

cultivar (JB Asano), while not changed in another cultivar (Tobak) as influenced by split N application. These results strongly indicated that split N application caused important changes in grain protein composition, which might play key roles in determining baking quality of the resulting wheat flour.

The composition of wheat flour protein is highly complex (Shewry et al., 2003). Most studies have determined grain protein composition using one-dimensional gel electrophoresis (1-DE) or reversed phase high-performance liquid chromatography (RP-HPLC). However, it is difficult to completely separate LMW-GS and some of the gliadins since they are overlapped in molecular weight (MW). Due to the difficulties in separating individual grain proteins by 1-DE or by chromatographic methods, the use of two-dimensional gel electrophoresis (2-DE) allows the visualization, comparison, as well as the subsequent identification of individual proteins. Many studies have used 2-DE to determine influences of environmental factors (e.g. high temperature, drought or fertilization) on wheat grain protein composition (Altenbach et al., 2011; Dupont et al., 2006a; Grove et al., 2009; Hurkman et al., 2013; Wrigley et al., 1984; Yang et al., 2011; Zörb et al., 2009). Particularly, a representative study on wheat flour proteome changes as affected by post-anthesis fertilization (PAF) was conducted by Altenbach et al. (2011) using 2-DE. They found that the proportions of 54 unique proteins were significantly altered by PAF. Increases were predominately lied in  $\omega$ -gliadins, HMW-GS and serpins as well as some  $\alpha$ -gliadins, whereas decreases were mainly observed in  $\alpha$ -amylase/protease inhibitors, farinins, purinins, puroindolines, LMW-GS, globulins, defence proteins and enzymes as influenced by PAF. However, since the post-anthesis fertilizer was applied additionally as a mixture containing N, phosphorus (P) and potassium (K) in that study, it was not possible to determine the effect only from N fertilization. In comparison, studies on split N application effect on grain protein composition using 2-DE have not been reported.

In this paper, wheat flours from two N fertilization treatments (with and without split N application) were selected from our previous study using two cultivars (Chapter 2). The method of 2-DE was conducted to determine the alterations in flour protein composition as well as to identify the specific proteins as influenced by split N application. The objectives of this study were (i) to provide more detailed information on grain protein composition of two wheat

cultivars, (ii) to evaluate the changes in individual proteins as influenced by split N application, (iii) to find unique proteins that might have higher correlations with baking quality of wheat flour.

## **4.2 Materials and Methods**

### Experimental design

Two winter wheat cultivars Tobak and JB Asano, belonging to different baking quality classes according to the German Federal Office of Plant Varieties, were used in this study. Tobak (baking quality class B) had similar loaf volume but lower raw protein concentration comparing to JB Asano (baking quality class A).

The N fertilization experiment was conducted in the year of 2011-2012 in Mitscherlich pots (Chapter 2). Nitrogen fertilization treatments N2 and N3 were selected from the previous study and renamed in the present study as follows: early-N treatment, 2 g N pot<sup>-1</sup> in two doses with 1 g N pot<sup>-1</sup> applied before seeding and 1 g N pot<sup>-1</sup> at EC30 (beginning of stem elongation) (Lancashire et al., 1991); split-N treatment, 2 g N pot<sup>-1</sup> in three doses with 1 g N pot<sup>-1</sup> applied before seeding, 0.5 g N pot<sup>-1</sup> at EC30 and 0.5 g N pot<sup>-1</sup> at EC45 (late boot stage). Other nutrients including P (0.6 g pot<sup>-1</sup>), K (2.3 g pot<sup>-1</sup>), S (0.5 g pot<sup>-1</sup>), Mg (0.33 g pot<sup>-1</sup>), Ca (1.19 g pot<sup>-1</sup>) and the minor elements Cu (10 mg pot<sup>-1</sup>), Zn (15 mg pot<sup>-1</sup>) and Mn (30 mg pot<sup>-1</sup>) were applied before winter wheat seeding. Each treatment was replicated five times. Winter wheat was sown on 30 November 2011 and harvested on 6 August 2012. Nitrogen fertilization was conducted on 25 November 2011, 2 April 2012 and 30 May 2012, respectively. Fungi disease and insects were well controlled by spraying fungicide Capalo three times and insecticide Biscaya once during winter wheat growth.

### Grain yield, N and S concentration

Grain yield (g pot<sup>-1</sup>) was determined as the dry weight of kernels in each pot. Grains were milled in a Titan laminated mill using a 500 µm sieve (Retsch, Haan, Germany). Nitrogen and sulphur (S) concentrations were determined by a CNS elemental analyser (Flash EA 1112 NCS, Thermo Fisher Scientific, Waltham, MA, USA). Crude protein concentration of wheat flour was calculated by multiplying the N concentration by 5.7.



### Micro baking test (MBT)

The MBT with 10 g wholemeal flour of each sample was performed according to Thanhaeuser et al (2014) and the detailed procedures were described in (Chapter 2). Briefly, the moisture of each sample was measured using infrared moisture analyser (MA35, Sartorius). The water absorption and dough development time were then determined using a micro-farinograph (Brabender, Duisburg, Germany). Afterwards, the MBT was performed and the loaf volumes were measured using Volscan Profiler 600 (Stable Micro Systems, Godalming, UK).

### Protein extraction for two-dimensional gel electrophoresis (2-DE)

The wholemeal flour was further ground to a fine homogeneous powder under liquid N using a mortar. Protein extraction from wholemeal flour for 2-DE was performed using a dithiothreitol (DTT) - trichloroacetic acid (TCA) - acetone precipitation method according to Zörb et al. (2009) with several modifications. Proteins were extracted by adding 1.6 mL buffer A (10% TCA in acetone with 50 mmol L<sup>-1</sup> DTT) to 100 mg flour. After vortexing shortly, samples were incubated in an ice-cold ultrasonic bath for 17 min and stored at -20 °C overnight. Samples were then centrifuged (16,000 × g, 15 min, 4 °C) and the supernatant were discarded. The precipitant was resuspended in 1.5 mL ice-cold buffer B (50 mmol L<sup>-1</sup> DTT, 2 mmol L<sup>-1</sup> EDTA, in acetone) and incubated in ice-cold ultrasonic bath for 15 min, and then incubated in -20 °C for 90 min before centrifugation (16,000 × g, 15 min, 4 °C). This procedure was repeated and the final pellets were lyophilized under N<sub>2</sub>. To dissolve protein, pellets were resuspended in 1 mL buffer C (8 mol L<sup>-1</sup> urea, 2 mol L<sup>-1</sup> thiourea, 4% CHAPS, 30 mmol L<sup>-1</sup> DTT, 20 mmol L<sup>-1</sup> Tris base) with 5 µL protease inhibitor cocktail (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany), and shaken (2 h, 33 °C) and incubated in an ice-cold ultrasonic bath for 15 min before centrifugation (16,000 × g, 30 min, 4 °C). Finally, supernatant was collected and stored at -20 °C for future use.

Protein concentration of each sample was determined using a 2D Quant protein quantification kit (GE Healthcare, Munich, Germany).

### Isoelectric focusing (IEF) and SDS-PAGE

To separate flour proteins, 2-DE was performed according to O'Farrell (1975) with several modifications. For samples from each biological replicate, two technical replicates were conducted (10 gels for each treatment within each cultivar, 40 gels in total). Commercially purchased immobilized pH gradient (IPG) strips (11 cm, pH 3-10, linear, SERVA, Heidelberg) were used for IEF, which was performed using a Protein IEF Cell system (Bio-rad). The IPG strips were placed in a tray and 200  $\mu$ L protein solution (105  $\mu$ g protein, 1  $\mu$ L protease inhibitor cocktail, 1  $\mu$ L IPG buffer pH 3-10, 1  $\mu$ L bromophenol blue, adjust to 200  $\mu$ L using buffer C) was applied. Strips were covered with paraffin oil. Isoelectric focusing was carried out at the following conditions: 12 h rehydration; 200 V (sloping upward), 0.5 h; 200 V (constant), 0.5 h; 500 V (sloping upward), 0.5 h; 500 V (constant), 0.5 h; 1000 V (sloping upward), 0.5 h; 1000 V (constant), 0.5 h; 4000 V (sloping upward), 0.5 h; 4000 V (constant), 1.5 h; 8000 V (sloping upward), 0.5 h; 8000 V (constant), 3.5 h at 20 °C. After the first dimension, strips were covered with paraffin oil and stored at -20 °C at least overnight before being used for the second dimension.

The second dimension was performed using medium sized (18 cm  $\times$  16 cm  $\times$  1.5 mm) SDS-PAGE gels (12.5%). IPG strips were rinsed with running buffer (25 mmol L<sup>-1</sup> Tris base, 192 mmol L<sup>-1</sup> glycine, 0.1% SDS) and slowly shaken for 20 min in 5 mL equilibration buffer (50 mmol L<sup>-1</sup> Tris-HCl pH 8.8, 6 mol L<sup>-1</sup> urea, 30% glycerol, 2% SDS, 1% DTT). The strips were then incubated in 5 mL equilibration buffer containing 4% iodacetamide under slow shaking for another 20 min. After that, the strips were rinsed with running buffer again and then mounted onto the gel surface and sealed with 1% agarose containing 0.001% bromophenol blue. A molecular weight standard 'Rotimark 10-150' (RotiMark, Roth, Crailsheim, Germany) was added as the marker lane next to the acid side of the IPG strip. Two gels were run simultaneously in the vertical electrophoretic unit (SE600, Hoefer) at 50 mA and 17 °C for about 5 h. Gels were then fixed (40% ethanol, 10% acetic acid), stained (1 tablet PhastGel™ Blue R in 1.6 L 10% acetic acid) and destained (10% acetic acid). Gels were digitized by scanning on an image scanner (Epson Perfection V700) at 300 dpi and 16 bits per pixel.

Proteome analyses

Gel images were selected before analysis. For each biological replicate, the gel had a higher resolution within two technical replicates was chosen. Computer-assisted 2D analysis of each gel was done using the software Delta 2D 4.0 (Decodon GmbH, Germany). All selected 2D gel images (five replicates for each treatment) of each wheat cultivar were warped using a group warping strategy. Each gel pairs were carefully reviewed and protein spots were matched. Afterwards, a fused image (fusion gel) was created and individual protein spots were detected. The fusion gel was then used to delete artefacts and specks on individual gels before further processing. Subsequently, spot quantities were normalized by dividing each spot's volume by the total spot quantity on the image and the protein spots were quantified by their relative intensity on the gel image. The quantity of protein was denoted by % volume.

#### Statistical analysis

Data for grain yield, protein concentration, N/S ratio and loaf volume were presented as the mean value  $\pm$  standard deviation of five biological replicates. Significant differences were calculated using the Student's *t*-test and were compared at  $P < 0.05$  using the software SPSS 13.0 (Chicago, IL, USA). For the comparisons of the normalized volumes of protein spots, the Student's *t*-test was performed using the 2D Decodon software at a significance level of  $P < 0.05$ .

### **4.3 Results and discussion**

#### 4.3.1 Grain yield, protein concentration, N/S ratio and loaf volume

Grain yield of both winter wheat cultivars were not affected by split N application (split-N vs early-N treatment), ranging from 75 to 80 g pot<sup>-1</sup> (Table 1). However, grain protein concentration responded differently to split N application between cultivars, being not altered in Tobak, while significantly increased by 9.1% in JB Asano (Table 1). Despite of the inconsistent effects on grain protein concentration, the loaf volumes in both cultivars were significantly improved by split N application. Besides, the enhancement of loaf volume in JB Asano (10.5%) was higher than that in Tobak (5.4%) (Table 1). Grain protein concentration is widely used as the main criterion in predicting baking quality of wheat flour (Delcour et al., 2012). However, it was clearly shown that grain protein concentration did not correlate with

baking quality (loaf volume) as affected by split N application in Tobak (Table 1). Actually, we already demonstrated that grain protein concentration was not suitable in predicting baking quality of wheat flour as affected by split N application and late N fertilization in these cultivars from pot and field studies (Chapter 2 and 3). These findings highly implied that the alteration in protein composition caused by N fertilization might have played key roles in determining baking quality of wheat flour.

It is well known that a strong inter-dependence existed in plant N and S metabolism. In terms of grain protein composition responses to increasing N fertilization and/or under S deficient conditions, similar effects were usually achieved, being the  $\omega$ -gliadins (low in S) and HMW-GS (low to medium in S) increase and  $\alpha$ -,  $\gamma$ -gliadins and LMW-GS (rich in S) decrease in their proportions to total grain proteins (Daniel and Triboi, 2000; Wieser and Seilmeier, 1998; Wieser et al., 2004; Zhao et al., 1999). The criterion of N/S ratio higher than 17 is recognized as S deficiency in wheat grain (Randall et al., 1981). In the present study, the grain N/S ratio in both cultivars was between 11 and 12 (Table 1), indicating that there was no S deficiency in our study.

#### 4.3.2 Grain protein profiles (2-DE) and quantitative proteome analysis as affected by split N application

Wheat grain proteins were separated into individual protein spots through 2-DE. Generally, protein profiles were similar between Tobak and JB Asano. Only quantitative changes in spot volume were detected, whereas no absence of existing protein spots or presence of new protein spots occurred as influenced by split N application. Therefore, the 2-DE protein profiles for the split-N treatment were presented in Fig. 1 for Tobak and in Fig. 2 for JB Asano.

In Tobak, a total of 341 protein spots was determined. The average spot volume of 27 protein spots was significantly changed by split N application (Table 2), accounting for 7.9% of the total protein spots and for 10.4% and 11.0% of the total spot volumes in early-N and split-N treatments, respectively. Besides, within these 27 spots, the volumes of 10 protein spots were increased (five spots increased more than two-fold), while 17 protein spots were decreased (six spots decreased more than two-fold) by split N application. In JB Asano, 352 protein spots were

detected. The average spot volume of 35 protein spots (18 spots being up-regulated and 17 spots being down-regulated) was significantly changed by split N application (Table 3), accounting for 9.9% of the total protein spots. These significantly altered protein spots taken up 16.7% and 16.1% of the total spot volumes in early-N and split-N treatments, respectively. Besides, within these 35 spots, no spots were increased more than two-fold, while three spots decreased more than two-fold by split N application. By comparing cultivars, less protein spots were significantly influenced by split N application in Tobak (27) than in JB Asano (35). However, the magnitude of changes in spot volumes was higher in Tobak (11 protein spots changed more than two-fold) than in JB Asano (three protein spots changed more than two-fold) (Table 2 and 3).

These significantly changed protein spots might contribute to the alterations in baking quality of the corresponding wheat flour. Therefore, to better understand their functions in plant metabolism and baking quality, identification of these specific protein spots were needed. The identification of these significantly changed protein spots was on-going at Department of Physiology and Cell Biology (IPK, Gatersleben, Germany) and was not finished to date. Nevertheless, the resolution and distribution of the protein profiles in the present study (Fig. 1 and 2) were comparable to other studies using 2-DE to investigate responses of grain protein composition to various environmental conditions (Altenbach et al., 2011; Dupont et al., 2006a, 2006b; Flæte et al., 2005; Grove et al., 2009; Hurkman et al., 2013; Skylas et al., 2005; Vensel et al., 2014; Zörb et al., 2009). Therefore, it was still possible to characterize some of the typical protein spots into different protein classes according to the MW and IEF of protein spots distributed in the whole protein profile.

Some protein spots at the bottom of the 2-DE gels (MW between around 10-15 kDa) were relatively decreased by split N application in both Tobak (no. 159, 162, 164 and 167) and JB Asano (no. 267, 270, 272, 281 and 345) (Fig. 1 and 2). Other studies also found protein spots in this area of 2-DE gels decreased with post-anthesis fertilization or increased with S fertilization (Altenbach et al., 2011; Grove et al., 2009). These proteins were supposed belonging to  $\alpha$ -amylase/protease inhibitors, which were believed to be important in the defence mechanisms of the seed against insects and microbial pests while also to be the allergens associated with

baker's asthma for human (Feng et al., 1996; Sanchez-Monge et al., 1992). Besides, some protein spots which might be classified into groups of farinins, purinins and triticins in Tobak (no. 109, 139, 293 and 304) and JB Asano (no. 229, 231, 292 and 317) were also decreased by split N application (Fig. 1 and 2). Together with  $\alpha$ -amylase/protease inhibitors, all these proteins contained relatively high content of S-rich amino acids (6-12% cysteine plus methionine) as reported by Altenbach et al. (2011). Therefore, although the N/S ratio indicated that there was no S deficiency in the present study (Table 1), it seemed the changes in different protein classes as affected by split N application were related to the proportions of S-containing amino acids in the proteins from these classes.

However, in terms of gluten proteins, protein spots belonging to different protein types responded differently to split N application between cultivars. A group of protein spots which were supposed to be  $\omega$ -gliadins or HMW-GS in JB Asano (no. 59, 61, 62, 64, 66, 70 and 71), while only one protein spot (no. 285) in Tobak were significantly enhanced by split N application (Fig. 1 and 2). However, another group of protein spots which might belong to  $\omega$ -gliadins was increased in JB Asano (no. 91, 98, 106, 110, 119 and 120) while decreased in Tobak (no. 61, 214 and 217) as affected by split N application (Fig. 1 and 2). The most abundant protein subunits in wheat grain were  $\alpha$ -,  $\gamma$ -gliadins and LMW-GS, distributed mainly between 30-50 kDa in the protein profiles (Fig. 1 and 2). Within these protein spots, mainly increases in Tobak while both increases and decreases in JB Asano were achieved by split N application. These results were comparable with our previous study using 1-DE (Chapter 2) that the relative abundances of certain  $\omega$ -gliadins were significantly enhanced by split N application in JB Asano, whereas both trends of increases and decreases were observed in LMW-GS and  $\alpha$ -,  $\gamma$ -gliadins in both cultivars. However, since  $\alpha$ -,  $\gamma$ -gliadins and LMW-GS were highly overlapped in MW and IEF, it was impossible to distinguish them without the identification of individual protein spots.

Baking quality of wheat flour is highly dependent on the quantity and composition of gluten proteins. However, the changes in protein and baking quality might result from different aspects between these two cultivars. Generally, with the enhancement in grain protein concentration, the relative proportions of  $\omega$ -gliadins and HMW-GS increase, while that of  $\alpha$ -,

$\gamma$ -gliadins and LMW-GS decrease (Altenbach et al., 2011; Wieser and Seilmeier, 1998). Therefore, the changes in protein composition might mainly be attributed to the significantly increased grain protein concentration in JB Asano, thus lead to improved baking quality (Table 1). However, for Tobak, the improvement in baking quality was mainly due to the alterations in protein composition without significant changes in grain protein concentration. As described above, although less protein spots were changed by split N application in Tobak than in JB Asano, the changes were to a higher magnitude in Tobak (Table 2 and 3). It implied that these proteins which were relatively increased by more than two-fold (no. 220, 236, 251, 258 and 274) might positively determine baking quality to a higher extent, while those proteins (no. 139, 164, 165, 241, 264 and 293) which were relatively decreased by more than two-fold might be less important in baking quality. Therefore, further investigations should be focused on the characteristics and functions of these unique proteins to provide a better understanding of their relationships with baking quality.

The current study clearly demonstrated that split N application significantly improved baking quality of wheat flour. Grain protein concentrations and compositions of different cultivars may respond differently to split N application. The alterations in relative abundance of some proteins were more sensitive to split N application, which implied that the functions of these unique proteins might be more important in affecting baking quality of wheat flour.

### **Acknowledgements**

We appreciate the help from Dr. Britta Pitann and Stephanie thor Straten. Cheng Xue gratefully acknowledges the financial support provided by the China Scholarship Council.

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## Tables and figures

**Table 1** Grain yield, protein concentration, N/S ratio and loaf volume as affected by split N application. Different letters in the same column within the same cultivar represent significant differences of mean values  $\pm$  standard deviation of five independent pot replicates at  $P < 0.05$ .

Treatment		Grain yield (g pot <sup>-1</sup> ) <sup>a</sup>	Protein (mg g <sup>-1</sup> flour) <sup>a</sup>	N/S	Loaf volume (mL) <sup>a</sup>
<b>Tobak</b>	Early-N	74.5 $\pm$ 3.8 a	88.4 $\pm$ 1.7 a	11.8 $\pm$ 0.9 a	31.4 $\pm$ 0.6 b
	Split-N	75.5 $\pm$ 2.4 a	91.9 $\pm$ 5.5 a	11.1 $\pm$ 0.8 a	33.1 $\pm$ 0.6 a
<b>JB Asano</b>	Early-N	79.5 $\pm$ 3.9 A	87.0 $\pm$ 2.5 B	11.0 $\pm$ 1.3 A	29.5 $\pm$ 1.5 B
	Split-N	75.0 $\pm$ 2.2 A	94.9 $\pm$ 2.7 A	11.8 $\pm$ 1.6 A	32.6 $\pm$ 0.6 A

<sup>a</sup> Data of grain yield, protein and loaf volume were extracted from Chapter 2.

Table 2 Unique protein spots that changed significantly ( $P < 0.05$ ) by split N application in Tobak

Spot Number <sup>a</sup>	Mean Normalized Volume (%)		X-fold change
	Early-N	Split-N	Split-N vs Early-N
65	0.18	0.3	1.7
70	1.1	1.48	1.3
128	1.03	1.94	1.9
178	0.36	0.64	1.8
220	0.05	0.2	3.7
236	0.18	0.43	2.4
251	0.42	0.85	2
258	0.16	0.4	2.6
274	0.32	0.76	2.4
285	0.37	0.5	1.4
61	0.71	0.55	-1.3
109	0.36	0.25	-1.4
139	0.51	0.2	-2.6
151	0.19	0.13	-1.4
152	0.26	0.17	-1.5
159	0.73	0.46	-1.6
162	0.23	0.12	-1.9
164	0.32	0.14	-2.4
165	0.2	0.07	-3
167	0.19	0.12	-1.6
214	0.27	0.18	-1.5
217	0.31	0.22	-1.4
241	0.9	0.36	-2.5
264	0.27	0.11	-2.5
293	0.39	0.12	-3.1
304	0.24	0.18	-1.4
320	0.14	0.12	-1.2

<sup>a</sup> Spot numbers are assigned according to Figure 1.

Table 3 Unique protein spots that changed significantly ( $P < 0.05$ ) by split N application in JB Asano

Spot Number <sup>a</sup>	Mean Normalized Volume (%)		X-fold change
	Early-N	Split-N	Split-N vs Early-N
59	0.44	0.61	1.4
61	0.24	0.34	1.4
62	0.38	0.55	1.5
64	0.27	0.37	1.4
66	0.21	0.32	1.5
70	0.13	0.18	1.4
71	0.26	0.36	1.4
91	0.53	0.64	1.2
98	0.2	0.25	1.2
106	0.51	0.66	1.3
110	0.16	0.24	1.5
111	0.25	0.34	1.4
116	0.38	0.45	1.2
119	0.17	0.22	1.2
120	0.22	0.3	1.4
157	2.18	2.6	1.2
203	0.86	1.1	1.3
206	0.23	0.34	1.5
129	0.05	0.03	-1.9
165	0.28	0.2	-1.4
184	1.06	0.73	-1.5
199	0.38	0.28	-1.4
213	0.34	0.22	-1.6
222	0.13	0.06	-2.1
225	0.18	0.12	-1.5
229	0.06	0.03	-1.7
231	0.07	0.04	-1.8
267	0.69	0.62	-1.1
270	0.86	0.58	-1.5
272	0.74	0.47	-1.6
281	0.33	0.16	-2
292	0.1	0.06	-1.7
302	1.75	1.28	-1.4
317	0.33	0.07	-4.6
345	1.68	1.26	-1.3

<sup>a</sup> Spot numbers are assigned according to Figure 2.

### **Figure captions**

**Figure 1** Effect of split N application on grain proteome in Tobak. The red and green lines with numbers indicate the spots that were significantly increased or decreased by split N application, respectively. pI, isoelectric point.

**Figure 2** Effect of split N application on grain proteome in JB Asano. The red and green lines with numbers indicate spots that were significantly increased or decreased by split N application, respectively. pI, isoelectric point.

Figure 1

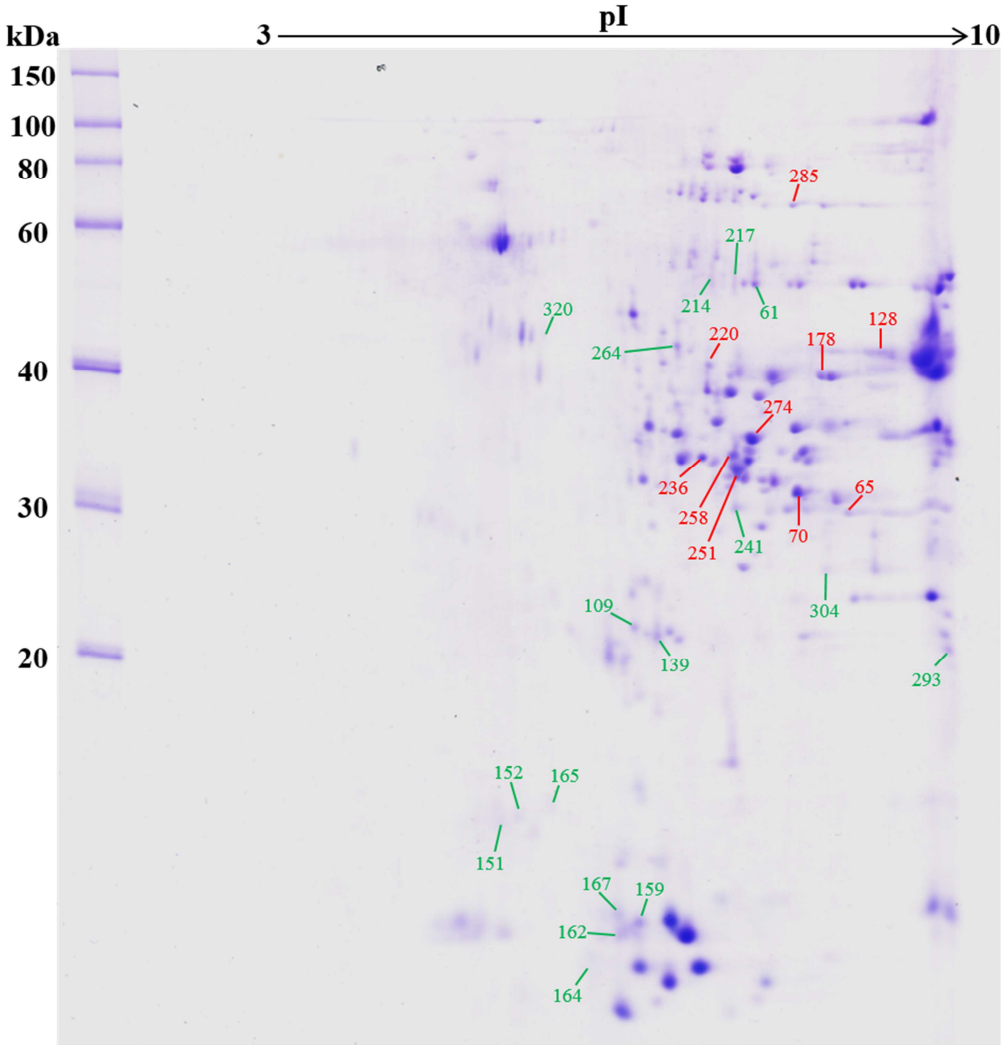
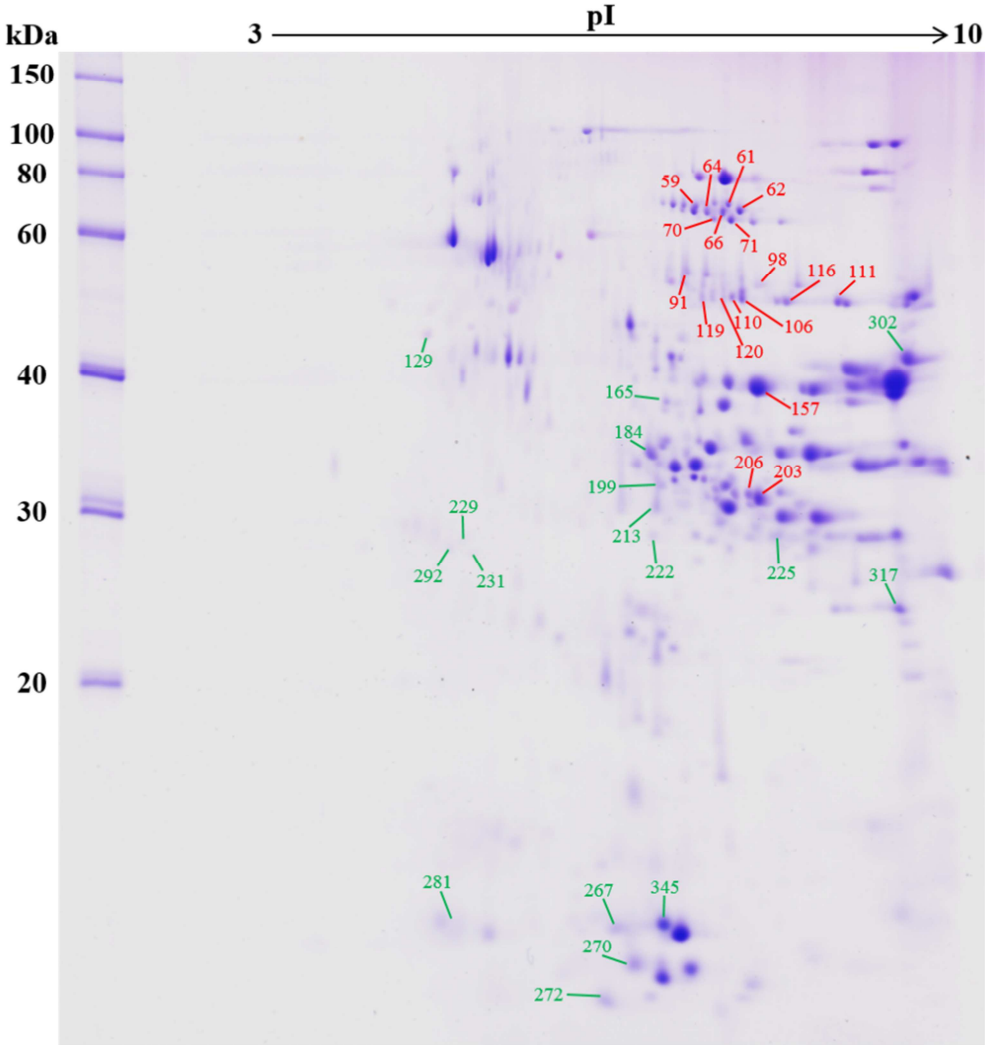


Figure 2





# **Chapter 5**

## **General discussion**

## **5. General discussion**

In order to sustainably produce crops with high yield and quality as well as to increase nitrogen (N) use efficiency for economic, nutritional and ecological aims, the skilful handling of N fertilizers is an important way in pursuing such goals. In wheat production, N fertilizers are usually applied in several doses at key growth stages to meet plant's N demand for canopy build-up and grain filling. Particularly, the application of additional N fertilizer at late growth stages (e.g. at heading or anthesis) is widely conducted in order to assure grain protein concentration (GPC), the main criterion for predicting baking quality of wheat. However, it raises the total costs (e.g. manpower, energy) of wheat production and the application of fertilizers at these late stages is also a practical problem for farmers. Therefore, the removal of the late N application or combining it with early N applications without exerting negative effects on grain yield and quality would benefit wheat producers. Increasing evidences showing that the relationship between GPC and baking quality is less evident for modern cultivars, providing a possibility that the late N fertilization might be dispensable for these cultivars. This thesis investigated the influences of late N fertilization on quantitative and qualitative changes of wheat grain protein, and thus baking quality, trying to give evidences on the feasibility of removing the late N application in quality wheat production.

Late N fertilization comprises two main aspects, N rate and N splitting. In this thesis, to avoid confusion between late N fertilization and N splitting as well as to differentiate effects from these two aspects, the term 'split N application' referred to the splitting of the same N rate distributed into several applications at different growth stages; while 'late N fertilization' meant applying additional N fertilizers at late growth stage on top of split N application.

### **5.1 Influence of late N fertilization on grain protein concentration**

Grain proteins are recognized playing crucial roles in affecting end-use quality of wheat products. The quality of wheat is ranked according to the GPC, the main criterion in evaluating baking quality of wheat flour, being higher water absorption capacity, more extensible and less tenacious dough and eventually larger loaf volumes often achieved with increases in GPC (Osman et al., 2012). Since most of the N present in wheat grain is in the form of proteins, thus

the GPC is usually enhanced by raising N fertilizer input (Benzian and Lane, 1981). This effect was approved in our study (Chapter 2, Table 1 and 2; Chapter 3, Table 2 and 3) that late N fertilization increased GPC in both pot and field experiments mainly through the effect of increased N fertilization rate which led to increased plant N uptake, while such effect was not observed by split N application without additional N amendment.

In terms of N sources for grain protein synthesis, the majority of grain N (60-95%) is derived from the N accumulated in vegetative organs before anthesis and remobilized later to the grain for protein synthesis, while the rest grain N comes from the lately absorbed N after anthesis (Triboi and Triboi-Blondel, 2002). Therefore, to increase GPC, both N sources need to be enhanced. It has been reported that N fertilizer applied at late stages favours protein synthesis in the grain over an increased yield (Sowers et al., 1994). Moreover, it was also demonstrated that split N application could enhance N remobilization from vegetative organs to grains, thus allowing an increase in GPC (Fuertes-Mendizábal et al., 2012). Results from our study (Chapter 2, Table 1) indicated that split N application could enhance N harvest index (NHI), especially, the late N harvest index was much higher than the overall NHI. These results indicated that the late N fertilizer can be more efficiently used for grain protein synthesis. However, this effect was not observed in the second pot experiment (Chapter 2, Table 1) or in the field experiments (Chapter 3, Table 2) and even the effect of split N application on NHI existed in the first pot experiment, it did not correlate well with the changes in GPC. Besides, it also seemed that influences of different N forms (nitrate-N or ammonium-N) and N fertilizer types (calcium ammonium nitrate or urea) on grain protein and baking quality mainly resulted from their effects on plant N uptake (Chapter 2 and 3).

Based on these findings from our study, it can be speculated that GPC depended mainly on the total available N amount for the plant, and the effects of different N managements on GPC might be eventually attributed to their influences on plant N uptake. It is reported that split N application could better match the requirement of plant N uptake, minimizing the excess of N in soil useless to the plant, thus reduces N losses from leaching or volatilisation and increases fertilizer N use efficiency (Alcoz et al., 1993; Ercoli et al., 2013). This effect of split N application could result in a similar effect as caused by increased N fertilization rate,

consequently, leads to a higher plant N uptake. However, this effect of split N application highly depends on environmental conditions (e.g. total precipitation and its temporal distribution, soil residual N and mineralization). Under conditions where precipitation is scarce at late growth stages, N applied at these stages may be not available to the plant, thus resulting in a negative effect in plant N uptake and GPC. In this case, the inconsistent results of split N application on GPC from literatures (Fuertes-Mendizábal et al., 2010; Garrido-Lestache et al., 2004, 2005; Schulz et al., 2015) might be explained from these aspects.

Therefore, under conditions that the plant N uptake could be assured, it might be possible to combine the late N fertilizer with early applications and reduce the overall N application times without decreasing GPC in quality wheat production. However, the baking quality of wheat flour is not only influenced by grain protein quantity but also highly affected by its composition (Veraverbeke and Delcour, 2002). Especially in our study, late N fertilization improved loaf volume of wheat flour mainly through the effect of split N application rather than increased total N fertilization rate (Chapter 2, Fig. 2; Chapter 3, Fig. 3) and the improvement in baking quality did not correlate to the enhancement in GPC. These results indicated that the changes in grain protein composition might determine variations in baking quality to a higher extent.

## **5.2 Influence of late N fertilization on protein composition**

Wheat grain protein consists of structural/metabolic proteins (albumins and globulins) and storage (or gluten) proteins (gliadin and glutenin). It has been reported that albumin-globulins accumulate from anthesis to around 20 days after anthesis (daa), while gluten proteins accumulate from approximately 6 daa to the end of grain-filling (Gupta et al., 1996; Panozzo et al., 2001). These gluten proteins play key roles in determining bread-making quality of wheat as gliadins mainly contribute to dough viscosity and extensibility, while glutenins provide dough with strength and elasticity (Wieser, 2007). In this study, with the enhancement in GPC, the concentrations and proportions of gliadins and glutenins were also increased (Chapter 2, Table 2; Chapter 3, Table 3), which agrees with other studies as modelled by Martre et al. (2006). However, although split N application did not affect GPC, the proportions of gliadin and glutenin fractions were increased by split N application, leading to improved baking quality in our study (Chapter 2, Table 2 and Fig. 2; Chapter 3, Table 3 and Fig. 3). This effect of split N

application was mainly observed in the first pot experiment and in the field experiments, while not in the second pot experiment. Anyway, it indicated that N fertilizer splitting could influence the N partitioning in the grain by changing grain protein composition, which was against literatures describing that the protein composition depends mostly on the protein content (Triboï et al., 2003). This effect of split N application might result from the different N sources and amino acids provided for grain protein synthesis. It has been reported that the amino acids that synthesized from the recently absorbed N were more readily available to be exported to the phloem to meet the demand of grain protein synthesis than that slowly released from the storage pool in the vegetative tissues (Yoneyama and Takeba, 1984). Besides, it is reported that glutamine and proline are the metabolically inexpensive amino acids, thus they may be good sinks for N when there is surplus N or (relatively) insufficient sulphur (S) (Dupont and Altenbach, 2003). Therefore, the amino acids composition might be altered due to the split N application, resulting in relatively increased S-poor (e.g. glutamine and proline) comparing to S-rich (e.g. cysteine) amino acids. Since gliadins and glutenins have higher glutamine/glutamic acid and proline while lower cysteine contents comparing to albumins and globulins (Cornell, 2012), therefore, the proportions of gliadins and glutenins were increased by split N application in our study.

Generally, the albumin-globulin concentrations are not affected by N fertilization and their proportions decrease with the enhancement in GPC (Triboï et al., 2003). However, the proportions of albumin-globulin fractions were not significantly altered by N fertilization in our study, accounting for 20-30% of the total grain protein (data not shown). Since split N application had no significant effect on GPC but increased the proportion of glutens, indicating the relative reduction of non-protein compounds and/or unextracted proteins in the flour. The unextracted proteins may still contain non-storage and storage proteins since the sequential protein fractionation method cannot extract all proteins in the flour. Besides, there might be losses of proteins in the precipitation process conducted during quantification. Therefore, the absolute amount of these protein fractions might be underestimated in our study. Nevertheless, the values and relative amounts of different protein fractions were in the same range comparing to other studies (Zörb et al., 2010). Moreover, since the same protein fractionation and

quantification methods were performed in both pot and field experiments in our study, comparisons between N fertilization treatments on protein composition were feasible and reliable. Therefore, we demonstrated that the proportional increases in gliadins and glutenins by split N application were not at the expense of albumin-globulins, but other N-containing compounds, e.g. free amino acids, amides, lipids.

Besides protein composition, gliadin and glutenin subunits composition was altered by late N fertilization as well (Chapter 2, Fig. 1; Chapter 3, Fig. 2). It is well known that a strong inter-dependence existed in plant N and S metabolism. In terms of grain protein composition responses to increasing N fertilization and/or under S deficient conditions, similar effects were usually achieved, being the  $\omega$ -gliadins (low in S) and high molecular weight glutenin subunits (HMW-GS) (low to medium in S) increase and  $\alpha$ -,  $\gamma$ -gliadins and LMW-GS (rich in S) decrease in their proportions to total grain proteins (Daniel and Triboi, 2000; Wieser and Seilmeier, 1998; Wieser et al., 2004; Zhao et al., 1999). In our study, the gliadin subunits composition was only significantly changed by late N fertilization in the first pot experiment, being the relative abundance of two and one  $\gamma$ -gliadins decreased in Tobak and JB Asano, respectively, and one  $\omega$ -gliadin increased in JB Asano (Chapter 2, Table 3). By contrast, the influence of late N fertilization on glutenin subunits composition was more obvious. Similarly, the relative abundance of glutenin subunits increased by late N fertilization mainly belonged to HMW-GS and D-type LMW-GS, while decreases existed only in B- and C-type LMW-GS (Chapter 2, Table 4; Chapter 3, Table 5). These changes matched well to the trends as described above from literatures. It should be pointed out that the N/S ratio  $> 17$  is regarded as S deficiency in wheat grain (Zhao et al., 1999). In our study, the N/S ratios were between 11-15 and 14-17 in pot and field experiments (data not shown), respectively, which proved no S deficiency in our study. However, it can be speculated that the N applied at late stages created a short period when N availability was much higher than that without this N application, resulting in a relatively high N/S ratio during this short period. If this is true, the synthesis of S-containing amino acids such as cysteine and methionine might be relatively reduced following this N application, whereas glutamine and proline synthesis were relatively enhanced as discussed above. Therefore, the components rich in glutamine and proline (e.g. gluten proteins,  $\omega$ -gliadins and HMW-GS) were

relatively increased in the grain. This assumption could be proved indirectly by studies on S fertilization (Moss et al., 1983; Wieser et al., 2004; Zörb et al., 2009), who demonstrated that S-rich proteins were significantly increased by S fertilization, whereas the S-poor proteins decreased.

Therefore, late N fertilization affected grain protein composition mainly through the effect of split N application, which increased the proportions of gluten proteins as well as certain subunits (e.g. x-type HMW-GS,  $\omega$ -gliadins), thus led to improvement in baking quality in our study. From the aspects of grain protein composition and baking quality, it seemed that the application of N fertilizers at late growth stages is indispensable in quality wheat production.

### **5.3 Factors may influence the effect of late N fertilization**

Although the effects of late N fertilization and split N application on protein concentration, composition and baking quality of wheat flour has been observed in our study. When making N fertilization strategies about whether a late N fertilization is necessary in wheat production, many other factors need to be considered.

It seemed GPC mainly depends on the plant N uptake (Chapter 2 and 3). Therefore, the total N fertilization rate needs to be considered. Logically, the effect of different application times becomes more evident with insufficient N supply. It has been reported that under sufficient N supply, even a single N application at early vegetative stages between tillering and stem elongation was sufficient to achieve high yield and GPC of winter wheat without increased risk of nitrate leaching (Schulz et al., 2015). Besides, environmental conditions during late growth stages of wheat can affect the decision of late N fertilization as well. For example, the late N fertilizer may not be taken up by the plants at locations have scarce precipitation in spring. In this case, a late N fertilization should be avoided.

Results from this study demonstrated that late N fertilization can influence baking quality of wheat flour mainly through the effect of split N application on N partitioning in the grain. However, it seemed that a few factors might regulate this effect of late N fertilization or split N application. It is noticeable that the effects of late N fertilization on grain protein composition was only observed in the first pot experiment through the effect of split N application, while no

such effect was shown in the second pot experiment (Chapter 2). Although we cannot demonstrate this difference strictly according to our experimental design, it seemed two main factors, total N fertilization rate and N application timing, might have led to this difference. The total N fertilization rate was increased from 2 to 2.5 g N pot<sup>-1</sup> and the timing of late N application was postponed from late boot stage (EC45) to heading (EC51) in the second comparing to the first pot experiment. Furthermore, results from field experiments (Chapter 3) provided further insights. Grain protein composition was altered when 220 kg N ha<sup>-1</sup> was split into three doses with the third N applied at late boot stage. However, when additional N was applied as the fourth N dose at heading, only GPC was increased whereas no further influences on protein composition and baking quality were detected. Based on these results, it was assumed that the timing of late N application seemed playing a crucial role in affecting protein composition and baking quality of wheat flour. It is generally considered that the post-anthesis N uptake is more important in improving protein and baking quality. Therefore, the late N fertilizers applied at heading or even anthesis are supposed to be more effective in quality wheat production. However, as discussed above, our results (Chapter 2 and 3) implied that certain stage (e.g. late boot stage) might be a key timing for N application, which determines grain protein composition and thus baking quality. After that stage, additional N fertilization might mainly influences protein concentration but not its composition. In our study, the N application at heading stage seemed already too late to improve baking quality of wheat. Therefore, further studies are needed to investigate whether this key stage exists and how it differentiates from other stages in affecting grain protein composition.

Cultivar differences should be also considered when making decisions on whether a late N fertilizer should be applied. It has been reported that the correlation between GPC and baking quality varied widely between different cultivars (Koppel and Ingver, 2010; Weegels et al., 1996). Generally, the two cultivars used in our study responded in a similar way to N fertilization management. However, the improvement of loaf volume in JB Asano was greater than that in Tobak (Chapter 2, Fig. 2; Chapter 3, Fig. 3). From the information according to German Federal Office of Plant Varieties, JB Asano (class A) is a cultivar that usually contains higher GPC at optimum baking quality as compared to Tobak (class B). It can be assumed that



JB Asano might be more dependent on GPC to achieve a high baking quality. These results indicated that measures for enhancing grain protein concentration and composition might be less necessary for cultivars such as Tobak in order to get optimum baking quality. Therefore, even this effect of late N application does exist, for cultivars whose baking quality is relatively stable within a certain range of GPC, the application of a late N fertilizer might be dispensable.

#### **5.4 How to predict wheat flour baking quality?**

Predicting the bread-making quality of any given wheat sample has been the open question for centuries. Many techniques have been developed trying to give answers to this question, but the goal still remains largely unattained. Loaf volume is the most direct and reliable parameter in evaluating bread-making quality of wheat flour. However, to conduct the baking test, large sample amounts and specific equipment are needed. Moreover, the process of conducting the baking test is time-consuming and inconvenient. Therefore, wheat producers and bakers have tried to find relatively simple and reliable parameters to predict bread-making quality. It is generally accepted that the endosperm storage proteins play the most important roles in determining wheat bread-making quality, being GPC positively correlated to the bread-making quality within one cultivar (Delcour et al., 2012). Therefore, market adjustments for wheat have been established worldwide based on GPC, with premiums commonly paid for protein concentrations above baseline levels (Woolfolk et al., 2002). However, results from our study showed that changes in GPC did not correlate well with variations in bread volume (Chapter 2 and 3), which clearly indicated that GPC was not an appropriate parameter in evaluating baking quality of wheat flour in our study. In fact, many studies have already demonstrated that large differences existed in the proportion of the variation in loaf volume that can be explained by protein concentration alone, especially between different cultivars (Johansson and Svensson, 1998; Koppel and Ingver, 2010; Pomeranz, 1965).

It has been reported that the quantity of glutenins could explain variation in loaf volume as well as, or better than the overall proteins, whereas the glutenin percentage in total protein explained the variation in loaf volume poorly compared with protein itself (Weegels et al., 1996). However, our results indicated that the proportions of glutenin (Chapter 2) or both gliadin and glutenin (Chapter 3) in total protein rather than their concentrations seemed determining bread

volume to a higher extent. Besides, it has been reported that although HMW-GS (especially x-type HMW-GS) are minor components in quantitative terms, they determine baking quality to a greater extent (Shewry et al., 2001; Wieser and Kieffer, 2001). Particularly in the present study, the x-type HMW-GS subunit 7 (MW 96-97 kDa) was enhanced by late N fertilization mainly through the effect of split N application (Chapter 2). Considering the relative abundance of this subunit that accounted for 34-36% of the HMW-GS in both cultivars, the relative change of this subunit may exert great effects on baking quality. However, this parameter alone could not completely explain the differences in bread volume as well. Moreover, by using two-dimensional gel electrophoresis (2-DE), we found that the relative abundances of several individual proteins spots were up-regulated by split N application (Chapter 4). It implied that the functions of these unique proteins might have exerted great influences on baking quality of wheat flour.

It seems unlikely that the bread-making quality of a given wheat flour could be predicted with certainty from a single test. Nevertheless, based on the findings from our study, we demonstrated that GPC was not suitable in evaluating baking quality (loaf volume) of wheat flour, particularly when evaluating the influences from split N application or late N fertilization. The proportions of glutenin and gliadin fractions and especially the abundant HMW-GS (allele 7) were regarded playing more important roles in determining wheat flour baking quality. Therefore, it can be speculated that the correlations between GPC and baking quality of wheat flour might exist over a broad range of GPC and cultivars. However, within a limited range of GPC, this correlation might not be enough to explain variations in baking quality of wheat flours. Therefore, in scientific studies just using GPC or single protein related parameter to predict baking quality of the resulting wheat flour is inappropriate and might result in misleading conclusions.

Although storage proteins determine baking quality to a great extent, it seems variations in baking quality could not be completely explained from the quantities and compositions of storage proteins. Actually, in addition to proteins, many other components (e.g. starch and lipid) in wheat flour are supposed to influence the baking quality as well. Starch, the most abundant component in wheat flour, could influence baking quality due to its physico-chemical

properties which are supposed to depend on starch granule structure and on the molecular composition of the starch polysaccharides amylose and amylopectin (Eliasson, 2012). Lipids are present in minor amounts in wheat flour. However, the composition, extractability and overall content of lipids highly determine bread volume of wheat flour (Pareyt et al., 2011). Studies on the interactions of the protein-lipid-carbohydrate might provide further insights in predicting baking quality.

### **5.5 Conclusions and perspectives**

Results from the current study suggested that split N application can change the N partitioning in wheat grain by increasing gliadin and glutenin proportions, thus improves baking quality of wheat flour. Although this effect of split N application might be blurred by total N rate effect or hidden by other environmental factors, it seems the practice of split N application as already performed for yield increments might be sufficient to achieve optimal baking quality. However, the application of additional late-N fertilizers on top of split N application mainly increases GPC, which may not lead to improvement in baking quality. Therefore, the practice of late N fertilization can be highly questioned. Further studies performed on additional locations and cultivars are needed to verify these results before this conclusion can be applied for wheat production in locations varying in environmental conditions and soil types. Besides, investigations are needed to find out the key growth stage which determines the N partitioning in wheat grain.

In wheat production, growers prefer to wait until spring to evaluate the potential productivity of their wheat crop before investing in the rest or additional N fertilizer in order to achieve wheats with optimum grain yield and protein content. However, when making such fertilization strategies, several factors should be considered. Based on results from the current study, the application of N fertilizers in spring is important in quality wheat production. However, we would like to suggest that this portion of N fertilizer should not be applied as late as has been usually considered (e.g. at heading) and the last soil N application given at late boot stage would be enough to produce wheat with optimum grain yield and baking quality. Besides, bearing in mind that S also determines baking quality of wheat flour to a great extent (Shewry, 2011), thus a balanced S and N fertilization should be maintained.

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# **Chapter 6**

## **Summary/Zusammenfassung**



## 6.1 Summary

Wheat is unique for the viscoelastic properties of its dough which could be processed into bread and other food products. Protein (concentration and composition) is crucial in determining bread-making quality of wheat flour. Applying additional nitrogen (N) fertilizers at late growth stages (heading or anthesis) is practically conducted to produce high-protein wheat. However, the application of N at late stages is inconvenient to conduct and raises the total costs in wheat production. Besides, there is increasing evidence that the relationship between grain protein concentration (GPC) and baking quality is less evident for modern cultivars. Therefore, measures to increase GPC (e.g. late N fertilization) might be dispensable for these cultivars. This thesis investigated how late N fertilization influenced baking quality of wheat flour through changes in GPC and its composition, thus to give evidences on whether the application of late N fertilizers still necessary in quality wheat production.

The main objectives of this study were (i) to reveal the effect of late N fertilization on the quantity and composition of wheat grain protein and baking quality, (ii) to distinguish whether this late N effect resulted from increased total N fertilization rate or from N splitting, (iii) to evaluate how N fertilizer types affect the protein and baking quality when applied as late N, (iv) to determine how cultivars belonging to different quality groups respond to late N fertilization. Based on these objectives, two pot experiments using two winter wheat cultivars were conducted. Moreover, a related field study (two years, two locations) was performed to check if results from pot experiments are applicable to wheat production.

Results from the first pot experiment showed that late N fertilization improved baking quality (loaf volume) of wheat flour by increasing GPC and changing protein composition (Chapter 2). Increasing N rate mainly raised concentrations of total protein and gluten fractions as well as gliadin/glutenin ratio. However, the improvement in baking quality mainly resulted from the effect of split N application (splitting of the same N rate into three doses). By split N application, both the quantities and proportions of gliadins and glutenins as well as certain high molecular weight glutenin subunits (HMW-GS) were increased, which seemed to be decisive for baking quality of wheat flour. The late N effects were greater when applied as nitrate-N than urea and these differences seemed to result from variations in plant N uptake. The late N effects were

more remarkable for the cultivar that was characterized by both, high loaf volume and GPC. However, under higher N rate and when the application of late N was postponed from late boot stage to heading in the second pot experiment, these late N effects were not found.

Results from field experiments (Chapter 3) demonstrated that split N application improved baking quality of wheat flour by enhancing the proportions of gluten fractions and certain x-type HMW-GS, which confirmed our findings from the first pot experiment. However, when the fourth N dose (additional N) was applied at heading, no further influence on baking quality was observed. This result together with that from the second pot experiment suggests that the late N applied at heading stage might be too late for quality wheat production.

Quantitative two-dimensional gel electrophoresis (2-DE) further revealed that split N application altered the relative abundances of certain individual proteins (Chapter 4). Therefore the functions of these unique proteins might exert great influences on baking quality of wheat flour and need further investigation.

In conclusion, grain protein concentration alone is insufficient for evaluating baking quality. The proportions of gliadins and glutenins as well as x-type HMW-GS correlate better with baking quality than the total protein amount. Split N application can change the N partitioning in wheat grain, thus influences baking quality. Therefore, the practice of split N application should be maintained in quality wheat production, whereas it seems possible to remove late N application at heading or to apply it earlier without deterioration in baking quality.

## 6.2 Zusammenfassung

Weizen ist einzigartig durch die viskoelastischen Eigenschaften seines Teiges, der zu Brot und anderen Nahrungsmitteln verarbeitet werden kann. Proteine (Konzentration und Zusammensetzung) sind von entscheidender Bedeutung für die Backqualität des Weizenmehls. Eine zusätzliche Stickstoff(N)-Düngungsgabe während später Wachstumsstadien (Ährenschieben oder Blüte) wird in der Praxis durchgeführt, um Hoch-Protein-Weizen zu produzieren. Allerdings ist die N-Gabe zu späten Wachstumsstadien nicht leicht durchzuführen und erhöht die Gesamtkosten der Weizenproduktion. Außerdem mehrten sich die Hinweise, dass die Beziehung zwischen der Kornproteinkonzentration (GPC) und der Backqualität bei modernen Sorten weniger erwiesen ist. Daher könnten Maßnahmen zur Erhöhung der GPC (z. B. die N-Spätdüngung) für diese Sorten überflüssig sein. In dieser Arbeit wurde untersucht, wie die N-Spätdüngung die Backqualität von Weizenmehl durch Änderungen der GPC und deren Zusammensetzung beeinflusst, um nachzuweisen ob die Applikation einer N-Spätdüngung für die Qualitätsweizenproduktion nach wie vor erforderlich ist.

Die Hauptziele dieser Arbeit waren (i) den Effekt der N-Spätdüngung auf die Quantität und die Zusammensetzung der Weizenkornproteine und auf die Backqualität aufzudecken, (ii) zu unterscheiden, ob der Effekt der N-Spätdüngung durch die erhöhte N-Düngergabe oder die Teilung der Gabe resultiert, (iii) zu bewerten, wie N-Düngerformen die Protein- und Backqualität beeinflussen, (iv) zu bestimmen, wie Sorten unterschiedlicher Qualitätsgruppen auf die N-Spätdüngung reagieren. Auf Basis dieser Ziele wurden zwei Gefäßversuche mit zwei Winterweizensorten durchgeführt. Darüber hinaus wurde eine zugehörige Feldstudie (zwei Jahre, zwei Standorte) durchgeführt, um zu überprüfen ob die Ergebnisse der Gefäßversuche auf die Weizenproduktion anwendbar sind.

Die Ergebnisse des ersten Gefäßversuches zeigten, dass die N-Spätdüngung die Backqualität (Brotvolumen) des Weizenmehls durch erhöhte GPC und veränderte Proteinzusammensetzung verbesserte (Kapitel 2). Die steigende N-Menge erhöhte hauptsächlich die Proteingesamtkonzentration und die Glutenfraktionen sowie das Gliadin-/Gluteninverhältnis. Dagegen resultierte die verbesserte Backqualität hauptsächlich

aus dem Effekt der N-Teilung (Aufteilung der gleichen N-Menge auf drei Düngergaben). Durch die N-Teilung wurden sowohl die Mengen als auch die Anteile der Gliadine und Glutenine als auch von bestimmten hochmolekularen Glutenin-Untereinheiten (HMW-GS) erhöht, was für die Backqualität entscheidend zu sein schien. Die Effekte der N-Spätdüngung waren größer, wenn sie als Nitrat-N statt als Harnstoff appliziert wurde, wobei diese Unterschiede offenbar durch Variation in der pflanzlichen N-Aufnahme zustande gekommen sind. Die Effekte der N-Spätdüngung waren bemerkenswerter für die Sorte, die sowohl durch ein hohes Backvolumen als auch eine hohe GPC gekennzeichnet war. Wenn allerdings wie in dem zweiten Gefäßversuch die N-Düngermenge erhöht und die späte N-Gabe statt zum Ährenscheiden zum Ährenschieben gegeben wurde, wurden diese Effekte der N-Spätdüngung nicht gefunden.

Die Ergebnisse der Feldversuche (Kapitel 3) zeigten, dass die geteilte N-Applikation die Backqualität des Weizenmehls durch erhöhte Anteile der Glutenfraktionen und bestimmter HMW-GS des x-Typs verbesserte, was unsere Ergebnisse des ersten Gefäßversuchs bestätigte. Wenn allerdings eine vierte N-Gabe zusätzlich zum Ährenschieben appliziert wurde, wurde kein weiterer Effekt auf die Backqualität beobachtet. Dieses Resultat zusammen mit dem des zweiten Gefäßversuches legt nahe, dass die N-Spätdüngung zum Ährenschieben für die Qualitätsweizenproduktion zu spät kommt.

Quantitative zweidimensionale Gelelektrophorese (2-DE) zeigte darüber hinaus, dass die geteilte N-Gabe die relativen Mengen bestimmter individueller Proteine veränderte (Kapitel 4). Daher könnten die Funktionen dieser besonderen Proteine großen Einfluss auf die Backqualität von Weizenmehl ausüben und bedürfen weiterer Untersuchung.

Abschließend kann gefolgert werden, dass die Kornproteinkonzentration allein unzureichend zur Beurteilung der Backqualität ist. Die Anteile der Gliadine und Glutenine sowie HMW-GS des x-Typs korrelieren besser mit der Backqualität als die Gesamtproteinmenge. Die geteilte N-Applikation kann die N-Aufteilung im Weizenkorn verändern und so die Backqualität beeinflussen. Die Praxis der geteilten N-Gabe in der Qualitätsweizenproduktion sollte daher beibehalten werden, während es möglich scheint, die N-Spätdüngung zum Ährenschieben

wegzulassen oder sie zu einem früheren Stadium zu geben ohne die Backqualität zu verschlechtern.

# Curriculum Vitae

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