FACULTY OF
BIOSCIENCE ENGINEERING

Outline of four degrees of diversification for understanding bread wheat quality

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"We know this world is good enough because it has to be.
Allow the hope that we will meet again out in the winter wheat.
Find me in the winter wheat."

– John K. Samson

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Dankwoord

"Ik ben een zalm"

Het is misschien wat onconventioneel om een dankwoord te starten met een verwijzing naar een eigenschap van jezelf, maar in deze tijden van melancholie en reflectie, biedt het me de ideale gelegenheid om aan te tonen waarom tal van mensen subiet de revue zullen passeren en een welgemeend woord van dank verdienen. Bovendien had ik zo meteen uw aandacht en wordt alvast dit deel van het doctoraat gelezen. Aldus: 'ik ben een zalm'. Los van mijn ongewoon studietraject wordt dit wel eens gebruikt als eufimisme om de manier waarop ik mijn onderzoek heb proberen uitvoeren te omschrijven: tegen de stroming in. Of zoals de persoon die subiet als eerste aan bod komt het onlangs verwoordde: 'maak jij niet alles wat moeilijk?'. En toegegeven: dat klopt. Doch, als u dit leest is er meteen voldoende bewijs dat moeilijk ook gaat. Al dient dat echter te worden genuanceerd. Dit soort moeilijk werkt alleen maar als je in een omgeving terecht komt waarin je altijd op de hulp van anderen kunt rekenen, ongeacht wat je poogt en onderneemt. En het is net hierom dat dit epistel van dankbetuigingen volgt; omdat ik te allen tijde op de onvoorwaardelijke en uitgebreide steun van tal van mensen kon rekenen, desondanks het continu tegen de stroom in gaan.

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voorraad staalpotten die je aan me hebt overgehouden.
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Tom Hellemans
February 17th, 2020

List of abbreviations

α	pasting rate
β	retrogradation/gelations rate
γ	gel stability
$\lvert G^* \rvert$	complex shear modulus
$\%\mathbf{REC}_{el}$	elastic recovery
aCL	average chain length
AL-L	Alveograph curve length
AL-P	Alveograph maximum required pressure
AL-PL	Alveograph configuration ratio
AL-W	Alveograph baking strength
AM	amylose
AMP	amylopectin
AS	area size
AUC	area under curve
BD	breakdown
BDB	National soil service of Belgium (Bodemkundige dienst van België)
BER	balanced error rate
BT	blend-trial
BWG	Biowanze gluten
CIMMyTss	CIMMyT sample set
CIRC	circularity
CLD	chain-length distribution
COH	crumb cohesiveness
CV	cross-validation
$\mathbf{CV}_{\mathbb{E}}$	environment-cross-validation
$\mathbf{CV}_{\mathbb{G}}$	genotype-cross-validation
CY-CLss	cross-year cross-location sample set
DAG	DAFA gluten
DDt	dough development time
$\mathbf{DEF}_{max}$	maximum deformation
DG	Glutomatic dry gluten
DP	degree of polymerization
DS	damaged starch
EI	expansion index
ER	overall extraction rate
FV	final viscosity
GI	Gluten Index
GLU	glutelin
GT	genotype-trial
$\mathbf{H}^2$	broad-sense heritability
HARD	crumb hardness
HFN	Hagberg falling number
HiAm	high-amylose
HiAm-WF	high amylose wheat flour
HMW:**LMWr**	ratio of HMW-GS to LMW-GS
HMW-GS	high molecular weight glutelin-subunits
HP-SEC	high-performance size-exclusion chromatography
HPAEC-PAD	high-performance anion-exchange chromatography with pulsed amperometric detection
HPLC	high-performance liquid chromatography
HS	holding strength

HtWr	height-to-width-ratio
LMW-GS	low molecular weight glutelin-subunits
LOO-CV	leave-one-out cross-validation
LPWF	low-protein wheat flour
LtWr	length-to-width-ratio
m-fold CV	m-fold cross-validation
MAE	mean absolute error
MC	moisture content
ML	moisture loss
ncomp	number of components
OvnS	oven spring
PCA	principal component analysis
PGr	prolamin-to-glutelin-ratio
PLS	partial least squares
PRO	prolamin
PROC	protein concentration
PTemp	peak temperature
PV	peak viscosity
RES	crumb resilience
rGCarea	relative gas cell area
RMSEP	root mean squared error of prediction
SBp	setback from peak
SBt	setback from trough
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SOFT	softening
SPAD	Soil Plant Analysis Development
SPRING	crumb springiness
STAB	stability
tan(δ)	phase shift angle
TEG	Tereos gluten
Tg	pasting temperature
Tg1	pasting temperature 1
Tg2	pasting temperature 2
TKW	thousand kernel weight
TSt	total starch
TW	test weight
VIP	variable importance in projection
VOLfc	loaf volume (per kilogram flour)
WA	water absorption
WA_{14}	water absorption at 14 % moisture
WA_{500}	water absorption at 500 BU
WAI	water absorption index
WBwg	Glutomatic water binding to the wet gluten
WEP	water extractable protein
WFR	water feed rate
WGC	Glutomatic wet gluten content
WpK	weight per kernel
WWF	waxy wheat flour
YLD	yield
Zadoks G.S.	Zadoks growth stage
ZEL	Zeleny sedimentation value

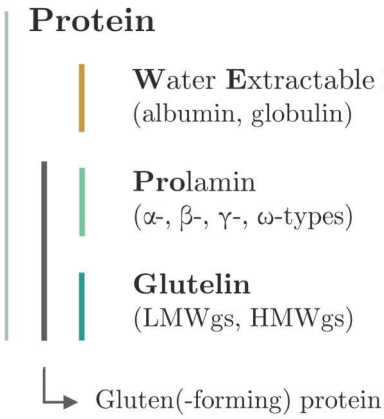
Definitions and guidelines

Throughout this dissertation, summarizing text blocks are used to further improve the understanding of the discussed topics at the time these are presented in the work. For parts which may not be in the reader's domain of knowledge and are therefore possibly difficult to follow or even irrelevant, these present the key elements that are otherwise more elaborately explained. **Axioms** show generally accepted ideas whereas **Statements** show concepts derived from own research findings. By combining these, research outcomes are translated to **Propositions** which act as provisional conclusions or encompass key attributes. Although all ideas presented in the text blocks can be derived from the full text, they may provide a slightly different insight as they act over all previous chapters. Moreover, as some chapters include divergent research questions, their conclusions are followed by **Highlights** which provide a summary the main findings.

Term	Definition
Wheat-to-bread chain	Within the scope of this dissertation, the processing chain from wheat to end-product is most commonly the one from wheat kernels to bread. However, this term is also used to refer to the more general processing chain including all stakeholders related to production, transformation and processing of wheat.
Cultivation conditions	The combination of environmental conditions (uncontrollable) and crop husbandry practices (controllable conditions) which are prevailing during the growth of wheat. 'Growing conditions' is sometimes used as alternative.
Genotype	Genotypes include all varieties within the same type (winter, spring) of wheat (*Triticum aestivum* L.).
Cultivar	A cultivar is different from a genotype as it is *commercially* available and has thus been tested in multi-annual and multi-locational field trials for its yield stability after which is was registered and included in a national variety list.
Model-system	A model-system is a simplified version of reality from which ingredients or processing steps are removed.
Fundamental	All attributes of a sample which provide a model-system independent insight in its compositional or functional properties.
Wholemeal	The term 'wholemeal' is used as a shortened and more distinct version of 'wholemeal flour'. It refers to the product obtained when integrally milling the entire wheat kernel.
Flour	The term 'flour' is used to describe 'refined flour' or 'white flour' which is the product obtained from separating the endosperm from the bran and germ in the kernel.
Gluten	The fraction of the proteins in wheat which contribute to the formation of a gluten-network; short for 'gluten-forming proteins'. During this dissertation, both terms ('gluten' and 'gluten-forming proteins') are used interchangeably.

Basic coding methods

Information on this page only applies if no details are provided in the caption of the figure or table



Color	CYCLss *Cultivar*	*Abb.*		Experimental *Cultivar*	*Abb.*	
	Atomic	ATM	1	WaxyDie	WxD	1
	Bergamo	BER	2			
	Cellule	CEL	3	1154.06	D11	2
	Collector	COL	4			
	Evina	EVI	5	Waxymum	WxM	3
	Gedser	GED	6	NX12Y48222	NXY	4
	Graham	GRA	7	Penawawa+Alpowa	P+A	5
	KWS Ozon	OZN	8			
	Mentor	MEN	9	Kanto 107//BaiHuo	KBA	6
	Popeye	POP	10			
	RGT Mondio	MND	11	Adelaide HiAm	ADE	8
	RGT Reform	REF	12	Arista HiAm	ARI	9
	RGT Sacramento	SCR	13			
	Rubisko	RUB	14			
	Terroir	TER	15	Kanto 107	KAN	7
	Tobak	TOB	16	Epi B	EPB	R
				Export flour	EXP	R

Yr.	Location	Coding		
2016	Tongeren	16.T		
	Ghent	16.G	●	
	Koksijde	16.K		
2017	Tongeren	17.T		
	Ghent	17.G	▲	
	Koksijde	17.K		
2018	Tongeren	18.T	⊠	
	Ghent	18.G	+	
	Koksijde	18.K	■	

Samenvatting

De parameters die de kwaliteit van tarwe (*Triticum aestivum* L.) bepalen, worden in vakliteratuur bekritiseerd sinds de helft van dit decennium en worden inmiddels ook in de tarweverwerkende industrie in vraag gesteld. Met een almaar toenemende vraag van consumenten voor artisanale ('*clean label*' of '*E-free*') producten in het bakkerijsegment, ligt de focus op de verkenning en het toepassen van het volledige potentieel van de onbewerkte grondstof. Wereldwijd is wintertarwe en de hieruit bekomen tarwebloem het meest gebruikt voor de productie van bakkerijproducten gezien het unieke deegreologische eigenschappen oplevert door de aanwezigheid van glutenvormende eiwitten. Echter, naast een toegenomen interesse in de aanwezigheid van minorcomponenten (lipiden, niet-zetmeel polysachariden, *etc.*), vindt er een paradigmaverschuiving plaats. De significante bijdrage van zetmeel in het tot stand komen van de deegeigenschappen wordt hierdoor gepromoot. Bovendien wordt deze ontwikkeling gesteund door de toenemende beschikbaarheid van tarwevariëteiten met een gewijzigde zetmeelsamenstelling. Beide aspecten vormen de fundamenten voor deze thesis waarin de variatie en functionaliteit van zowel eiwit als zetmeel worden onderzocht en worden gerelateerd aan broodkwaliteit.

Middels contacten met de industrie werden enkele van de voornaamste struikelbokken in de tarweverwerkende keten gedentificeerd en vertaald naar strategieën om de diversiteit inzake tarwekwaliteit te verbeteren. Deze worden gelinkt aan de opbouw van de keten welke in de introductie van het literatuuronderzoek beknopt wordt toegelicht. Daaropvolgend wordt een uitgebreid overzicht gegeven van de zetmeel- en eiwitgerelateerde eigenschappen, op moleculair en macromoleculair niveau, die bijdragen aan functionele eigenschappen en de eindproductkwaliteit van tarwegebaseerde producten. Een tweede deel focust zich op de landbouwpraktijken die kunnen worden toegepast om de kwaliteit te sturen en hoe omgeving dit kan benvloeden.

Het experimentele werk is voornamelijk opgebouwd rond tarwestalen verkregen van een genotype ($\mathbb{G}$) $\times$ omgeving ($\mathbb{E}$) studie waarin zestien variëteiten, opgegroeid in negen omgevingen (drie jaren, drie locaties) in Vlaanderen, zijn opgenomen. De studie identificeert de samenstellingseigenschappen en functionele eigenschappen van meel en bloem die best te relateren zijn met broodkwaliteit. Naast het inschatten van de bijdrage van elke fractie, wordt ook gepoogd de onderliggende mechanismen in kaart te brengen. Gezien de meeste analytische technieken ontworpen zijn om eiwitgerelateerde eigenschappen op te meten, dient tevens een verzameling analyses te worden samengesteld om een gebalanceerde set aan eigenschappen te verwerven. Derhalve werd een methodiek, gericht op de bepaling van de *samenstelling* van de tarwe-eiwitten, ontwikkeld waardoor een alternatief wordt geboden voor conventionele technieken (Zeleny sedimentatiewaarde, Glutomatic, Farinograaf, Alveograaf) en de daarbij ervaren tekortkomingen.

Door het combineren van *reversed-phase* HPLC en *machine learning* konden descriptieve en continue variabelen juist worden voorspeld op basis van de eiwitsamenstelling. Genotype kon worden voorspeld met

een hoge accuraatheid waartegen omgevingen slechter van elkaar onderscheiden konden worden. Dit wijst erop dat verschillen in de samenstelling voornamelijk een gevolg zijn van het genotypes. Deze conclusie werd deels bevestigd door de $\mathbb{G} \times \mathbb{E}$-studie waaruit tevens bleek dat de meeste eigenschappen onderhevig zijn aan een significant interactie-effect tussen beide factoren. Om een goede voorspelling van het volume—de voornaamste kwaliteitsindicator voor industrieel geproduceerd brood—te bekomen, dienden zowel eiwit- als zetmeelgerelateerde eigenschappen in het model te worden opgenomen. Bovendien werd opgemerkt dat metingen op meel aanvullende informatie opleverden tegenover dezelfde eigenschappen bepaald op bloem. Het insluiten van de eiwitprofielen bekomen via HPLC verbeterde niet alleen de juistheid van de voorspelling, het toonde ook aan verkozen te worden boven de eigenschappen bepaald via de conventionele technieken.

Aansluitend bij de $\mathbb{G} \times \mathbb{E}$-studie werden twee proeven uitgevoerd om een beter inzicht te verkrijgen in de impact van factoren die anders door 'omgeving' worden omsloten. In eerste instantie werd het gebruik van bemesting—meer specifiek stikstof (N) en zwavel (S) bemesting—om tarwekwaliteit te sturen onderzocht. Desondanks bemesting één van de meest conventionele praktijken is, werd via het literatuuronderzoek aangetoond dat veel onduidelijk bestaat over welke praktijken (tijdstip, fractionering en dosering) meest effectief zijn. Door de strenger wordende regelgeving aangaande de toegelaten stikstofgift, dringt de vraag of dit limiterend is voor de productie van kwaliteitstarwe in België zich ook op.
Daarnaast werd bodemtype in relatie tot plantengroei en korrelsamenstelling nagegaan gezien het ontbreken van literatuur hieromtrent. De hypothese werd gesteld dat dit een mechanisme kan bieden om de anders ernstige gevolgen van warmte- en droogtestress te verminderen.

Op basis van de bemestingsproeven werd aangetoond dat de huidige restricties niet noodzakelijk limiterend zijn voor de tarwekwaliteit In België. Er werd geen significante impact op opbrengst of het eiwitgehalte waargenomen bij een toenemende stikstofgift in drie en vier fracties. De applicatie van vier N-fracties aan een lagere hoeveelheid (30 % onder advies) was efficiënter dan meer N toe te dienen (30 % meer dan advies) in drie fracties. Het toepassen van S was in alle gevallen bevorderlijk voor de eiwitgerelateerde eigenschappen en de broodkwaliteit, zelfs in concentraties lager dan de aanbevolen dosis. De efficiëntie van de S-bemesting nam verder toe wanneer het tezamen met de derde N-fractie werd toegepast in vergelijking met een toepassing tussen de derde en vierde N-fractie aan het begin van de korrelvulling. Terwijl een meer intensieve bemesting (met zowel N als S) altijd tot een toename in het eiwitgehalte leidde, werd een algemene afname in de glutensterkte waargenomen voor N-bemesting waartegen S de sterkte *en* uitrekbaarheid van de gluten bevorderde.
Desondanks verschillen in de groei van de tarwe werden waargenomen tussen beide bodemsoorten, was de impact op opbrengsteigenschappen en korrelsamenstelling beperkt. Effecten van droogtestress werden verminderd door de kleibodem waardoor een verschuiving in de samenstelling van de glutenvormende eiwitten alleen werd opgemerkt voor alle variëteiten die een watertekort hebben ervaren. Bovendien leidde de warmtestress tot een verdubbeling van het *eiwitgehalte* in de korrel wat echter niet opweegt tegen de opbrengstverminderingen gerelateerd aan droogtestress.

In het tweede deel van deze thesis werden twee voorname functionele eigenschappen gerelateerd aan de interactie tussen (gluten)eiwitten en zetmeel, meer specifiek: waterbinding en het verstijfselingsgedrag. Beide werden op een meer fundamentele manier bestudeerd door gebruik te maken van extrusie. Deze *high-temperature short-time* verwerkingstechniek werd bovendien geselecteerd voor zijn huidige industriële

relevantie en de beperkte hoeveelheid literatuur beschikbaar over de applicatie van tarwe hierin. Twee afzonderlijke onderzoeken werden uitgevoerd. Ten eerste werd de eiwitconcentratie en -samenstelling in relatie tot het vochtgehalte in de *feed* bestudeerd. Er werd getracht een passend concentratiebereik of functioneel gedrag te bekomen waarbij expansie optimaal was terwijl obstructie van de extruder werd vermeden. Resultaten toonden aan dat zowel de eiwitconcentratie als de interactie met het vochtgehalte van de *feed* bepalend zijn voor de kwaliteit van de extrudaten. Eiwitsamenstelling zorgt voor een verschuiving in het moment waarop de macroscopische effecten optreden. Deze worden bovendien gerelateerd aan de viscositeit van de *melt* wat op zijn beurt voornamelijk met waterbeschikbaarheid en zetmeelverstijfseling in verband wordt gebracht. Dit is ook bevestigd door het tweede onderzoek waarin de zetmeelsamenstelling werd gevarieerd door het maken van blends. Bloem vrij van amylose (*waxy* tarwebloem) tot bloem met reguliere concentraties werd gebruikt. Een verlaging van de *melt* viscositeit ligt aan de basis van een toegenomen expansie met een optimum bij een beperkte afname van het amylosegehalte.
De in beide onderzoeken waargenomen verschillen in de textuur werden gerelateerd aan eiwit- en amyloseconcentraties en de toegevoegde hoeveelheid water tijdens de extrusie. Ook de breekbaarheid variëerde afhankelijk van de interne structuur van de extrudaten welke middels *X-ray micro-computed tomography* werd onderzocht.

In een finaal onderzoek werd de samenstelling van de zetmeelfractie verder uitgebreid door het insluiten van *partial waxy* en *high-amylose* tarwevariëteiten wat resulteerde in een amyloseconcentratie variërend van ≤ 1 tot 49 %. Deze variëteiten werden toegepast in een broodbereidingsproces om hun mogelijk meerwaarde voor de productie van bakkerijproducten bloot te leggen. Een adequate kruimstructuur werd waargenomen voor slechts één *waxy* variëteit. Dit werd gerelateerd aan een hogere deegsterkte wat opnieuw wijst op de interactie met de eiwitsamenstelling. Analoog aan de extrusie-experimenten, werd tevens geconcludeerd dat een minimale hoeveelheid (7 %) amylose benodigd is voor het bekomen van een toegenomen broodvolume met een gelijkaardige kruimstructuur als broden op basis van reguliere tarwebloem. De kruimhardheid van het *partial waxy* brood bleef lager over een bewaringsperiode (afgesloten) van vier dagen. Broden op basis van *high-amylose* tarwebloem waren daarentegen aanzienlijk kleiner en hadden een significant hardere kruim.

Deze thesis biedt een gedetailleerd inzicht in mogelijke strategieën om tarwekwaliteit te verbeteren. Een applicatie-gedreven selectie van genotypes is één van de geprefereerde methoden. Hiervoor is evenwel een nauwgezette kennis van bepalende eigenschappen om een kwalitatief eindproduct te verkrijgen een vereiste. Het huidige begrip hiervan is echter te beperkt om reeds allesomvattende doelen voor veredeling op te stellen of om landbouwpraktijken zo toe te passen dat ze gegarandeerd tot een verbeterde kwaliteit aanleiding geven. Huidige resultaten tonen weliswaar aan dat het momenteel heersende gedachtegoed om het eiwitgehalte te verhogen en tezelfdertijd hoge opbrengsten te behouden, moet worden herzien. Dit biedt bovendien geen garantie op een goede bakwaardigheid. De bijdrage van zetmeeleigenschappen dient daarenboven tevens als cruciaal te worden beoordeeld. In dit licht is het aanbevolen om niet uitsluitend op momenteel beschikbare technieken voor kwaliteitsbeoordeling te berusten. Hoge-resolutie data en vooruitstrevende mathematische modeleringstechnieken moeten worden gecombineerd wanneer de relatie met bakkwaliteit wordt onderzocht of voor het verbeteren van een diversificatie richting de variërende toepassingsgebieden van tarwe.

Summary

The attributes defining the quality of wheat (*Triticum aestivum* L.) are under discussion in professional literature since the mids of this decade and are now being questioned in the wheat-processing industry as well. With a continuously increasing consumer demand for artisanal products ('*clean label*' or '*E-free*') in the bakery segment, the focus lies on the exploration and employment of the full potential of the raw input material. Globally, winter wheat and the therefrom derived refined flour is most frequently used for producing bakery products due to its unique dough rheological properties resulting from the presence of the gluten-forming protein. However, besides an increased interest in the presence of minor components (lipids, non-starch polysaccharides, minerals, bran, *etc.*), a paradigm shift emphasizing the significant contribution of starch to this behavior is most certainly taking place. This development is further supported by the increasing availability of wheat cultivars with an altered starch composition. Both aspects form the foundations for the current dissertation in which the variability and functionality of both protein and starch are investigated and related to bread quality.

Through contacts with industry, some of the main hurdles in the wheat processing chain were identified and translated to a framework of strategies to improve diversity in terms of wheat quality. These are related to the buildup of the chain which is outlined in the introduction of the literature review. Thereafter, a comprehensive overview of starch- and protein-related parameters on a molecular and macromolecular level is provided to show their relevance for explaining functional behavior and the end-product quality of wheat-based products. A second part focuses on the crop husbandry practices which can be used to steer wheat quality attributes as well as how environment may influence this.

The experimental work is primarily arranged around the wheat samples from a genotype ($\mathbb{G}$) $\times$ environment ($\mathbb{E}$) study comprising samples from sixteen cultivars grown at nine environments (three years, three locations) in Flanders. The study identified the compositional and functional variables of wholemeal and refined flour relating best to bread quality. Apart from an estimation of the contribution of each fraction, this aided in uncovering underlying mechanisms. As most analytical techniques are designed to measure protein properties, an analytical toolbox had to be established to obtain a balanced set of parameters. Hence, a composition-based approach for analyzing wheat proteins was developed to overcome the shortcomings described in literature for conventional protein-oriented techniques (Zeleny sedimentation value, Glutomatic, Farinograph, Alveograph).

By combining reversed-phase HPLC and machine learning techniques, descriptive and continuous variables could be accurately forecasted on the basis of the protein composition. Genotype could be predicted with high accuracy whereas environments were more difficult to distinguish, indicating that protein composition is mainly influenced by genotype. This was partially confirmed by the $\mathbb{G} \times \mathbb{E}$-study wherein for most parameters, a significant interaction-effect was noted. For obtaining an accurate prediction of the loaf volume—the main quality indicator of industrially produced bread—both protein- and starch-related attributes had to be included in the model. Furthermore, it was shown that measurements on wholemeal

flour provided supplementary information compared to their analog measurement on flour. Inclusion of the chromatographic data did not only improve the accuracy of the predictions, it was also preferred to parameters obtained through conventional techniques.

Next to the $\mathbb{G} \times \mathbb{E}$-study, two additional trials were conducted to obtain a more detailed insight in the influence of factors otherwise embodied by the factor 'environment'. Firstly, the use of fertilization, more specifically nitrogen (N) and sulfur (S) fertilization, to steer wheat quality was investigated. The literature review revealed that it remains unclear which practices (timing, fractionation and dosage) are most effective, despite fertilization is one of the most commonly applied strategies for quality improvement. With legislation becoming more restrictive in terms of the allowed N dosages, its possibly limiting effect on wheat quality in Flanders was investigated.
Secondly, soil type in relation to plant growth and kernel composition was investigated in more detail as literature on this is lacking. Its interaction with heat- and drought-stress was also studied as it was hypothesized to provide a mechanism allowing to reduce the otherwise detrimental effects on wheat quality.

From the fertilization trials, it was shown that prevailing restrictions are not necessarily limiting for wheat quality. No significant increase in wheat yield or the protein content was observed at increasing N-dosages at three and four N-fractions. Applying four fractions at lower dosages (30 % less than the suggested dosage) was also more effective than applying 30 % more N in three fractions. Application of S was always beneficial for protein-related attributes and bread quality, even at amounts lower than the suggested dosage. Its impact further increased when it was administered together with the third N-fraction instead of separately (between the third and fourth N-fraction) at the beginning of the kernel filling stage. Whereas more intensive fertilization treatments (both N and S) increased the protein content, a general decrease in gluten strength was perceived for N-fertilization whereas S stimulated gluten strength *and* its extensibility. Although differences in plant growth were observed when comparing both soil types, impact on yielding parameters and kernel composition was limited. However, clay soil showed to mitigate effects related to drought-stress whereas a shift in the composition of the gluten-forming proteins was noted for all cultivars which experienced a water shortage. Heat-stress furthermore lead to a doubling of the protein *content* although this did not outweigh the yield deprivation resulting from drought.

In the second part of this dissertation, two main functional properties related to the interaction between (gluten) protein and starch, more specifically water binding and pasting behavior, were studied in a semi-fundamental way by the use of extrusion processing. This high-temperature, short-time processing technique was also chosen for its current relevance in industry and the limited amount of research available on the applicability of wheat therein. Two separate studies were performed. Firstly, protein concentration and composition in relation to the moisture content of the feed was studied. It was attempted to find a suitable concentration range or functional behavior at which expansion was optimal while preventing ceasing of the extruder. Results showed that protein content and the interaction with the feed moisture content was determining for the quality of extrudates. Protein composition on the other hand promoted or delayed shifts in the studied macroscopic effects. Most of these were traced back to the melt viscosity with water availability and starch gelatinization being the two most important underlying mechanisms.
This was also confirmed in the second study in which the starch composition was varied by making blends, going from virtually no amylose (waxy wheat flour) to regular amylose contents (regular wheat flour).

The lowering of the melt viscosity promoted expansion with an optimum at slightly reduced amylose concentrations. In both studies, textural differences were observed and related to the protein and amylose concentrations and the amount of water added during extrusion. Also the brittleness varied according to the internal structure of the extrudates, revealed using X-ray micro-Computed Tomography.

A final study further broadened the compositional differences in the starch fraction by including partial waxy and high-amylose wheat cultivars resulting in amylose concentrations ranging from ≤ 1 to 49 %. These were applied in a breadmaking process to shed light on their potentially added value in bakery products. The presence of an adequate crumb structure for a single waxy cultivar was related to its higher dough strength, again indicating an interaction with protein composition. Analog to extrusion experiments, a minimal amount of amylose (7 %) is required to obtain an increased loaf volume with a similar crumb structure as regular wheat flour based breads. Crumb firmness of partial waxy wheat breads remained lower during a four day storage period (lowered staling rate) whereas breads from high-amylose wheat flour were significantly smaller and had a markedly firmer crumb.

To conclude, this dissertation provided a detailed insight in possible strategies for wheat quality improvement in a chain-wide perspective. Application driven genotype selection is one of the preferred strategies although a prerequisite is the profound knowledge on determinative factors to obtain high quality end-products. Notwithstanding, current insights are too limited to select all-encompassing breeding targets or to apply crop husbandry to accurately steer wheat quality. However, findings show that the current mindset of increasing the protein content while maintaining a high yielding potential is outdated and does not guarantee a high baking quality. Starch properties have to be considered as equally important. It is therefore also recommended not to rely solely on currently available techniques for quality screening. High-resolution data and advanced mathematical modeling have to be combined when studying the relation with baking quality or to improve diversification towards wheat's various fields of application.

Introduction

The enduring increase in the production of winter wheat (*Triticum aestivum* L.) results from its broad field of application in a variety of industries. Belgium is one of the main countries in Europe in which the major stakeholders in the so called '*wheat-to-bread*'-chain (farmers, mills and food producers) are remarkably well represented due to the heritage of farming and a thriving food industry. Mainly the cereal processing and bakery industry are well represented, combined accounting for 14.2 % of the market share (Federatie van de Belgische Voedingsindustrie (Fevia), 2019). Moreover, many of the produced (frequently wheat-based) foods are globally renowned and thus, exported. For the production of bread—the main staple food which is by dietary guidelines also recommend to be consumed on a regular basis—wheat remains the most important raw materials due to its unique properties. Additionally, although it has a prominent cultural value for most Belgians, a shift in the purchasing behavior from artisan bakery (small-scale) to supermarket (industrially produced) is clearly noticeable.

To meet the desired production capacity and to produce such high-quality products, wheat is imported from neighboring and/or top producing countries such as France, Germany, the United States, Canada and Russia. Through this chain globalization, wheat has become a commodity good resulting in the need for an enhanced standardization in terms of price setting and quality screening. Mainly the approaches applied for the latter are however proven to be insufficient resulting in production-line downtime, defects in end-products and, eventually, economic losses and food waste (AIBI International Association of Plant Bakers, 2017). At the same time, the bakery industry is on the verge of a movement towards the implementation of '*Industry 4.0*' which is required to remain competitive. At right angles to this is the high uncertainty associated with the application of wheat—a highly fluctuating raw material—in a wide diversity of complex food products on an industrial scale.

The four degrees of diversification

The search for approaches to tackle the currently faced hurdles in industry brings researchers around to world to a primary research question: how is wheat quality defined? However, to answer this question, an improved understanding of the relation between key quality attributes, perceived differently by all main stakeholders in the chain, is required. In essence, the impact of cultivation conditions (both controllable and uncontrollable) on wheat composition has to be understood more thoroughly as well as how this relates to end-product quality. Four approaches—denoted as the 'four degrees of diversification' (Figure 1)—connected to the primary topic and its underlying questions are investigated in this dissertation.

Firstly, wheat quality can be defined by the application in which it will be applied. In this case, it already has to be known which compositional and functional attributes of the wheat are determining for obtaining the desired end-product quality. Nevertheless, selecting the genotype ($\mathbb{G}$) possessing these properties does

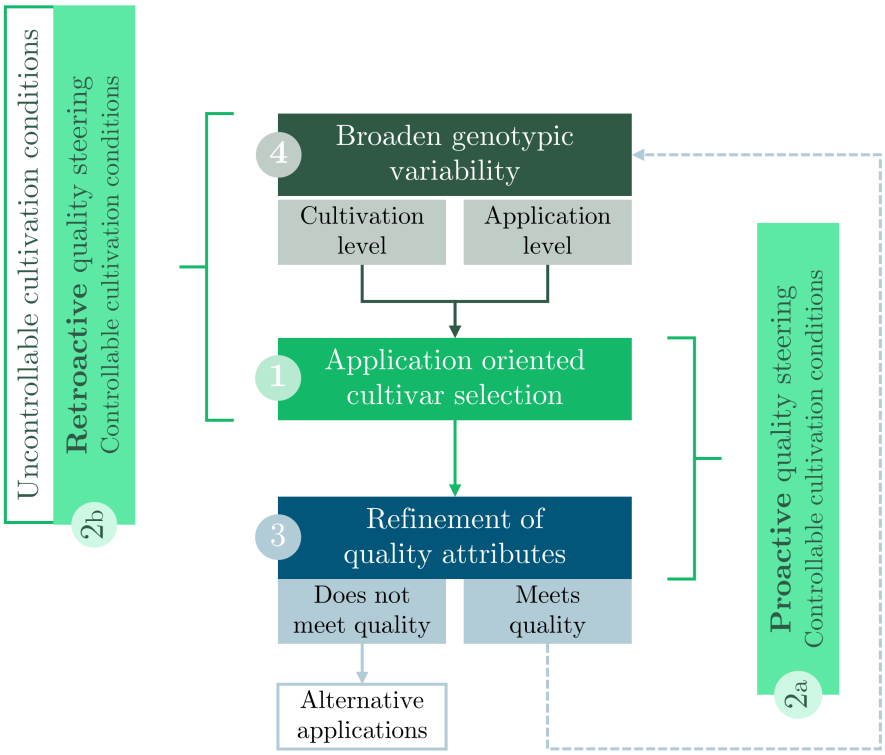


Figure 1: Schematic overview of the 'four degrees of diversification', approaches for improving wheat quality, which form the basis of the current dissertation.

not directly assure its optimal expression as environmental conditions ($\mathbb{E}$) may influence these. Thus, a second question urges itself: which of the (relevant) quality attributes are dependent from $\mathbb{E}$-effects and to which extent? In light of farmers, it may be more relevant to know how wheat quality attributes can be steered using cultivation properties (proactive, 2a) or how the latter can be applied for circumventing unfavorable, uncontrollable environmental conditions (retroactive, 2b). Thirdly, a refinement of the knowledge of the relation between composition and end-product quality will remain consistently important. To allow a more nuanced diversification at earlier stages (before arriving at the food producer), this is preferably acquired in a model-system independent (*i.e.* fundamental) way. This also forms a prerequisite for the development of techniques for quality screening which remains a challenge due to the compositional complexity of wheat and its derivatives (wheat flour or wholemeal). Finally, the vulnerability of wheat to climate change demands for genotypes which are more resilient against extreme weather events (besides pests and diseases). In light of the first degree of diversification, these quality attributes on a cultivation level should be accompanied by a broadened genotypic variability towards the application. For example: to produce 'clean label' or 'E-free' foods, it may be beneficial to use currently unexplored compositional properties of specific wheat genotypes.

Research outline

The first part of the literature review provides an elaborate overview of the compositional attributes of wheat which have been linked to functional properties and bread quality. In this way, a solid basis is obtained for testing the outcomes of the experimental work performed in this research against. Moreover,

the environmental impact on conventional quality attributes is discussed in the second part of **Chapter 1**, thereby building upon some of the ideas discussed in the primary part of the chapter. A schematic overview of the complete research outline can be found in Figure 2.

To be able to properly screen wheat composition and functionality, an adequate toolbox of analytical techniques should be available. As this is one of the main hurdles on an industrial level—and therefore also included in one of the 'four degrees of diversification'—it was attempted to develop a novel approach through the combination of a fundamental technique with advanced data processing. In **Chapter 2**, a validation of the technique is described followed by a *proof-of-principle* to illustrate the potential of the presented approach.

Subsequently to the two more supporting chapters, **part I**—which covers three chapters related to experimental work on wheat cultivation—of the dissertation is started. **Chapter 3** uses a comprehensive dataset obtained from a cross-year cross-location field trial to elucidate on the factors determining for the quality prediction of wheat-based bread. In industry, the general consensus is that protein properties are virtually solely determinative for the bread quality. However, by using an extensive set of protein and starch related attributes, supplemented with data from the earlier described approach, the overall contribution of the starch fraction to bread quality can be estimated more accurately.

Secondly, as protein content remains important, the feasibility to use fertilization to steer both content and quality is investigated in **Chapter 4**. Outcomes are coupled with legislative restrictions and it is discussed if the latter impede the production of high-quality wheat in Flanders. By including two harvest years for the nitrogen fertilization trials, stability of wheat quality traits is further investigated.

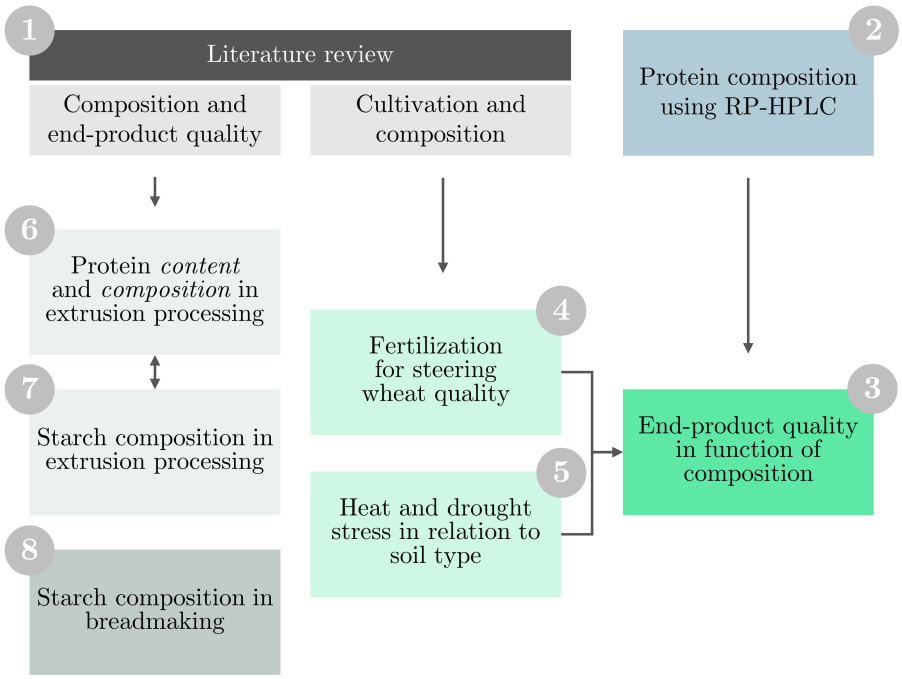


Figure 2: Schematic overview of the outline of the research. Colors refer to the main '*degree of diversification*' to which the chapter is related.

To conclude the first part of the dissertation, **Chapter 5** discusses how heat and drought stress may impact wheat growth and composition using a semi-fundamental greenhouse setup. Outcomes of this research

are used to partially cover the impact of uncontrollable parameters during wheat cultivation and how these interact with the soil type.

Supplementary to the horizontal view on the chain from cultivation to end-product, **part II** covers a more delineated approach in terms of uncovering the relation between composition and functionality in the next three chapters. A main focus was on the interaction between starch and protein in wheat and how a broadened diversity in the former can be used to enable functional attributes. This was illustrated in both breadmaking and extrusion processing to emphasize that an increased genetic variability may also promote the use of wheat in novel applications. Meanwhile, it is accentuated that this demands for a reconsideration of common assumptions on wheat quality throughout the wheat-processing chain.

Undesirable viscoelastic properties of the melt can be measured through quality attributes of the end-product. As the presence of gluten-forming proteins in wheat may greatly affect these properties, it was studied how protein content and composition influence extrudate quality in relation to the feed moisture content. In this way, **Chapter 6** contributed to the development of basic outline of compositional attributes under which wheat could be applied in extrusion processes.

Chapter 7 continues on this by separately investigating how a variation in the amylose concentration might counteract the possibly detrimental effects of wheat gluten proteins. Furthermore, an elaborate screening of the so called 'waxy' genotypes applied in these experiments provided insight in the phenotypic variability on a starch compositional level. Finally, the potential of such genotypes for producing 'ready-to-eat' snacks with improved sensory characteristics was evaluated.

An even broader diversity in the starch composition was attained by studying waxy, partial waxy, regular, and high-amylose wheat genotypes in the final chapter (**Chapter 8**). Besides testing their performance through breadmaking, the feasibility to apply conventional analytical techniques on these raw materials was investigated. Lastly, research outcomes provide an alternative insight in the reason why protein properties mainly contribute to predictions of bread quality attributes in both previous and current research.

CHAPTER 1

Composition and functionality of wheat in relation to bread quality and cultivation conditions

Part of this chapter (from Section 1.4) is adapted from: Hellemans, T.*, Landschoot, S.*, Dewitte, K., Van Bockstaele, F., Vermeir, P., Eeckhout, M., & Haesaert, G. (2018). "Impact of crop husbandry practices and environmental conditions on wheat composition and quality: a review," *Journal of agricultural and food chemistry*, 66(11), 2491-2509.

*Both authors contributed equally to this publication.

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1.1 Wheat production and processing

Wheat (*Triticum aestivum* L.) is embedded in a variety of cultures and even religions throughout the world. The basis of its renowned status lies in its high yielding potential, its adaptability to a broad range of cultivation conditions (and thus, its global availability) and the diversity in both its composition and nutritional value (Shewry, 2009). Another key element that has contributed to the persistent success of wheat is the presence of gluten proteins, which provide unique dough rheological properties upon mixing with water. This further emphasizes wheat's applicability to a broad range of food products including: breads, biscuits, cakes and pastries, but also pasta, noodles, *etc.* (Woychik et al., 1961; Day et al., 2006).

In the past few decades, modification of the structure of gluten proteins by novel processing technologies has been increasingly explored. In this way, their functionality was altered thereby broadening their applicability as emulsifiers and texturizers. As such, they provide an alternative for milk and soy proteins (Day, 2011). Wheat gluten can also be used for protein enrichment in both food and feed products (Day et al., 2006) or can form the basis for the production of texturized vegetable proteins (*i.e.* meat analog products) (Smetana et al., 2015; Malav et al., 2015; Kumar et al., 2017). Moreover, with (wholemeal) wheat flour containing 65–80% starch, wheat also forms the preferred ingredient for the production of a wide diversity of staple foods, as dietary guidelines recommend that a majority (40–75 %) of the energy intake occurs through the consumption of (complex) carbohydrates (Buyken et al., 2018).

Besides gluten and starch, which occur as both individual byproducts or as the main components of wheat flour, waste streams of wheat processing can be of high value when valorized correctly. Ravindran and Jaiswal (2016) provided an overview of the waste streams in plant processing coming from agricultural practices and food production as well as from the distribution and consumption of both the raw and processed materials. For wheat, both the straw and husk are considered as the main waste streams, even though these were found to be the ideal candidates for second generation biofuel production. In addition, the bran (another byproduct) can be considered as a valuable additive for producing fiber enriched foods or extracting fructans and arabinoxylan (and its derived oligosaccharides) from. On their turn, these can be used for stabilizing purposes in the production of (silver) nanoparticles or can be applied in health promoting foods due to their prebiotic effects (Grootaert et al., 2007; Singh et al., 2015; Joelsson et al., 2016).

> Global wheat cultivation has a historical background although the application of wheat or its waste side-streams in food and non-food products continues to expand. Within the food industry, the presence of gluten-forming proteins provide a unique advantage thereby promoting the application of wheat in a variety of bakery products.

Despite the broad applicability of wheat, to date, a poor diversification on the basis of the screened quality attributes exists. This partially results from the way wheat and its derivatives (wholemeal and flour) are being analyzed. Frequently, only the main uses are taken into consideration (Alexopoulou, 2010). For example, within the current wheat processing industry, three main fields of application are distinguished: wheat for the production of (a) high-demanding bakery applications (bread), (b) less-demanding bakery

products (biscuits, cakes, pastries), and (c) animal feed. Discrimination between these classes is currently made on the basis of easily determinable wheat quality attributes which also form the basis of commonly used grading systems for wheat classification.

In the 1870's, grading systems were developed to overcome some of the hurdles of local and national but mainly international wheat trading. By roughly classifying wheat using generic compositional and yielding attributes, it was attempted to uniformly assess quality (Kennett et al., 1998). However, uniformity within a grade could not be established due to differing environmental (mainly soil and climate) conditions. In addition to a lack of clear objective, the standard measures and the therefor used instrumentation provided only low-resolution data. Together with the concept itself, classification thus promoted an overall quality reduction through inadequate diversification (Hill, 1990). Nowadays, optimized grading systems are used for international trading purposes and are supplemented with additional measures for quality estimation defined in the receiving specifications. This accounts for both millers and food producers as their end-products (flour (blends) or food products, *resp.*) are highly dependent on the stability of the incoming material (wheat or (wholemeal) wheat flour, *resp.*).

Apart from physical quality characteristics (physical soundness, cleanliness, *etc.*), attributes enclosed in receiving specifications are the protein content, Zeleny sedimentation value and enzyme activity (*i.e.* α-amylase) (Hellemans et al., 2018). Depending on the application, these are supplemented by the Farinograph water absorption, the Alveograph configuration ratio and the Alveograph baking strength. According to Huen et al. (2018b), bakery companies rely on values provided on analytical certificates which typically include 5–10 parameters. In contrast, the US wheat marketing system relies primarily on the wheat kernel hardness and the growing season despite physicochemical and rheological tests are widely used in industrial R&D-settings (Chapman et al., 2012). However, as these currently used measures fail to describe wheat quality sufficiently, deficits of the end-products are frequently observed even when the receiving specifications are met (Guzman et al., 2016; Gwirtz et al., 2007). This implies the enduring presence of three major shortcomings: (a) currently used quality attributes (both compositional and functional) do not provide enough predictive power to accurately forecast end-product quality, (b) the screened characteristics are too stringent, not taking into account the complexity of wheat quality and (c) functionality related to processing is not fully covered. Probably even more determining is that quality is defined differently by each stakeholder in the wheat-to-bread chain with only a small overlap between subsequent links.

In contrast, it is inherent to the globalization of the supply chain that quality cannot be measured in detail before buying the raw material. As is illustrated in Figure 1.1, the vertical and extensive build-up of the chain and the distinct drivers promote the existence of physical and intellectual bottlenecks. Although this is the main reason to retain the use of grading systems, various stakeholders call for a more holistic view on quality through alternative approaches. One of the opportunities lies in the way of providing chain-wide feedback. Possible flows are shown in the top frame of Figure 1.1. Currently, the limited number of feedback loops which are solely between consecutive stakeholders, forms a major drawback in efficient quality enhancement. Alternatively, the breeder could (indirectly) obtain feedback from the final actor (*i.e.* the consumer) and use this to adapt its goals in terms of the delivered product. In an ultimate situation, however, information would be shared between all stakeholders (including third parties) in the chain, thereby supporting the search for a *common* goal.

> Grading systems simplify international trading and supports top-level diversification, but do not ensure an enhanced selection in terms of wheat quality as underlying parameters are too unspecific to be related to end-product quality.
> For industrial mills, a mismatch exists between the quality desired by the food producer and the quality of the incoming product. Breeders and farmers perceive wheat quality entirely different than the industries which are eventually using the derivatives of their products. The use of grading systems partially circumvents this imbalance but also reduces diversification at the same time.

To optimize the efficiency of quality screening within the current chain situation, a large-scale study (FLOURplus) investigated which (combination of) parameters and analytical methods used across Europe, reflect(s) baking quality best. As the majority of applied techniques tests dough quality empirically, it makes them more suitable to rapidly and easily predict baking quality (*i.e.* using a single technique without advanced data processing). On the other hand, they are limited to a narrow range of end-products (*i.e.* only leavened white bread) as they mimic a specific formulation and processing (Chin and Martin, 2014; Salimi Khorshidi et al., 2018). Moreover, numerous analytical parameters were found to be significantly correlated, making them provide no supplementary information. Only specific combinations of parameters resulted in a satisfactory prediction of bread quality (AIBI International Association of Plant Bakers, 2017).

The fact that emerging, industry-relevant methods (such as the Glutopeak and solvent retention capacity) provide little to no additional information for improving the accuracy and robustness of the prediction, emphasizes the need to search for alternative approaches (Huen et al., 2018b). This can be attained by developing a fundamental (*i.e.* model-system independent), analytical technique for determining key compositional attributes *or* by applying advanced data modeling techniques on past data in order to build powerful models (Stojceska and Butler, 2012; Bouachra et al., 2017; Peressini et al., 2017; Huen et al., 2018a; Salimi Khorshidi et al., 2018). However, in the current market situation (vertical), a consistent information flow between all stakeholders—mainly between elevators, flour mills and food producers—is lacking (Lefeber and Landschoot, 2019). This encourages the loss of details on the overall quality of the final material (Hamprecht et al., 2005). Limitations by the virtue of the wheat-to-bread chain's complex structure have to be overcome when thinking about implementing the aforementioned approaches (Kennett et al., 1998).

> The complexity of the wheat-to-bread chain and consequently, the lack of a consistent, downstream information flow impede the use of 'big data' approaches for quality forecasting. Analytical techniques are also oriented towards specific industries (or fields of application) and are therefore not model-system independent.

Besides the limited time and possibilities for quality screening upon receipt (at the level of processors) and the presence of information gaps between stakeholders, poor year-to-year quality consistency (*i.e.* genotype stability) forms a major hurdle for wheat processors. Shewry (2009) states that this is one of the main challenges for future wheat cultivation. As these fluctuations are a result of genotype-

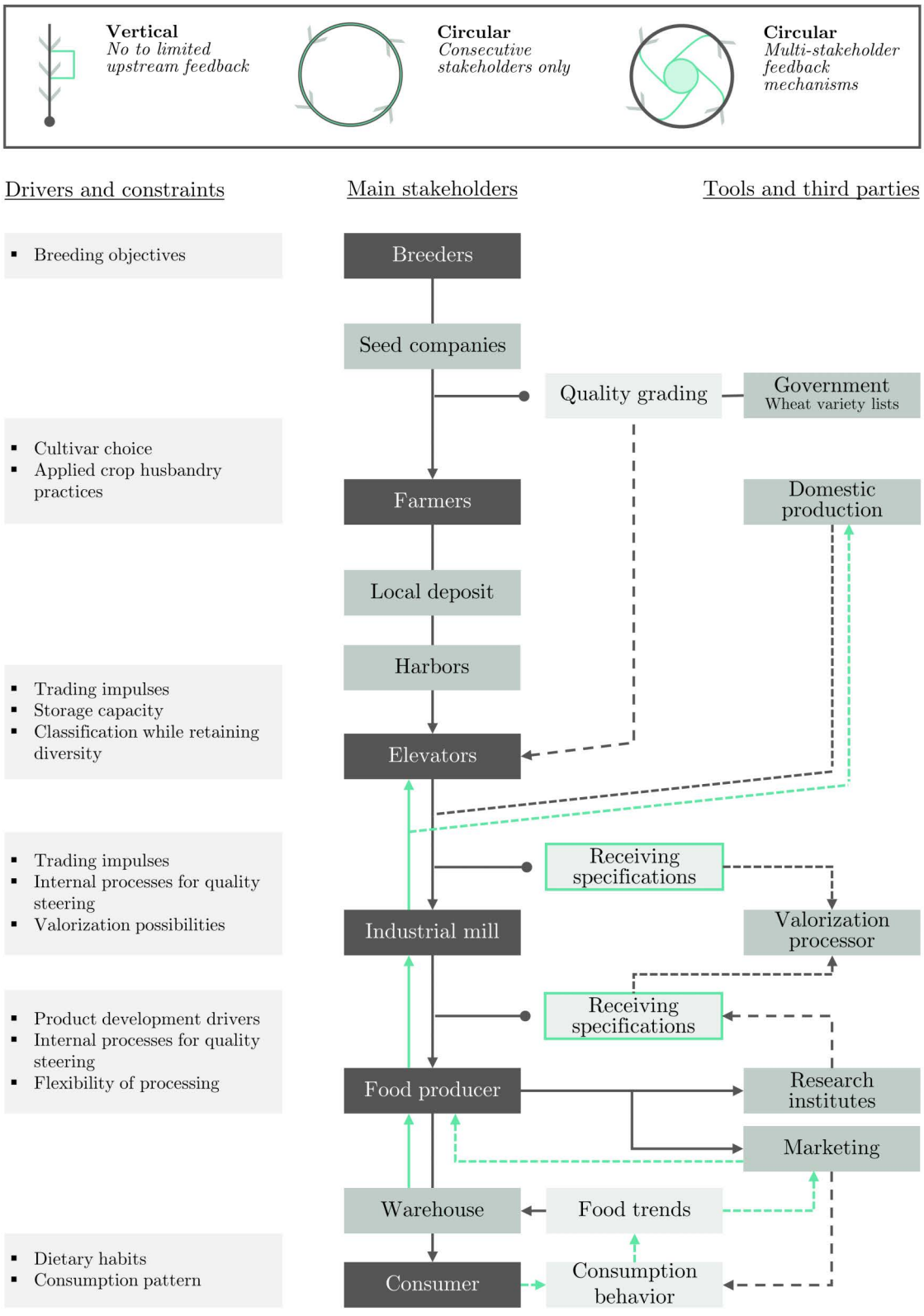

Figure 1.1: Simplified flow chart of the wheat-to-bread chain with an indication of the most common feedback loops (- -) in the current *vertical* market situation. Main drivers and constraints per stakeholder are indicated in the left boxes. Alternative situations for data flows (circular with subsequent or distributed feedback) are illustrated in the top frame.

environment ($\mathbb{G} \times \mathbb{E}$)-interactions and global climate change continues to increase environmental variation, trait stability will become even more important in wheat breeding (Peterson et al., 1997; Huang et al., 2016).

Strategies to counteract this type of variation can also be implemented at the level of industrial mills or food producers by means of blending, *i.e.* mixing batches of wheat flour to obtain specific compositional or functional attributes. Recently, the trend has been the establishment of a direct triangular relation between the farmer (domestic production), the industrial mill and the food producer. The latter can administer its own laboratory tests or, even more desirable, can establish which wheat cultivars possess the most desirable end-use characteristics (Kennett et al., 1998). Such feedback mechanisms, illustrated by the green dashed arrows in Figure 1.1, are of great importance to efficiently match wheat quality to the specific needs of the food processors. However, bypassing the consecutive construct of wheat producer, elevator, mill and food producer may entail additional costs and risks and. Therefore, it will require an extensive knowledge of the relation between wheat composition and end-product attributes.

Despite the divergent approaches to improve the sustainability of the wheat processing chain—including (method development for) enhanced quality screening, redefining breeding goals, more nuanced classification and diversification, *etc.*—it is crucial to fully understand, in a model-system independent way, which compositional and functional attributes are determinant in making an accurate prediction of the baked good's end-product quality.

> When optimizing the chain, extensive knowledge of which attributes to screen to which level of detail is required as this forms the basis of quality enhancement on all stakeholder levels, independent from the used approach.

This literature review provides an overview of the major starch and protein related attributes, how these correlate with the empirical techniques for flour quality screening and, eventually, how they influence bread quality. In a second part, it is elaborately described how controllable and uncontrollable cultivation conditions affect (basic) wheat quality attributes. In this way, a full overview of the factors influencing end-product quality, starting on the field, is provided.

1.2 Compositional attributes

Within the compositional attributes, two levels can be distinguished: properties on a molecular level and elements which are constituted on a *macro*molecular level (Figure 1.2). For starch, amylose and amylopectin (and their fine-structural properties) are considered to be on the former level whereas features related to the granular structure are classified on the second level. Effects caused by interactions (between granules or with other components) result in specific functional behavior, therefore being distinguished from inherent compositional attributes.

For proteins, the presence (and ratio) of specific fractions and the molecular weight-distribution is placed under molecular attributes whereas higher protein structures (tertiary and quaternary level) and the formation of the gluten network are classified under macromolecular properties. The latter can also be

divided in spontaneous (as part of the biosynthesis) and mediated (by the addition of shear and/or the presence of other components) occurrences.

Apart from compositional attributes, fundamental functional properties which provide additional insights in interactions between protein, starch and water are discussed. The majority of the functionalities require a difference in energy which has to be applied under the form of temperature (increase or decrease) or shear (mixing). Eventually, in breadmaking, dough extensibility and elasticity are considered majorly determining and vary according to differences on a compositional (molecular and macromolecular) and functional level.

Prior to zooming in on the two main fractions in wheat (flour), the contribution of minor wheat constituents to wheat quality is discussed. This will aid in framing the interactions which possibly take place between both groups and further nuances the origin of observed effects.

1.2.1 Minor constituents

Wheat lipids form a complex group of components with varying polarities which occur as starch (internal) lipids or as non-starch (free) lipids. Despite their low levels in wheat flour (2–2.5 %) endogenous lipids exert a profound role in bread making and (Chung et al., 2009; Pareyt et al., 2011). During dough development, their redistribution may alter dough rheological characteristics by the incorporation of triacylglycerols and free fatty acids in the gluten network, thereby acting as plasticizer (Gerits et al., 2013). Furthermore, amylose-lipid complexes can form during processing or may be present in starch granules, thereby impeding there water absorption. According to Tang and Copeland (2007), this retards retrogradation and lowers enzymic hydrolysis making them highly suitable for the production of foods with a low glycemic index.

The group of 'non-starch polysaccharides' belong to the dietary fibers and are mainly represented by arabinoxylan, arabinogalactan and β-glucan. They are mainly present in the bran portion and in the aleurone layer of wheat and are thus removed by flour extraction (Saeed et al., 2011). Arabinoxylan, which is the paramount component in wheat (60–69 % to 88 % of the total non-starch polysaccharides in bran or flour respectively) can be further divided in a water-extractable and an unextractable variant. Whereas the former may positively influence bread quality (loaf volume, crumb structure and texture), the latter has a negative effect (mainly gas cell coalescence) (Courtin and Delcour, 2002).

Also influenced by milling is the ash content of the wheat flour. Apart from a limited nutritional benefit, the presence of minerals may affect the color of both the flour and, therefrom resulting, the crumb color. High ash contents are generally related with paler and less attractive colors. Moreover, the presence of specific mineral components (Phosphorus, Potassium and Magnesium) may influence starch pasting properties as is seen for potato although concentrations are considerably lower in wheat (flour) (Zaidul IS et al., 2007). Literature on this relationship and the possible impact on bread quality is however lacking.

When considering wholemeal flour, bran properties becomes majorly important. A primary influence is attributed to the generally larger particle size which has been correlated negatively with gluten-network development. The effect was attributed not to dilution of the gluten but to fibre-gluten interactions and particles piercing gas cells and disturbing the gluten network(Noort et al., 2010). Furthermore, an increase

in enzymes (lipases, proteases and amylolytic enzymes) may lower the processability and end-product quality. Contrastingly, Saeed et al. (2011) postulates that bran addition may improve bread quality through addition of arabinoxylan and lipids. The concentration is however determinative in this case with an optimum depending on the hydration rate of the flour and the further processing.

1.2.2 Protein

From a nutritional perspective, proteins are considered to be the most important nutrients for humans and animals as they form a source of essential amino acids and provide energy for the body (Schaafsma, 2012). Wheat has, from this point of view, a low protein quality due to an inadequate amount of lysine compared to the physiological requirements for adults (Shewry and Hey, 2015). However, proteins play a crucial role in the production of various foods, mainly in bread making, thanks to their unique functional properties which are determined by both the overall concentration as well as by their composition. Both are influenced by genotype ($\mathbb{G}$) and environment ($\mathbb{E}$) and by their mutual interaction. Qualitative variation has a mainly genetic origin and is reflected by polymorphisms as well as by a variation in the presence/absence of different protein units and subunits. In contrast, the $\mathbb{E}$-effect (*e.g.* growing season, site, fertilization) mainly impacts quantitative variation, *i.e.* total protein concentration and contents of the different units (Triboi et al., 2000). A review of the environmental factors affecting protein concentration and composition is given in Section 1.4 (page 34).

1.2.2.1 Protein concentration

For the broad-sense heritability (H^2) of protein concentration (PROC)—the ratio of total genetic variance to total phenotypic variance—differing values are reported in literature. According to Vogel et al. (1976), one third of the variation in the PROC can be attributed to variations in genotype ($H^2 = 33\,\%$). Pearson et al. (1981) and Surma et al. (2012) reported lower values of 19 % and 24 %, respectively, whereas Reif et al. (2011) and Würschum et al. (2016) found notably higher values of 87 % and 85 %, respectively. Although the observed variation may result from the genotypes and environments included in the analysis, it can be postulated that 13–81 % of the PROC is determined by environmental conditions.

In general, protein concentration of wheat for food purposes ranges from 10 % to 15 % (dry weight) (Shewry and Hey, 2015). Comparison of 212 600 lines from the World Wheat Collection, demonstrated that a broader range (from 7 % to 22 % protein) can occur. However, the screened lines were more diverse in their field of application and were collected from different areas over multiple years (Vogel et al., 1976). In contrast, the HEALTGRAIN project analyzed 150 wheat lines which were grown under the same conditions. A variation from 12.9 % to 19.9 % was noted for wholemeal whereas flour had a lower PROC ranging from 10.5 % to 19.0 % (Ward et al., 2008).

The difference between protein contents of wholemeal and flour can be attributed to the mill type and kernel size. Both play a vital role in the relative proportion which is lost during milling. According to Baasandorj et al. (2015), large kernels have a consistently higher flour yield (*i.e.* higher starch concentration) compared to small kernels reducing the relative amount of proteins (the so-called ‘dilution effect’). These researchers also concluded that flour milled from the small kernels contained a higher concentration of prolamin

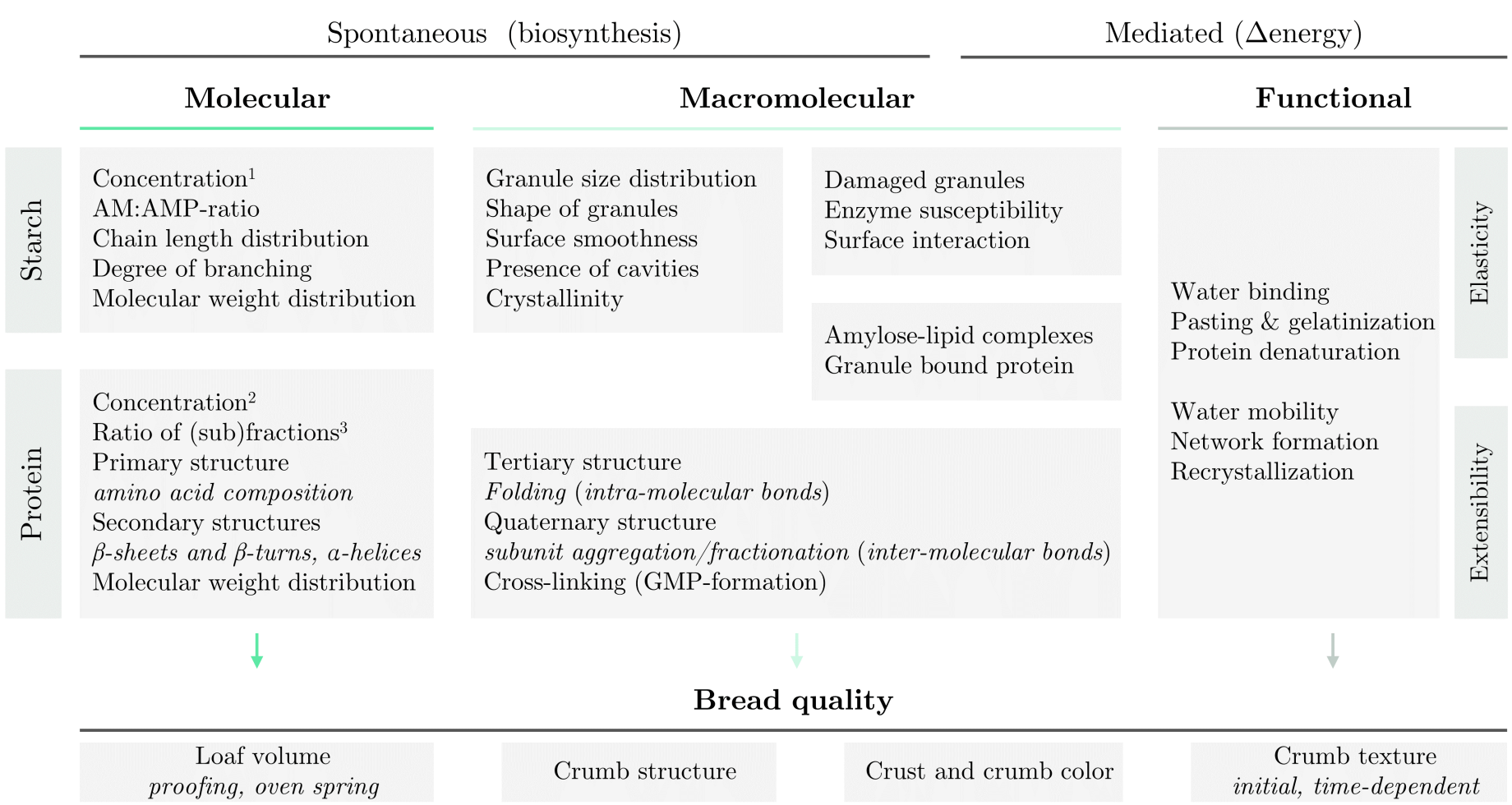

Figure 1.2: Overview of the factors influencing bread quality divided in starch and protein-related attributes on a molecular, macromolecular and functional level.
[1] Concentration of both the total starchand the starch fractions, amylose and amylopectin.
[2] Total protein content (PROC) as well as the concentration of all subfractions (prolamin: α-, β-, γ-, and ω-prolamin and glutelin: high- and low-molecular weight glutelin-subunits (HMW-GS and LMW-GS).
[3] Comprises both the prolamin-to-glutelin-ratio and the ratio of HMW-GS to LMW-GS.

(PRO). In addition, it was seen that the difference between the PROC of wholemeal and flour was greatest for the samples with lower protein contents (Ward et al., 2008).

1.2.2.2 Protein composition

Proteins of the cereal endosperm are complex, heterogeneous mixtures which show a high degree of polymorphism between genotypes. They are usually classified according to their solubility. In the early stages of research on wheat proteins, Osborne (1907) found that a sequential extraction gave rise to four different fractions: albumin (soluble in water and dilute buffers), globulin (soluble in salt solutions), PRO (soluble in 70–90 % ethanol) and glutelin (GLU) (soluble in dilute acid or alkali) (Figure 1.3). Albumin and globulin, which together form the water extractable protein (WEP) of wheat endosperm, represent 20–25 % of total grain proteins and are mainly enzymes involved in the metabolic activity of the wheat kernel (Zilić et al., 2011). Nutritionally, these non-gluten proteins have a very good amino acid balance.

PRO and GLU respectively make up approximately 30 % and 50 % of the total PROC in wheat. In literature, some controversy exists about their name originating from the idea that 'gluten' refers to the macromolecular structure formed during mixing upon water addition. Therefore, in this review, PRO and GLU are collectively referred to as gluten-forming proteins or 'gluten' in short (Wrigley et al., 2006). Moreover, 'gliadin' is the specific name for prolamin in wheat whereas 'glute*n*in' is the wheat-specific name for glute*l*in.

In addition, Shewry et al. (1986) proposed an alternative classification of the gluten-forming proteins in wheat based on the amino acid sequences and structure. A differentiation between three groups of closely related proteins was made: high molecular weight prolamins (subunits of GLU with molecular weights of 77–160 kDa), S-poor prolamins (ω-PRO) and the S-rich prolamins. The latter group comprises three kinds of PRO: α, β and γ, and the low molecular weight (LMW) GLU (subunits of 23–68 kDa) (Branlard et al., 2001). The correspondence with the Osborne nomenclature is illustrated at the bottom of Figure 1.3.

Prolamin structure PRO are a heterogeneous mixture of monomeric polypeptides with a molecular weight ranging between 28 and 70 kDa (Payne et al., 1982). As mentioned above, PRO are further divided into α-, β-, γ- and ω-PRO, although, based on their DNA sequences, α- and β-PRO can be combined. A complete review on the primary structure and nanostructure of PRO aggregates is provided by Urade et al. (2018).

In hexaploid wheat, the genes encoding for PRO are clustered at six loci, with Gli-A1, -B1 and -D1 located on the short arms of group 1 chromosomes (1AS, 1BS and 1DS) and Gli-A2, -B2 and -D2 on the short arms of group 6 chromosomes (6AS, 6BS and 6DS) (Payne, 1987). The number of genes encoding for the various subunits has been studied by Huo et al. (2018a,b) for the reference wheat 'Chinese Spring'. It was seen that 47 genes were responsible for the synthesis of α-PROs, 14 for γ-PRO and 19 for ω-PRO. Of these, 26 α-, 11 γ- and 5 ω-PRO encoded full-length proteins, while the remaining genes were either partial sequences or pseudogenes. The high number of genes involved in PRO synthesis are believed to result from unrestrained substitution, deletion and insertion events during evolution in the absence of a strong selection pressure (Shewry et al., 1984).

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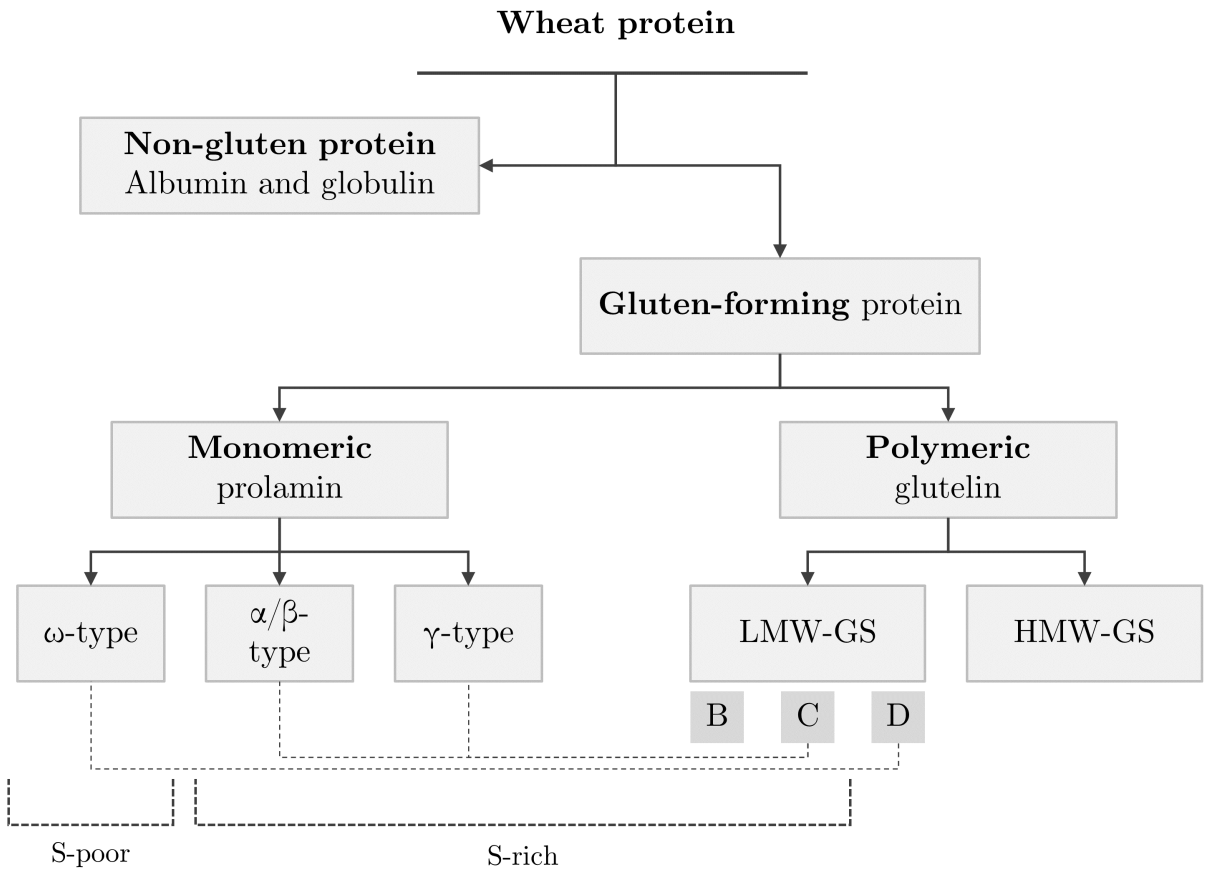

Figure 1.3: Nomenclature of the gluten-forming protein in wheat according to Osborne (Osborne, 1907) and Shewry (Shewry et al., 1986).

Mani et al. (2010) revealed a statistically significant difference in the accumulation of all PRO fractions due to the $\mathbb{E}$-effect controlling their expression. According to Daniel and Triboi (2000) the proportion of ω-type PRO in total PRO increased with both temperature and nitrogen fertilization whereas the α-PRO increased with the temperature and decreased with the nitrogen supply. γ-PRO, on the contrary, decreased at higher temperature and increased with the nitrogen fertilization.

Glutelin subunits By reducing the disulfide bonds, the quaternary structure of GLU (Section 1.2.2.3) gets lost and separate subunits (tertiary structure) are obtained. These can be classified in high molecular weight glutelin-subunits (HMW-GS) and low molecular weight glutelin-subunits (LMW-GS) on the basis of their molecular weight. The diversity in these subunits, mainly in the HMW-GS, forms a key factor during wheat processing as it is believed to substantially determine the gluten elasticity. HMW-GS have high contents of proline and glycine and low contents of lysine with an unusually high content of glutamic acid. They consist of non-repetitive N- and C-terminal domains flanking a central repetitive domain that confers the elasticity to the gluten-forming proteins (Gianibelli et al., 2001). It furthermore has to be noted that most bread wheat cultivars contain only six HMW-GS genes while the numbers of LMW-GS genes are much higher (Altenbach et al., 2019). In addition, a considerable allelic variation among different wheat cultivars has been found for each protein group (Metakovsky et al., 2019). Apart from the $\mathbb{G}$-effect, Naeem et al. (2012) has shown that sulfur availability and heat stress are the environmental characteristics that impact GLU composition most. Differences mainly exert through the influence on the

polymerization. Although Johansson et al. (2013) supports that protein polymer size and its complexity is highly determinative, they attributed this to all environmental factors affecting crop development *before* anthesis. Variations resulted to starch-protein accumulation interrelations.

On the basis of N-terminal amino acid sequences, the LMW-GS can be further divided into three subgroups, called LMW-s, LMW-m, and LMW-i types, according to the first amino acid residue of the mature protein: serine, methionine, or isoleucine, respectively (D'Ovidio and Masci, 2004). An alternative subdivision of the LMW-GS in a B-, C-, and D-type has been proposed by Jackson et al. (1983) according to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) mobility. Following the findings from Lafiandra et al. (2004), B-type subunits are considered typical LMW-GS whereas C- and D-types correspond mostly to modified PRO that become part of the GLU polymer (quaternary structure) because of mutations in the number and/or position of cysteine residues. In this way, the formation of intermolecular bonds is promoted.

Ratio of gluten proteins and subunits Depending on the extraction procedure and the method of fractionation, as well as on the nomenclature used for distinguishing the different protein fractions, the prolamin-to-glutelin-ratio (PGr) can vary from 0.49 to 4.0 (Wrigley et al., 2014). Moreover, as is shown in a study from De Santis et al. (2017) on durum wheat (*Triticum durum*), genotype also influences the PGr. Modern genotypes were characterized by a decrease in the PGr as a result of the averagely two times higher expression of B-type LMW-GS compared to the old group of durum wheat genotypes. Research from Johannsson et al. (2001) who also studied the effect of genotype on gluten strength, demonstrated that variation between cultivars was not caused by an increase in total polymeric protein (*i.e.* GLU) but resulted from in a shift from LMW-GS to HMW-GS (according to the nomenclature of Shewry et al. (1986)). Therefrom, it was concluded that a lowered PGr is related to gluten strength. Various researchers also found proof that the total nitrogen content (or PROC) of the grain is significantly positively related with the PGr (Triboi et al., 2000; Johannsson et al., 2001; Fuertes-Mendizábal et al., 2010). It is generally accepted that both $\mathbb{G}$ and $\mathbb{E}$ influence the amounts of the polymeric and monomeric proteins (Zhonghu and Wang, 2014).

1.2.2.3 Gluten network formation

When preparing dough (water + flour) or, more specifically, bread dough (water + sodium chloride (NaCl) + flour + yeast), the gluten-forming proteins in the flour can provide a matrix for holding starch granules and encapsulating air and CO_2 produced during yeast fermentation. PRO and GLU have the ability to form a continuous macromolecular network which gives rise to the crucial viscoelastic behavior. However, proteins have to be hydrated and energy has to be added through mixing before gluten network formation can take place. In essence, hydration of gluten-forming proteins is a prerequisite for the development of the network, and in turn also impacts dough structure and its rheological properties (McCann and Day, 2013).

Wang et al. (2015) found that a maximum efficiency for gluten to form a fully developed network was obtained at a PGr of 1. Higher amounts of PRO might act as a barrier to the network formation while, at low concentrations, the stabilizing effect of PRO is lost. However, their mode of action might be

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significantly different in dough as their agglomerated structure is dependent on their interactions with water and other low molecular weight compounds in the system. Although recent findings from Urade et al. (2018) have shown that PRO are soluble in pure (neutral pH and degassed) water—enabling researchers to analyze PRO in conditions close to those in real dough—a major discrepancy on the role of PRO during gluten network formation remains existing. Consequently, gluten network formation itself is not well understood. An extremely simplified concept of gluten network formation is presented in Figure 1.4. The concept of the glutelin macropolymer is however one of the most widely adopted models and is therefore discussed briefly below.

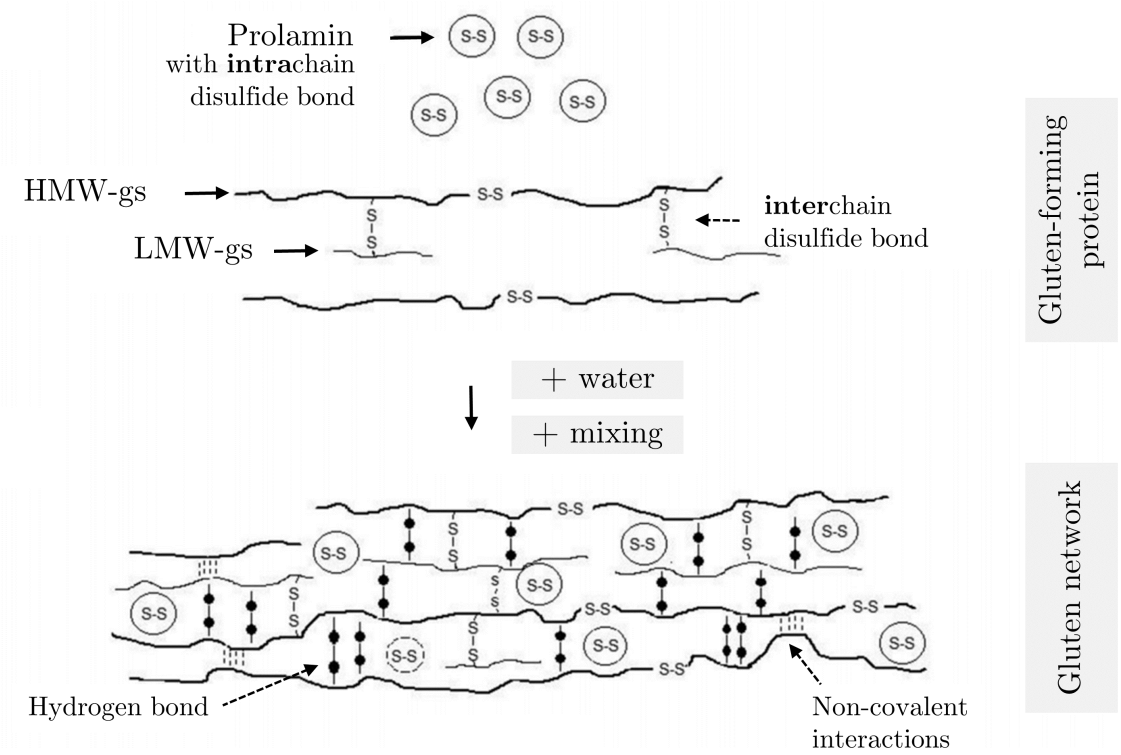

Figure 1.4: Highly simplified illustration of the position of prolamin (monomeric) and glutelin (polymeric) in the gluten network and how this is established. While mixing stimulates the formation of interchain bonds (disulfide bonds, hydrogen bonds or other non-covalent interactions), it also contributes to an elongation of the otherwise folded protein.
Figure adapted from Ortolan and Steel (2017).

Glutelin macropolymer By cross-linking via disulfide bonds, the HMW-GS and LMW-GS can form a glutelin macropolymer. Following the model proposed by Graveland et al. (1985), HMW-GS linked by disulfide bonds form the backbone of the glutelin macropolymer while oligomers of LMW-GS are attached to this. On its turn, the glutelin macropolymer intermingles randomly with individual particles of PRO through non-covalent interactions (Tuhumury et al., 2014; Wang et al., 2015). Don et al. (2006) stated that the formation of GLU particles stable in an SDS-solution requires the presence of HMW-GS as this affected the size and degree of polymerization of the glutelin macropolymer.

Although several models are proposed, no true consensus on the structure of the glutelin macropolymer and its presence in food systems has been reached (Don et al., 2003; Mueller et al., 2016). The basis for this lacuna lies in the required sample preparation and fixation techniques. The quantity and structure was also found to be dependent on both mixing time and intensity as well as on the presence of other

components. Notwithstanding the challenges in studying structural differences of glutelin macropolymers, changes in protein composition as a result of $\mathbb{E}$- and $\mathbb{G}$-effects, were also related to its quantity. Don et al. (2005), for example, found that heat stress lowers the relative amount of HMW-GS incorporated in the glutelin macropolymer. Jiang et al. (2009), on the other hand, related water stress (both drought and waterlogging) to a decreased glutelin macropolymer content.

In general, it can be concluded that polymorphisms alter the formation of a macromolecular structure. The difficulties related to studying the network formation on a molecular scale, requires to apply empirical and fundamental rheological techniques on the formed dough (*i.e.* network) to deduct its structure.

In contrast, the secondary structure of the gluten proteins may also become increasingly important. Findings from Wang et al. (2015) and Urade et al. (2018) emphasized the large contribution of α-helices, β-turns and β-sheets to the viscoelastic behavior of the gluten network in dough. Elasticity it thought to be the result of the high level of glutamine residues in all contributing proteins which can form both inter- and intra-molecular hydrogen bonds (Tuhumury et al., 2014; Ortolan et al., 2017). Covalent cross-linking of polymeric proteins on the other hand may only explain the resistance to extension but does not contribute to the elastic behavior of dough.

Overall, breaking and formation of bonds—caused by thiol-disulfide ($SH-SS$) interactions between PRO and GLU—result from the energy added through mixing and thus, the duration and intensity of this process. Kuktaite et al. (2007) studied the effect of mixing time on gluten network formation. It was seen that for most of the tested flours, the amount of water bound to the gluten network increased due to overmixing. However, no effect was observed for the storage modulus (a measure related to dough elasticity) of the gluten network for any of the flours. Also, the addition of sodium chloride has an impact on the gluten network. Some studies found a decrease in elasticity with an increase in the NaCl concentration (Angioloni and Dalla Rosa, 2005; Lynch et al., 2009; McCann and Day, 2013), while others reported the contrary (Beck et al., 2012). Tuhumury et al. (2014) attributed these contradictory effects to both the complex and concentration-dependent effects of NaCl addition on gluten forming proteins as well as on the nature of small deformation rheology measurements. In addition, NaCl delays the formation of the gluten network resulting from the reduced hydration rate. This delay may impact the unfolding and alignment of protein polymers and subsequently, the gluten network structure (McCann and Day, 2013; Ortolan et al., 2017).

> Wheat proteins are extremely complex in their buildup with a considerable diversity in their primary structure. This extends over all levels which introduces more variability, also on a functional level. There is still much uncertainty on the form in which gluten and non-gluten protein are present during and after gluten network formation. Main remaining questions are the position of the water-extractable protein in the network and the macromolecular structure which is eventually obtained. The total concentration (of the protein in wheat and its subfractions) and their mutual ratio is considered determinative.

1.2.3 Starch

Starch generally accounts for about 65–85 % of the dry weight of the wheat kernel and comprises a mixture of two glucose polymers, amylose (AM) and amylopectin (AMP). It distinguishes itself from the free mono-, di-, and oligosaccharides present in the wheat kernel (approximately 7 % of the total amount of carbohydrates) by both its high molecular weight and its granular appearance (Wang et al., 2014b; Shewry and Hey, 2015). It is the long-term energy storage polymer of wheat (Wu and Gilbert, 2010).

1.2.3.1 Starch concentration

With starch being the main component in the wheat grain, it determines grain weight to a great extent. Together with the number of fertile tillers (*i.e.* spikes) per plant and the grain number per unit area (or per spike), it directly impacts wheat yield (González et al., 2014; Yan et al., 2010). Increasing demands for wheat as a valuable starch source requires to exploit alternative strategies for maximizing the yield. Two key aspects are being studied: (a) enhancement of carbohydrate production in the leaves (*i.e.* increasing source capacity) and/or (b) improvement of the utilization of photoassimilates by sink organs (*i.e.* enhancing sink strength) (Bihmidine et al., 2013). Various researchers already found proof that, in wheat, source capacity is sufficient to meet the sink demands (Zhang et al., 2010b; González et al., 2014; Xie et al., 2015), emphasizing the need to investigate sink-related attributes to maximize the starch yield. Moreover, Yan et al. (2010) stated that inferior grain filling did not result from limiting assimilates concentrations. The opposite was however postulated by Tuncel and Okita (2013) who have observed that a stagnation of the seed weight which originated from limitations at source (*e.g.* nutrient supply in leaves) and/or transport (*e.g.* transport of nutrients into sink organs). In contrast, when abundant assimilates are present, the physical properties of the sink (*i.e.* grains) were found to be limiting for their capacity. One of these properties is the number of cells in the endosperm which is considered most determinative. Additionally, kernel size does also affect yield, though not as primarily as grain number (Slafer et al., 2015). Physiological features such as the sink strength (capacity of non-photosynthetic structures to compete for import of photoassimilates), unloading at phloem and retrieval, utilization and storage by sink cells are also determining (Bihmidine et al., 2013). Furthermore, both the duration of the filling stage as well as the filling rate (typically slow-fast-slow (Shewry et al., 2012)) were found to be varying between cultivars and environments with higher starch contents at an earlier initiation of the accumulation time and an increased rate (Yan et al., 2010; Xie et al., 2015).

In recent years, the strong, negative correlation which exists between grain yield (and thus, starch content) and grain protein concentration is studied as both a high yield and protein content are desired quality attributes. This relation is believed to exist as a result of the inter-relationship between carbon and nitrogen metabolism at the canopy level. Apart from this physiological point of view, a nitrogen (*i.e* protein) dilution effect by carbon-based compounds may also contribute to this correlation (Bogard et al., 2010). Despite the strong negative correlation, some wheat cultivars show to have a higher protein content than expected based on their yields. This phenomena is referred to as grain protein deviation (Mosleth et al., 2015). For its determination, environment can be included as factor in the regression analysis (Monaghan et al., 2001) or the residuals from a regression analysis per environment can be used (Bogard et al., 2010). In light of chain-wide quality improvement, grain protein deviation can be used to identify genotypes

which combine a high grain yield and high protein content and can be linked to genetic information to specifically breed for this trait (Thorwarth et al., 2018).

1.2.3.2 Starch composition

Starch consists of two polysacharrides, AMP, which is a highly branched molecule with α-(1→4)-linked D-glucose backbones, and AM, a primarily linear chain of D-glucose units with virtually no α-(1→6)-linkages (Pérez and Bertoft, 2010). Both molecules form the foundations of a complex and not yet fully unraveled macromolecular structure which is illustrated in Figure 1.5.

Amylose:amylopectin-ratio The occurrence of natural mutations in wheat genes leading to higher proportions of AMP or AM is low, as they have to be present on all three (ABD) wheat genomes to have a significant impact on the starch composition (Shewry et al., 2013). Therefore, starch from bread wheat flour only shows a narrow variation in the amylose:amylopectin ratio being about 1:3.

In recent years, the interest in wheat with an altered starch composition—ranging from waxy (no AM) or partial waxy (low AM-concentrations) to high-amylose (increased AM contents) starch—is drastically increased. Firstly, this is a result of an enlarged interest for industrial application as more is known about their applicability and functional uses and secondly, due to the broadened (commercial) availability.

In general, the AM:AMP-ratio can be altered through breeding approaches as a lot of research has been done on the enzymes (and thus, genes) involved in the synthesis of both molecules. However, mainly lowering AM is well understood compared to increasing the AMP-content. For the latter, synthesis is catalyzed by four enzymes, namely ADP-glucose pyrophosphorylase (AGPase), starch synthase (SS), starch-branching enzyme (SBE) and starch-debranching enzyme (DBE). AM synthesis is controlled solely by granule-bound starch synthase (GBSS) encoded by Wx-A1, Wx-B1 and Wx-D1 located on chromosomes 7AS, 4AL and 7DS, respectively (Nakamura et al., 1993).

Lowering the AM content can be done by focusing on the three loci, giving eight possible combinations of alleles. When all three alleles are present, the flour has an AM content of 20–25 %. Types with one or two null alleles (partial waxy) have an AM content of 1.7–5.0 % and the type with null alleles on all three loci has an AM content of 0.6–0.7 % (waxy) (Chen et al., 2016). Developing high-amylose wheat is more complex. Slade et al. (2012) used TILLING (Targeting Induced Local Lesions in Genomes) to detect mutations in the genes coding for the starch branching enzyme. By combination of these new alleles through breeding, a high-amylose wheat cultivar containing 47–55 % AM was developed. Regina et al. (2015) were able to elevate AM content to 85 % in wheat. This was done by combining deletions and SNPs resulting in wheat genotypes with altered expression levels of the genes encoding for the starch branching enzymes (SBEs).

Molecular fine structure Major differences exist between the two molecules which make up starch. However, neither on the basis of the characterization definition (*e.g.* relative occurrence in the starch fraction) nor on the structural definitions (parameters describing their molecular fine structure), an unambiguous distinction can be made (Vilaplana et al., 2012). This may also be attributed to the presence

of intermediate components, *i.e* polyglucans with structures that are in between that of AM and AMP (Bertoft, 2017).

The presence of an overlap is also indicated by the molecular weight-distribution of AM and AMP. Closely related to this parameters is the degree of polymerization (DP) which is used for describing both the length of the side chains (if any) as well as for indicating the total number of D-glucose units in the molecule. The latter is self-evidently correlated with the molecular weight of the starch components. According to Beckles and Thitisaksakul (2014), AM has a molecular weight of 10^6–10^8 Da and a DP of 200–10 000 D-glucose units whereas AMP is considered to be larger (molecular weight of 10^8–10^9 Da). Copeland et al. (2009) and Wu and Gilbert (2010) however reported a lower molecular weight for AM (10^5–10^6 Da) despite the upper limit (from 1000–10 000 D-glucose units) was equal. AMP, having a DP of more than one million according to these authors, has a higher molecular weight of 10^8 Da. The presence of AMP with extra long chain branches, intermediate branched components, branched AM and immature clusters in maize and rice starch was already shown by Vilaplana and Gilbert (2010; 2014) using highly complex 2D-high-performance size-exclusion chromatography (HP-SEC).

> Because of the ambiguous definition of the two main starch components, amylose and amylopectin, their absolute proportion in starch and the therefrom resulting ratio (normally 1:3 in wheat) are frequently varying. Moreover, analytical approaches can yield different results due to an unsystematic extraction or interaction with minor components (mainly lipids).

Branch chain-length distribution

AMP consists of numerous chains which are much shorter than the main chains found for AM or its branches. The chains of AMP are divided into two major groups, short and long chains, generally using a DP of 36 as cutoff-point (Wu and Gilbert, 2010). As short chains predominate in wheat starch, an average chain length of 17.7 was reported for wheat in a review by Bertoft (2017). These findings are in accordance with results from Singh et al. (2009a) who found that the most abundant side chain had a DP of 11. Moreover, 41.1–49.1 % of the chains were found to be smaller than DP 13 and 93.0 % than DP 25. An even lower average DP (5.7) was found in a study from Kalinga et al. (2014).

Yasui et al. (2009) compared the DP of AMP from low-amylose (waxy) and non-waxy bread wheat. It was concluded that the proportions of side-chains with DPs 6–12, 13–24, 25–36, and 37–60 were in the ranges 26.5–27.5 %, 43.6–44.1 %, 13.6–14.2 %, and 11.0–11.7 %, respectively. The difference in chain-length distribution profiles of waxy, low-amylose and non-waxy AMP was negligible Schirmer et al. (2013); Yasui et al. (2009). In contrast, Yoo and Jane (2002) found that (partial) waxy wheat cultivars contained less to no extra long chains for AMP (DP $\geq$ 77). Apart from the varying $\mathbb{G}$-effect, $\mathbb{E}$ did appear to affect the distribution pattern (Zhang et al., 2017). They found that AMP form wheat grown in moderate drought conditions contained a lower percentage of short branch chains, a higher percentage of long branch chains, and a higher average chain length (aCL) relative to those in AMP from well-watered wheat.

Degree of branching

Despite α-(1$\rightarrow$6) linked branches could be observed in both AM and AMP molecules, large differences in the quantity and the structure of the branches exist. AMP is a highly-branched molecule with 4–5 %

α-(1→6) branched α-(1→4)-glucans, whereas for AM, the degree of branching is lower than 0.5 % (Copeland et al., 2009; Mukerjea and Robyt, 2010; Hazard et al., 2012; Wang et al., 2014b). However, 26–44 % of the AM molecules in wheat starch is branched (Bertoft, 2017) with 3–11 chains of approximately 200–700 D-glucose residues per molecule (Copeland et al., 2009). The degree of AMP branching is determined by the expression levels of the starch branching and debranching enzymes. However, by down-regulation of starch branching enzymes, the amylose content in wheat can also increase (Hazard et al., 2012).

In contrast to AMP, which consists of three types of branch chains (A, B and C-chains), no evidence was found that AM side-chains are linked with other chains than the main D-glucose polymer (Vilaplana and Gilbert, 2010). Each AMP molecule has a single C-chain, which carries the sole reducing end of the molecule and which is substituted by B- or A chains. These B-chains are branched by A-chains or other B-chains at O-6 of a glucosyl unit whereas A-chains are linked with B- or C-chains by their reducing ends through α-(1→6) linkages. The latter chain type is not branched and are therefore called unsubstituted chains (Jane, 2014; Copeland et al., 2009). According to the review of Bertoft (2017), the number of side chains in wheat AMP is 14.2. Naguleswaran et al. (2014) and Kalinga et al. (2014) reported the degree of branching as number of branch points against the DP (≈17). Respectively, values ranging between 1.7–6.9 % and 15.7–16.8 % were reported.

Starch granular structure The structure of native starch can be organized in four length scales: the molecular scale (∼0.1 nm), the lamellar structure (8–9 nm), the growth rings (∼0.1 µm), and the whole granular morphology (µm-level) (Zhang et al., 2013a). On a molecular scale, pairs of AMP branches, mainly those with a DP of 10–20 units, unite into double helices which build up the crystalline lamellae (Bertoft, 2017). Per turn, 6 glucose residues are required resulting in a length of 4∼6 nm. AM can be spread among these crystallites, although it is primarily present in the amorphous lamellae being randomly deposited amongst the AMP skeleton (Tetlow and Emes, 2014; Beckles and Thitisaksakul, 2014). No cross-linking of AM-chains with other AM-chains was observed (single helical state) ruling out the old hypothesis that AM-chains occur in bundles (Pérez et al., 2009). Following Beckles and Thitisaksakul (2014), it are mainly the α-(1→6) branch points of AMP which are found in the amorphous region.

The combination of both regions (amorphous and crystalline) results in a lamellar structure. The recent elucidation of a substructure of 'blocklets' provides however an alternative view on their presence. Although not fully understood, this flexible semi-crystalline structure consists of a single or a few AMP molecules and was revealed using scanning and transmission electron microscopy and atomic force microscopy (Beckles and Thitisaksakul, 2014). In their comprehensive review, Tang et al. (2006) proposed the existence of so-called 'defective' blocklets which originate from the different lengths of side chains when intermediate components are present. It are these type of blocklets which form the amorphous lamella (soft shells) whereas the 'normal' blocklets construct the crystalline lamella (hard shells). Using small angle X-ray scattering, the size of blocklets was estimated resulting in a range from 10–60 nm. The alternating occurrence of the lamellar structure results in the visual presence of growth rings (100∼400 nm) which were found to be thicker around the hilum of the granule while becoming thinner towards the periphery (Bertoft, 2017). In case of wheat granules, void spaces or cavities were observed in the granule

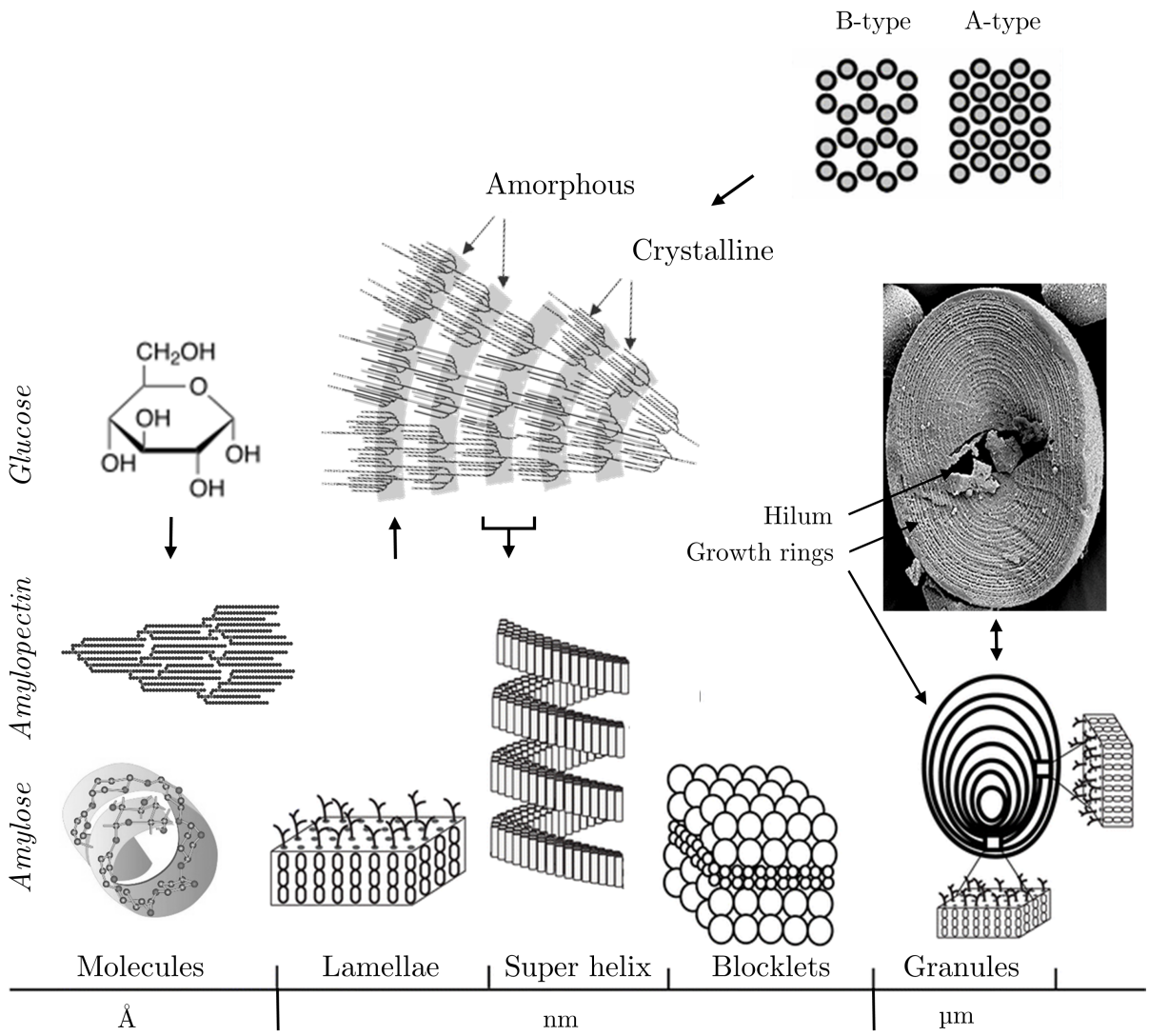

Figure 1.5: Schematic overview of the buildup of starch granules starting from glucose. The lamellae are composed from amylose and amylopectin molecules and organized in an alternating structure in which super helices of amylopectin can be observed. Together, these form blocklets which on their turn are the building blocks of the A- and B-type granules. Depending on the arrangement of the amylopectin molecules, A- or B-type crystals are obtained.
Figure adapted from Vu and Marletta (2016).

surface (Kim and Huber, 2008). This was also related to the presence of intermediate starch components by Beckles and Thitisaksakul (2014).

> The impact of genetic and environmental effects on starch properties varies considerably in literature. Moreover, molecular and macromolecular properties of starch in the wheat endosperm (*i.e.* flour) are highly interrelated and are complex in their physical appearance. Hence, higher structural conformations are not yet fully understood although significant progress is made in recent years.

Crystallinity

Clustered AMP side chains are organized in the double helix conformation that subsequently forms crystalline structures which can be divided into three types: A, B and C. In A-type crystalline starch,

glucose helixes are packed densely into a monoclinic unit cell containing eight water molecules whereas B-type crystalline starch is packed less dense. In the latter, the double-helices are packed in a hexagonal unit cell containing 36 water molecules. In this crystalline lattice, the water molecules fill up a channel, which does not exist in the A-type. C-type crystalline starch consists of a combination of A- and B-type crystallinity (Bertoft, 2017). Despite most cereal starches give the so-called A-type pattern, high-amylose variants yield the B-type pattern (Pérez et al., 2009). Finally, V-type crystallinity is distinguished from the aforementioned types by various researchers. It occurs as a results of the interaction between AM and lipids, thereby forming so-called amylose-lipid complexes.

Generally, a decrease in crystallinity with endosperm development (days after anthesis) was observed (Zhang et al., 2013a). There was no *in vivo* evidence that $\mathbb{G}$ or $\mathbb{E}$ had an impact on the *type* of crystallinity despite findings on the positive effect of elevated temperatures during growth on formation of double helices (Tester and Karkalas, 2001). Furthermore, it was shown that the *magnitude* of crystallinity of native starch granules ranges from about 15 % for high-amylose starches, over 36 % for conventional wheat starch to about 45–50 % for waxy starches (Copeland et al., 2009). However, no such correlation could be established on the basis of the results from Blazek et al. (2009). In addition to this varying $\mathbb{G}$-effect, a minor $\mathbb{E}$-effect was observed by Nhan and Copeland (2014).

Granule size distribution and shape

In wheat, both bi- and trimodal size distributions for the starch granules are reported with A-types granules being the largest and B- and C-types being consequently smaller. However, some ambiguity exists in literature on the cut-off value for the discrimination between A- and B-types ranging form 5–15 µm. For C-type granules, 5 µm is most commonly used. However, as C-type granules occupy only a limited volume fraction ($\approx$3 %), their distribution pattern generally does not allow them to be discriminated from B-types (Zhang et al., 2010a).

During grain filling, A-type granules are formed first in the developing endosperm whereas the B-type granules are formed later during the kernel development (Park et al., 2009). As this is a result from differences in their biosynthesis, the molecular organization and architecture of AM and AMP fractions was found to be varying between each granule type (Copeland et al., 2009). For example, Salman et al. (2009) observed that shorter branch chains of AMP may disrupt the order of the granule structure, resulting in lobed or compound starch granules and small granules. A structural difference between AMP molecules of the two types of granules was also reported by Zhang et al. (2013a) who, in earlier research, attributed these differences to the activity of starch synthase enzymes (Zhang et al., 2010a). Wei et al. (2010) visualized the formation of the different granule types in function of grain development time. They concluded that the large A-type granules formed and developed in the large amyloplasts (sometimes with a single granule per amyloplast) while small amyloplasts—in which numerous granules were formed and developed—came from protrusion of the large amyloplast envelope.

Besides size (and volume fraction), the shape of the main granule types differs with a disk-shaped, spherical and irregular form for respectively A-, B-, and C-type granules (Tang et al., 2006; Wei et al., 2010). This differing morphology is also accompanied by a varying specific surface area and alterations in the surface characteristics (presence of protrusions, depressions and pores) of the heterogeneous outer layer of starch granules (Tang et al., 2006).

As both crystallinity and granular structure are dependent on the AMP chain-length distribution (CLD) and higher structural conformations, differences in the X-ray diffraction patterns were observed for A- and B-type granules. According to Wei et al. (2010), crystallinity of the larger A-type granules was lower than that of small B- and C-type starch granules which was contradicted by Zhang et al. (2013a). According to these researchers, the crystalline lamellae of the B-type granules are more densely packed compared to A-type granules.

> Starch is abundantly present in wheat and can vary to a large extent on both the molecular and macromolecular level. Although commonly two molecules (amylose and amylopectin) are defined, a wider diversity in terms of branching, molecular weight, branch length, *etc.* can be expected resulting from alterations in their complex biosynthetic pathway which involves numerous enzymes. As these also contribute in the formation of starch granules, variability in their properties is expected to be even larger. As such, functional characteristics (*e.g.* pasting behavior) can be related to properties of each of the structural level.

Although there is a general understanding of the impact of compositional and structural attributes (AM:AMP-ratio, AMP CLD, *etc.*) on the physiochemical characteristics of the granules, much uncertainty exists on how this impacts functionality (relative crystallinity, pasting temperature range, gelatinization enthalpy, swelling power, rigidity towards (enzymatic) degradation, *etc.*) (Geera et al., 2006; Copeland et al., 2009; Zhang et al., 2013a; Jane, 2014).

1.3 Relation with functionality and end-product quality

Compositional properties of starch and protein are closely related to their own and each other's functionality. In native wheat flour, both components are already interacting to a limited extent. Upon adding water and energy (under the form of temperature and/or shear), numerous transformations take place with a profound interaction between both components. Depending on the molecular composition of both fractions, their macromolecular attributes and the applied processing, specific functional outcomes can be observed on a macroscopic level. Although a wide variety of analytical techniques is present to characterize wheat at each level (molecular, macromolecular, functional), the complexity of the numerous interactions and the structural changes which take place simultaneously often obscure the true origin of a relation.

The next sections provide a comprehensive overview of the current knowledge on the contribution of compositional attributes (separately or in interaction) on main functional and end-product quality attributes. In doing so, it is attempted to separate intermingled or closely related properties thereby focusing on the primary attributes.

1.3.1 Functionality

It is inherent to functional properties that interactions between two or more components occur, thereby displaying a specific isolated characteristic which majorly contributes to the eventual end-product quality.

In this light, water is a crucial element to obtain such functionality as both starch and proteins bind and, depending on the amount present in the mixture, compete for it continuously. In general, two conditions can be distinguished: functionality measured in an excess of water or in water-limited circumstances. Bread dough can be considered as an example of the latter situation. Although the former is often considered to be more fundamental, its close relation with effects occurring in the end-product and their contribution in forecasting end-product quality makes them valuable to measure.

1.3.1.1 Water binding and pasting behavior

According to Copeland et al. (2009), a better understanding of the location of AM in starch granules is required to relate structural (macrostructural level, Figure 1.2) attributes to properties that involve absorption of water. In this light, swelling and, eventually, gelatinization (pasting) are the main events taking place when applying heath and/or shear. Besides the use of functional attributes for explaining mechanisms contributing to end-product quality, they have proven to be valuable to differentiate between growth environments. This may not be evident using conventional chemical properties. Besides starch, proteins also bind water, even at room temperature. However, shear has to be applied to allow proper hydration of the unfolded proteins. Additionally, while the AM:AMP-ratio is likely to have an important bearing on the functional properties of starch, AMP fine-structure may vary accordingly. Thereby, uncertainty is introduced into the prediction of functional properties based on the AM content alone as is a common parameter in structure-functionality research.

Starch gelatinization is induced by heating starch suspended in water, thereby disrupting the hydrogen bonds between hydroxyl groups allowing the latter to form new hydrogen bonds with water molecules (Li et al., 2014). As such, granules will start to swell. This leads to the simultaneous occurrence of various alterations on a macromolecular level, most frequently quantified by the loss of birefringence or crystallinity in general. Driving factors are mainly the granular characteristics such as the size, shape and the molecular architecture (especially the AMP CLD) (Schirmer et al., 2013). As AMP binds considerably more water, the relative proportion can also be considered determinative.

Conclusive evidence has been found that pasting starts at the hilum of the granule—as this is believed to be the least organized region—and proceeds toward the periphery (Singh et al., 2003; Chen et al., 2011). More recent research from Cai and Wei (2013) has however provided clear proof that crystallinity disruption and swelling began from the surface for starch granules free of AM. This difference may be related to the otherwise inhibitory activity of AM and amylose-lipid complexes on water binding which is nonexistent in waxy starches. Although lipids can also bind in the long branch-chains of AMP, this is found to be less common than the complexation with the helices in AM (Schirmer et al., 2013). In this way, the loss of granular rigidity is promoted despite the overall higher crystallinity of waxy starch granules. The high susceptibility to thermal and mechanical breakdown also facilitates granule rupture during pasting measurements. Contrastingly, wheat flours containing high AM concentrations (HiAm-WF) have a markedly lower swelling power due to the relatively high concentration of amylose-lipid complexes and the lower content of AMP in the granules. Therefore, these starches generally require temperatures higher than 130 °C before pasting takes place (Wang et al., 2014a).

According to Van Hung et al. (2008), differences in water absorbing properties of high-amylose (HiAm) cultivars were also related to the fine structure of AM and AMP. Although it was shown that the shape of high-amylose wheat starch granules was similar compared to normal wheat starch (also spherical and oval), this molecular difference contributed to the presence of significantly smaller granule sizes in high amylose wheat flour (HiAm-WF). It also observed that the surface of the granules was damaged and cracked with mainly the larger granules having a rough and porous surface (Van Hung et al., 2008; Schirmer et al., 2013). Although water could penetrate the outer layer of the starch granules easier, the densely packed structure prevented the water to bind to the AMP.

Pasting is claimed to be mainly a starch related functional property. However, interaction effects with proteins (mainly gluten-forming proteins) are observed by various researchers. At the basis of this interaction lies the hydrogen bonding which takes place between the amino group of the glutamin (present in high quantities in the GLU-fraction) and the second or the third hydroxyl of the glucose molecules of the starch (Wang et al., 2017b). Hence, both the total concentration and the composition of the gluten can alter the water binding properties and may in this way impact the pasting behavior. This was also shown in the study by Chen et al. (2010) who found a linear decrease in peak viscosity (PV), holding strength (HS) and final viscosity (FV) upon increasing protein concentrations (10–18 %, 2 % increments) with the strongest influence from the gluten with the highest Gluten Index (GI). Breakdown (BD) and pasting (Tg) and peak temperature (PTemp) were also affected by the type of gluten. Also, the added amount impacted the pasting behavior but to a lesser extent. Findings were in accordance with more fundamental research by Jekle et al. (2016) who related an increased Tg to a barrier effect of gluten around the starch granules, thereby hindering diffusion of water into the granules (Figure 1.6). The decreased gelatinization intensity (*i.e.* loweredPV), on the other hand, was related to competition for water between protein and starch, as well as to hindered granule-granule interactions. The latter lowered the mechanic granular disruption. According to León et al. (2010), the expression of HMW-GS (specifically x-types 1Ax1 and 1Dx5) had lowering effects on the PV and the peak temperature (PTemp). This may illustrate cross-linking of the gluten even in non-limiting hydration conditions, thereby affecting water binding due to its competition for water with starch. In this light, polymorphisms (*e.g.* the presence of HMW-GS 5+10 instead of 2+12) which greatly affect the cross-linking behavior, may markedly alter the water availability and mobility required for starch pasting under conventional conditions.

Despite pasting properties can be considered as valuable predictors for breadmaking quality, the origin of this relationship might come from the impact of protein properties on the pasting curves. Barak et al. (2013) postulated that the negative relation between loaf volume and PV might come from the fact that flours with a higher PROC inhibit swelling of starch granules, thus lowering the PV. In this way, not the lower PV but te higher protein content lies at the basis of this effect. In addition, pasting measurements are performed in excess of water compared to mechanisms occurring in dough under water-limiting conditions.

> The pasting behavior is mainly defined by starch properties but may be subjected to interaction between starch granules and gluten-forming protein. At the same time, the water availability is also determining in the pasting behavior with different mechanisms taking place in an excess of water or at limiting concentrations. Protein content and composition can also impact the occurrence these

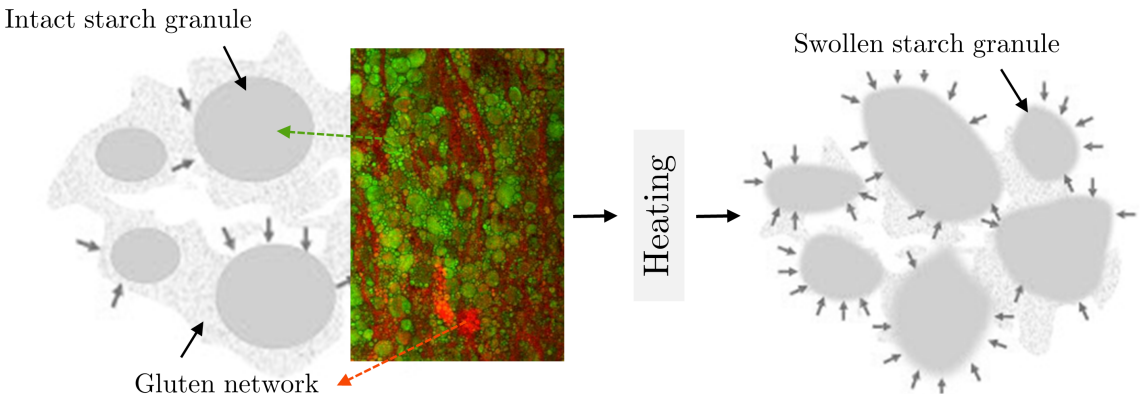

Figure 1.6: Simplified overview of a dough system in which the gluten network formed around the starch granules, inhibits water (small light-gray arrows) to access the granules thereby preventing them to swell when heat is applied. Depending on the protein content, this may also occur in excess of water.
Figure adapted from Jekle et al. (2016). Confocal laser scanning microscopy figure is obtained from Van Bockstaele (2011).

mechanisms by binding water or by creating a barrier around granules. In both situations, waer will not reach the starch granules impeding their swelling.

1.3.1.2 Dough rheology

In the domain of dough rheology, two types of methods can be distinguished: empirical and fundamental techniques. The former use subjective parameters to describe the dough viscoelastic behavior (*e.g.* consistency, elasticity, extensibility, *etc.*) whereas results from the latter are more objective and abstract. Both are however valuable as shown in a review from Van Hung et al. (2006) who related the dough strength of flour with low and high AM contents to the baking quality. They postulate that by measuring the dough rheology, interaction effects and functionality developed during processing are captured. This concept is also supported by Van Bockstaele et al. (2008) who used both types of techniques to predict the loaf volume.

According to findings from Purna et al. (2011), the optimum mixing time (*i.e.* the dough development time (DDt)) decreases non-linearly with decreasing AM contents in the flour. However, in the study from Ramachandran et al. (2016) using near-isogenic lines for waxy genes (Wx-A1, Wx-B1 and Wx-D1), no similar trend was observed. Moreover, Purna et al. (2011) found contradicting results for the water absorption which increased for only one of the two waxy wheat cultivars at a blending ratio of 45 % (in regular flour). The latter difference was assumed to result from a variation in the starch damage as a result of varying kernel hardnesses.

Yi et al. (2009b) reported that the gluten network of normal wheat flour was less extensible than in waxy wheat flour dough. In the latter, starch granules were mostly buried in the gluten matrix but the gluten themselves were not as evenly dispersed and did not cover the starch granules as uniformly or entirely as in normal wheat flour. McCann et al. (2018) observed similar effects for high-amylose wheat flour which contained more relatively small granules (with rough surfaces and irregular shapes). They postulated that a jamming effect occurred due to the formation of pockets of concentrated starch granules within a protein network, thereby gravely impacting the viscoelasticity. The storage modulus (G', measure for dough

elasticity) increased and strain hardening effects—defined as the phenomenon that the stress required to deform a material increases more than proportional to the strain (van Vliet, 2008)—upon uniaxial extension were larger.

The PROC of flour shows an inverse relationship with G' and the loss modulus (G", measure of energy dissipated) up to approximately 14 % protein on dry matter (Khatkar, 2005). Upon increasing relative proportions of PRO in the flour, a shortened mixing time (DDt) and a lower stability (STAB) were obtained. At concentrations higher than 0.5 % (on flour basis), G' decreased while the phase shift angle ($\tan(\delta)$) continuously increased. Upon increasing the concentrations from 0 to 0.5 %, an increase in the elasticity was however noticed (Zhang et al., 2008; Barak et al., 2015). This is in partial contrast with an early view that PRO act as plasticizers in gluten networks so that increasing the PGr causes a decrease in elasticity (Popineau et al., 1994; Song and Zheng, 2007). However, at such low concentrations, PRO addition may promote network formation.

Conformations taking place at the secondary level of the PRO, are also assumed to affect dough rheology. It is suggested that the number of β-sheets is correlated with dough stability and β-turns is related to extensibility. At the basis of this phenomena lays the content and position of the disulfide bonds with the overall content being significantly correlated with dough stability (Gao et al., 2016). The β-sheet content and α-helix:β-sheet ratio were, according to findings from Liu et al. (2016), also significantly correlated to the percentage of unextractable polymeric proteins ('UPP').

HMW-GS may first affect the size distribution of GLU polymers through differences in the number of cysteins. Findings from Ktenioudaki et al. (2010) however showed the involvement of both LMW-GS and HMW-GS in this process. From this, it can be concluded that LMW-GS and HMW-GS contribute to overall dough strength but enrichment with the former indirectly promotes the number of physical cross-links. The balance is however crucial as both the extensibility and strength of the dough are of importance in determining bread quality. Generally, an increase in the proportion of unextractable polymeric proteins—independent from the way in which this is established—results from a shift towards higher molecular weights which gives rise to a higher resistance against extension (Naeem et al., 2012). Also Zhang et al. (2008) observed a strong correlation between this proportion and the maximum resistance.

Alternatively, the higher structural orders (tertiary and quaternary levels) making up the gluten network can be used to forecast dough behavior. Using confocal laser scanning microscopy and network analysis, Gao et al. (2018) have shown that the lacunarity—which indirectly reflects the size distribution of starch granules embedded in the gluten network—is positively correlated with the percentage of unextractable polymeric proteins, Farinograph DDt and STAB. Moreover, the more end-points, the higher the water absorption and protein weakening is.

> The interplay of protein and starch in their native form results in unique functional properties in the dough. These can be generally reduced to differences in the visco-elastic behavior which can be measured using small or large rotational, uni- or biaxial deformations. The resulting parameters closely relate to elasticity and extensibility. The molecular composition and macromolecular structure are equally important for the eventual functional behavior. Moreover, preprocessing steps (*e.g.* milling) will influence how these interactions comes to expression during processing.

Proposition

For both protein and starch, properties at multiple levels can be defined as single molecules form macromolecular structures. Thereby, interactions between molecules from the same class (starch-starch, starch-protein) as well as from different classes (also including lipids, non-starch polysaccharides, *etc.*) occur. As such, not only the concentration of all single components may be relevant, but also the properties (compositional or functional) of the superimposed structures. For measuring these interaction-effects and the phenomena occurring during processing (mixing, heat treatment), functional measurements can be used. Properties derived from conditions dissimilar to the ones in the eventual system provide a fundamental insight in mechanisms and may be related to various applications. In contrast, model-system dependent measurements enclose more of the true effects taking place but can probably not be related to effects taking place under different conditions.

1.3.2 Bread quality

The schematic overview in Figure 1.7 builds on the proposed distinction between molecular, macromolecular and functional properties as discussed in the previous sections. It attempts to illustrate how the main traits are involved in determining the final quality of bread. Two crucial factors, highlighted in red, are considered main general properties which encompass diverse underlying mechanisms and result in various outcomes. These nodes and their outcomes are further subdivided according to the major processing steps in breadmaking. Initial drivers for the water absorption and network formation are split using the distinction between molecular and macromolecular attributes. Also determining interaction effects are separately included.

Although no real starting point is defined in the diagram, the AM:AMP-ratio and the molecular weight-distribution of the gluten protein can be considered as the most fundamental compositional attributes related to the bread quality. Together with various starch granular properties, these alter the dough viscoelastic properties (McCann et al., 2018) which, on their turn, have been related to bread quality (Autio et al., 2001; Van Bockstaele et al., 2008). In addition, it is generally accepted that the presence of GLU alleles 5+10 and 2+12 are associated with respectively good and poor bread making quality as they promote or hinder network formation. In contrast to the established beneficial status of GLU (especially the HMW-GS), the contribution of PRO is unclear but may not be neglected (Barak et al., 2013).

The high contribution of protein related attributes in comparison to starch properties can be partially attributed to the limited inclusion of both aspects when studying baking quality. Moreover, applied research on the impact of molecular starch properties on bread quality is lacking. Nevertheless, an attempt is done to provide a brief overview on the end-product quality attributes which are most frequently investigated and which (combination of) (macro)molecular variables mainly impacts them.

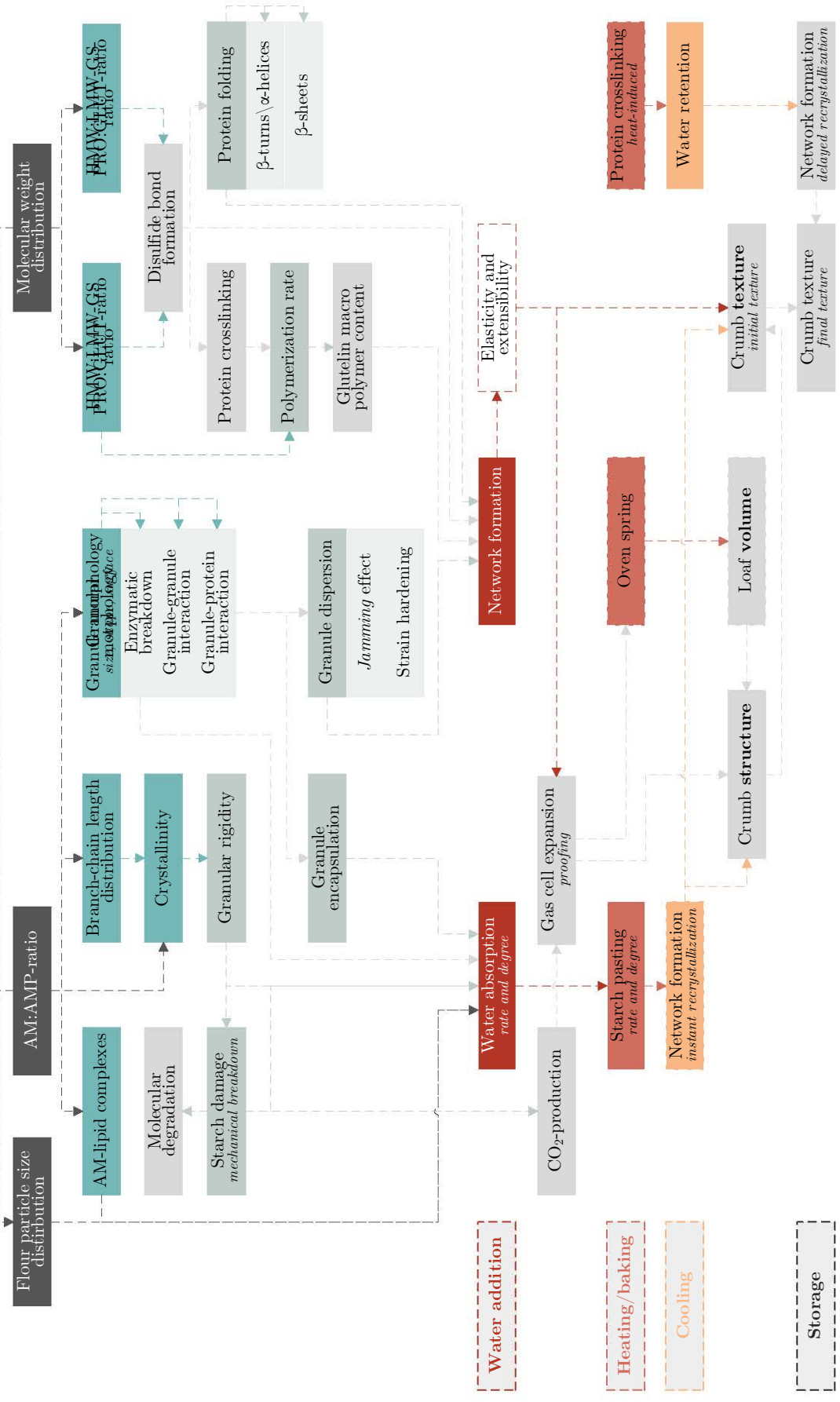

Figure 1.7: Schematic overview of the factors on a molecular, macromolecular and functional level which contribute most to bread quality (*i.e.* loaf volume, crumb structure and crumb texture). Central nodes 'water absorption' and 'network formation' form the key elements through which complex quality traits of the bread are developed.

1.3.2.1 Loaf volume

The loaf volume develops as a result of expanding gas—both air entrapped during mixing and CO_2 produced by yeast fermentation during proofing—inside the cells in the dough. Firstly, the walls of the gas cells in the dough have to withstand (rapid) expansion during proofing and baking without collapsing (Wilde, 2012). Secondly, the dough cohesiveness (*i.e.* elasticity) has to be high enough to promote an upwards expansion. Thirdly, a too high resistance against expansion or a too elastic dough will not expand as the pressure in the gas cells will not be high enough. Finally, the (partially) gelatinized starch should provide enough structure, together with the denaturated protein, to take over the structure created by the (gluten-forming) proteins (Mills et al., 2003). Although multiple possibilities in terms of composition may give rise to suitable conditions, the balance remains highly delicate.

Protein properties A major controversy exists about the relation between protein content of flour and the specific loaf volume as the strength of correlations between both appears to be highly variable with R^2-values ranging from 0.01 to 0.86 (Gabriel et al., 2017; Zörb et al., 2018). According to Gabriel et al. (2017), this broad range is related to weather conditions, local site conditions, level of fertilization and wheat variety. It was also shown that for protein contents above 12 %, which represent the relevant range for trading, the predictive power of the protein content for loaf volume drops to 0.15 whereas 59 % of the variability was explained when looking at the entire set of 591 samples. Therefore, Zörb et al. (2018) concluded that the strategy of increasing raw protein content to achieve higher baking quality is exhausted and that a shift towards breeding for protein *composition* would be opportune.

Besides PROC, the concentration and ratio of the different protein fractions was found to be important in loaf volume development. Addition of albumins to wheat starch resulted in a significant increase in specific volume, assumedly due to their ability to bind carbon dioxide, their low denaturation temperature (which aids in stabilizing the crumb structure in early stages of baking) and the overall improved foaming capacity (Ziobro et al., 2013). However, the specific role of non-gluten proteins in wheat flour remains unclear in case also gluten-forming proteins are present (Veraverbeke and Delcour, 2002). The PGr was found to be negatively associated with the specific loaf volume (Barak et al., 2013). This was however contradicted by Wieser and Kieffer (2001) who concluded that loaf volume was influenced by the total amount of gluten protein more than by the proportion of different types of subunits. In their research, no significant differences between PRO and GLU with respect to their effects on bread volume could be detected.

In the study from Dhaka and Khatkar (2015), a significant and strong correlation between the ratio of HMW-GS to LMW-GS (HMW:LMWr) and loaf volume was found. The authors attributed this relation to the higher proportion of large size polymers (which have a higher molecular weight). This also supports the finding that HMW-GS (mainly HMW-GS pair 5+10) promotes cross-linking during gluten-network formation. Additionally, the suitability of the PRO and GLU content (separately) and the concentration of the glutelin macropolymer to predict the baking performance of wheat flour was shown by Barak et al. (2013). These authors found r-values varying between 0.71 and 0.80. Contrastingly, research of Jonnala et al. (2010) found only a weak correlation between loaf volume and percentage of unextractable polymeric proteins in waxy bread wheat genotypes whereas the total *polymeric* protein content of the

flour and the PROC showed to be correlated significantly. It was also shown that correlations were only present when optimal mixing times (instead of a fixed mixing time) were used during breadmaking.

Starch properties Another important factor in the already difficult balance described in the introduction of this section, is the ability of gas to escape from crumb to crust during baking in a controlled way. The necessity of this mechanism is clearly illustrated by the application of waxy wheat flour (WWF) in breadmaking. It is generally accepted that a minimal concentration of AM in the flour is required to obtain breads which do not show post-baking collapse. The latter phenomena, also called the 'keyhole effect', can be ascribed to the decreased pasting temperature (Tg) of waxy and partial waxy flours. Gas cells in the dough expand with increasing temperature but remain impermeable as starch granules already fused by the low pasting temperature. Cell walls expand to their maximum but fail to rupture. In essence, gas cells only coalesce and form a single hole instead of an uniformly distributed crumb (Purna et al., 2011). In contrast, wheat flours with higher Tg will have a gas-continuous cell wall obtained by cross-linking of proteins and partially gelatinized starch granules. Small ruptures will emerge at the time the structure is already strong enough to remain stable.

This phenomena can however not be generalized. For example, Blake et al. (2015) observed in their research that, upon decreasing the AM content, pasting was delayed under water limiting conditions. This was attributed to the greater water uptake of the waxy starch granules during swelling and a reduced overall water ability, together driving complete melting of the crystallites to higher temperatures. Moreover, a relation with the water binding capacity of the gluten protein is proposed by Wang et al. (2004) (as described further below).

Low-amylose wheat flour (with AM contents $\leq 10\,\%$) contains more damaged starch (Garimella Purna et al., 2015) which is more readily accessible for digestion during yeast fermentation. Furthermore, AMP is more susceptible for degradation by α-amylase. In this way, more CO_2 is produced resulting in increased loaf volumes (Van Hung et al., 2006). This is however not the sole mechanism behind the negative correlation between the AM content and loaf volume. The generally higher water absorption (Purna et al., 2011; Mouliney et al., 2011; Schirmer et al., 2013) and meanly larger granule size (Bertolini et al., 2003; Zhang et al., 2013b; Ramachandran et al., 2016) may positively influence dough rheology, making dough more extensible and less rigid while maintaining the elasticity. Hence, expansion during baking is promoted (Blake et al., 2015).

A few studies have shown that, using WWF in blends with normal wheat flour, a slight increase in the loaf volume can be obtained without the detrimental effects as these described above (Van Hung et al., 2006; Blake et al., 2015; Ramachandran et al., 2016). Although assumedly between 15–35 % AM, it is unclear at which concentration and under which conditions (gluten composition, water content, damaged starch content, *etc.*) this increase can be obtained. Moreover, in a study by Ramachandran et al. (2016), no volume increase was observed for a partial waxy line with 20.9 % AM compared to breads made from a regular near-isogenic line with $\approx 28\,\%$ amylose. This apparent contradiction may also be attributed to the different pasting behavior of a blend of waxy and regular wheat flour and a partial waxy wheat flour with the same AM concentration.

Besides its contribution to the nutritional value of bread (reduced digestibility through lowered accessibility), the starch functionality of HiAm-WF (with AM contents ranging from 35–85 %) is markedly

different (Regina et al., 2012). By altering the starch synthesis, granular properties were also affected. However, this is dependent on the pathway through which starch composition is modified (Regina et al., 2015). Mainly the volume fraction and arrangement of the starch granules in the protein matrix differed from the regular *suspension model* proposed by Tanner et al. (2011). In essence, a decreased dispersion of starch granules in the gluten network at higher amylose concentrations was noted. After visualization and quantification using confocal laser scanning microscopy, this was related to an increased dough strength due to a '*jamming*' effect. Clusters of starch granules in the gluten network inhibit the flow under shear conditions thereby also negatively impacting the loaf volume. Moreover, HiAm-WF contained more irregular shaped granules with rougher surfaces.

> Even when effects related to protein would be taken into account, no consistent trends between the amylose content and functional properties can be established. Differences between waxy, partial waxy, regular and high-amylose flours extent to the molecular fine structure and granular architecture of the starch fraction. This impedes a straightforward comparison between the different types. This also supports the different behavior of flours who naturally contain a certain amylose concentration compared to blends of flours containing the same content of this starch component.

1.3.2.2 Crumb structure

During baking, the expanded gas cells will set in the starch-gluten matrix, resulting in the formation of a sponge structure (bread crumb) instead of a foam structure (bread dough) (Mills et al., 2003). In order to create the desired structure and texture of the final product, it is crucial to control the size and number of gas cells created in dough, and to retain their stability throughout the bread making process (Wilde, 2012). In order to obtain the latter, mainly gas cell coalescence must be prevented during dough proofing and oven spring, together with an equal growth of the gas cells (so-called Ostwald ripening) (van Vliet, 2008). Naturally, an optimum between the dough weight, loaf volume and crumb coarseness is present as compact breads are dense and have a closed structure whereas large volumes are accompanied by an open and airy structure (Yi et al., 2009a).

Results from Ramachandran et al. (2016) have clearly shown that, independent from the protein properties, the number of larger gas cells (coarser crumb) increases at decreasing AM contents with detrimental effects ('keyhole shape') when concentrations are below 15 %. Similar effects can be obtained by adding high concentrations of malt flour thereby increasing the α-amylase activity in the dough. The crumb structure would become more opened as AMP is broken down to a larger extent. As the therefrom resulting dextrins and glucose will not contribute to the formation of a rigid gluten-starch network, gas cells will coalesce (Lee et al., 2001).

Tronsmo et al. (2003) stated that crumb structure was *solely* dependent on protein quality since no starch related attributes were included in the predictive model for related quality measures. This is partially supported by van Vliet (2008) who stated that deformation will be *primarily* governed by the properties of the protein. They reasoned that it cannot be governed by native starch as this is much stiffer than the gluten and as it occupies a significant fraction ($\sim$0.35–0.40) in gluten films (*i.e.* gluten network present in dough). However, the phenomenon of strain hardening is believed to be essential for the expansion of gas cells in

dough (Tronsmo et al., 2003). Strain hardening results from the formation of multi-branch structures in the gluten network which decreases the mobility of material components (*e.g.* starch granules), leading to an increased stress level during the deformation (McCann et al., 2016).

Independent from the uncertainty on the drivers behind dough rheology, dough viscoelastic behavior is considered indicative for the crumb structure of bread. Earlier views however considered that bubble expansion was independent from these properties during the early stages of proofing. Although during mixing, later findings have shown that higher viscosities tend to produce smaller bubbles. On its turn, this is considered generally important for their growth during proofing and thus, final crumb structure (Mills et al., 2003).

1.3.2.3 Crumb texture

Crumb texture is a diverse and complex sensory feature of the bread crumb which is defined by crumb firmness (hardness), adhesiveness (stickiness of the crumb) and springiness. Moreover, the difference in the hardness provides insight in the retrogradation rate (physicochemical shelf-life). One of the main difficulties when studying texture is controlling the impact of loaf volume and crumb structure. This is supported by early research from Fessas and Schiraldi (1998) who concluded that differences in crumb firmness might be mainly due to the structure formed in the course of proofing and baking, rather than to starch retrogradation and moisture loss.

When the water absorption of the starch increases, more water will retain in the crumb after cooling, resulting in an increased adhesiveness. Interestingly, the moisture content (MC) of the crumb from WWF based bread was only markedly higher after seven days of storage compared to breads produced using regular wheat flour Lee et al. (2001). In this light, Ramachandran et al. (2016) found that near-isogenic lines who had a null allele on the D-genome, produced a significantly less sticky crumb than other singlenull lines. However, the AM content of a single-null line will only be limitedly reduced.

The rate at which retrogradation of AM occurs in processed foods is considered to be important for stickiness, as well as the ability to absorb water and the digestibility (Copeland et al., 2009). Moreover, initial crumb firmness has been attributed to the rapid re-association of the amylose fraction (minutes to hours) and is considered a prerequisite for obtaining a desirable crumb structure and texture. Breads produced with WWF had an equal firmness after storage compared to conventional wheat flour based breads due to the delayed retrogradation of AMP (hours to days) (Purna et al., 2011).

The increase in firmness in function of time is frequently solely attributed to the retrogradation properties of AMP. Contradicting results were found by Lee et al. (2001) who compared breads made from waxy *flour* with mixes from regular and waxy *starches* (forming the basis of reconstituted flours). The enthalpy of retrogradation decreased (indicating that less retrogradation occurred) as the proportion of waxy wheat starch in blends was increased. More retrogradation was noted for breads baked with waxy wheat flour. The latter finding was attributed to the amount of water available. However, they also found that initial crumb firmness was lower, even at the lowest incorporation (10 %) of waxy starch in regular wheat starch. No difference in the initial crumb firmness was seen by Mouliney et al. (2011) although breads with incorporation of 20 % WWF were 27 % softer after five days of storage. This was against expectations as higher relative AMP concentrations are assumed to result in more retrograded starch after storage.

Moreover, when the proportion of chains with a DP of 6–9 increased, a decrease in the retrogradation rate was observed by Inokuma et al. (2016).

By performing conventional viscosity measurements on freeze-dried dough, Collar (2003) showed that PV, Tg, and the setback during cooling can be considered as valuable predictors of bread firming behavior during storage. As the starch composition (AM:AMP-ratio and AMP CLD) is related to granule rigidity, swelling power and pasting behavior, it can be postulated that compositional attributes are also valuable in forecasting end-product quality and, more specifically, the retrogradation rate in bread during storage.

Another possibly determining factors may be the mobility of water as hypothesized by Wang et al. (2004). From their research, it was concluded that besides water availability, the water retention capacity of the gluten may also contribute greatly to effects on crumb texture in function of time (*i.e.* retrogradation). Also initial firmness was found to be positively correlated with the PGr. As GLU showed to be correlated stronger, it was postulated that the these contribute more to bread firmness and the development thereof (Collar, 2003).

Highlights

- The amylose-to-amylopectin ratio is a fundamental and determinative attribute for the functionality of the starch fraction besides the fine structure of each component.
- The presence of hybrid components and the ambiguous definition of the starch components results in a measuring error due to an overlap between both components.
- Much uncertainty on the relevance of the secondary structure of the gluten-forming proteins in gluten network formation and dough development remains. Also the position of prolamins is still unclear.
- Functional properties differ according to variation of both the molecular and macromolecular structure of a single component (protein or starch) and their mutual interaction.
- Functionality can be roughly narrowed down to network formation (gluten-starch network), water binding and pasting/gelatinization behavior. These effects require energy addition in the form of mixing or heating and will be highly dependent on the amount of water available for each component.
- Bread quality is related to protein content in a limited range although contradicting findings are reported. Additionally, it is thought to be mainly dependent on the gluten-protein composition and the amount of amylose.
- Literature on the combined effect of differences in the protein content and composition and starch composition is available on bread quality is lacking.

1.4 Cultivation conditions to steer yield and composition

Last decade, breeders mainly focused on yield increases, with grain quality being a secondary breeding objective (Battenfield et al., 2016; Sanchez-Garcia et al., 2015). Average yields (10 year average, 2008–2017) for Northern and Western European countries ranged from 6.5 to 7.2 ton ha^{-1} with Ireland, Belgium and the Netherlands as the countries with the highest yields (9.2, 8.8 and 8.7 ton ha^{-1} respectively). These are generally higher in comparison with central America, which tends to have an average lower yield (up to 5.2 ton ha^{-1}), mainly due to the use of spring wheat. Also recently emerged grain trading countries such as Ukraine, Poland and the Russian Federation are characterized by lower yields (2.5 to 4.6 ton ha^{-1}) but are leading exporters due to their vast area for wheat cultivation. Globally, average wheat yield (winter and spring wheat under intensive and non-agricultural practices in suited or inadequate growing conditions) reaches only 3.3 ton ha^{-1} (FAOSTAT database, 2019). Although yields globally tend to increase, in many countries a yield stagnation is experienced. On one hand, this can be attributed to climate change, especially global warming (Asseng et al., 2015), and the therefrom resulting higher occurrence of extreme weather conditions. On the other hand, intensive genetic selection which has led to genetic erosion in modern breeding pools, partially contributes to this phenomenon. According to Reif et al. (2005), wheat's genetic diversity was narrowed from 1950 to 1989, but was again enhanced from 1990 to 1997. This occurred through the introgression of new genes or alleles provided by landraces which are a valuable source of genetic diversity. These races can be used to breed varieties adapted to local environmental conditions according to the origin of the landraces (Lopes et al., 2015). Using a SNP-based diversity map, Cavanagh et al. (2013) characterized the impact of breeding on genomic and geographic patterns of genetic diversity. It was concluded that most of the diversity present in the modern varieties was also present in landraces, with a 6 % reduction in the population size. Based on microsatellites, Huang et al. (2007) observed a dip in the genetic diversity of European winter wheat varieties in the 1960s in comparison to the genetic diversity in the earlier 1940s. On the other hand, in the later decades, a quantitative increase of genetic diversity was found. So, according to these authors, modern plant breeding has resulted in changes of alleles present in the wheat germplasm which rather led to an improvement of the genetic diversity.

Additionally, the results of several studies corroborate the evidence that a considerable breeding progress, especially with respect to yield, was achieved during the last decades. Brisson et al. (2010) and Oury et al. (2012) demonstrated that the stagnation of bread wheat yield in France did not correspond to a slowing down in genetic progress. It appeared that since the end of the 1980s the genetic progress has been partly or totally counterbalanced by the adverse effects of climate change. Also Laidig et al. (2017) concluded that, based on analysis of German wheat trials, winter wheat breeding progress remains to be very successful with a large gain in grain yield (24 %). On the contrary, location specific environmental policies mainly focus on the restriction of the use of fertilizers. For example, in the European Union, Nitrogen and Phosporus fertilization became strictly regulated which could also contribute to the stagnation of wheat yield (Peltonen-Sainio et al., 2009).

Last years, the wheat demand is rising, partly as a result of population growth, but also because of an increasing consumption per capita. Many people consume wheat based foods on a daily basis as this substantiates the dietary guideline for carbohydrate intake. In China, wheat consumption increased more

than six-fold from just over 19 mt in 1962 to 123 t in 2012. In the EU wheat consumption has been fairly flat in recent years. The figure of 121.5 t in 2012 represents an increase of only 7 % over the figure in 1999. Wheat consumption in the USA increased from 16 t in 1962 to 38 t in 2012 which is less than one third that of the EU (Curtis and Halford, 2014). However, for these figures, no distinction between wheat used for human or animal consumption and (second generation) bioethanol production is made. Nevertheless, in the USA and Europe (mostly in France and the UK), wheat straw is used instead of the wheat kernel as this results in a higher ethanol yield (Gupta and Verma, 2015). Therewith, Semenov et al. (2014) stated that, considering the limitations on expanding crop-growing areas, a significant increase in crop productivity will be required to achieve the projected need to raise the world food supply by 70 % by 2050. Nevertheless, to meet the more specific demands of the food industry, to prevent food losses and to optimally utilize the available resources, the production of high quality wheat with distinct properties is equally important.

> Despite wheat yields are already high in West-European countries, stagnation may preclude that production meets the rising demand. Besides wheat for human consumption, other fields of application (animal feed, bio-ethanol, side stream valorization, *etc.*) contributes to the prevailing shortage.

Besides yield gain, there is a continuously growing interest in the cultivation of high quality and nutritious wheat. Since wheat derivatives (whole meal, white flour, extracted starch and gluten) are applied in a wide range of industrially produced foods, differing compositional characteristics are desired. This is further augmented by the diversified processing techniques (*e.g.* extrusion or working with preferment in bread making) related to these novel or conventional areas of application. Moreover, quality is also a multidisciplinary concept due to the various stakeholders involved in the wheat breeding, cultivation and processing chain (gray boxes in Figure 1.8). While farmers generally look at yield and production costs, millers and industry are mainly interested in processing quality and the resulting functional properties (Dupont and Altenbach, 2003). Consumers on the other hand want a tasty end-product (Troccoli et al., 2000) and their increased awareness of food related health issues, authenticity and sustainability has led to an enlarged food diversity (Guzman et al., 2016). In addition, food losses manifesting on both the industry and household level, require a more detailed understanding of the various spoilage mechanisms. Physicochemical degradation (staling), which is most commonly observed in bakery products, is still far from being fully elucidated. As also described in Section 1.3.2.3, starch and starch–gluten interactions play a central role in the staling mechanism (Fadda et al., 2014) and most of the quality aspects of bakery products. Nevertheless, the market value of all wheat grain is mainly determined by its protein concentration despite the mandatory diversity in wheat composition for the numerous fields of application(Oury and Godin, 2007).

Both yield and quality traits of wheat depend on its genotype ($\mathbb{G}$-effect), but is also clearly influenced by the environment ($\mathbb{E}$-effect) and the mutual interaction ($\mathbb{G} \times \mathbb{E}$) between both factors (Kaya and Akcura, 2014; Nuttall et al., 2017; Williams et al., 2008). Various studies have reported the significantly stronger effect of environmental conditions on wheat yield and composition in comparison with the effect of the genotype. It is estimated that the improvement of the genetic stock contributes for 30–50 %, while agricultural techniques contribute for 50–70 % to increasing yields (Jolnkai, 1985). More recent studies approve that the environment is the predominant source of yield variation (Kaya and Akcura, 2014;

Mohammadi et al., 2015). This implies that when the same wheat variety is grown in environments with differing meteorological conditions and/or varying crop husbandry practices, a heterogeneous group of products, all with different compositional properties and consequently a specific quality, can be obtained.

A thorough understanding of the driving factors governing environmental variations in grain quality is a prerequisite to cultivate wheat with specific properties in a sustainable and repeatable way. Especially in an industrial process, stable wheat quality is desired as this impedes a continuous adjustment of ingredients. Moreover, proper dough handling is crucial for preventing production line downtime. In that evidence, industry is majorly interested in how specific characteristics can be controlled during growth or which varieties are less susceptible to environmental fluctuations. Extensive research has been performed in order to estimate the effect of a wide range of environmental factors on the composition of the harvested wheat grain. In the full version of the review (Hellemans et al., 2018), a comprehensive overview is given of all attributes summarized in Figure 1.8. A reduced selection, thereby focusing on the studied agricultural topics in the remainder of this dissertation, is presented in the next sections of the literature review.

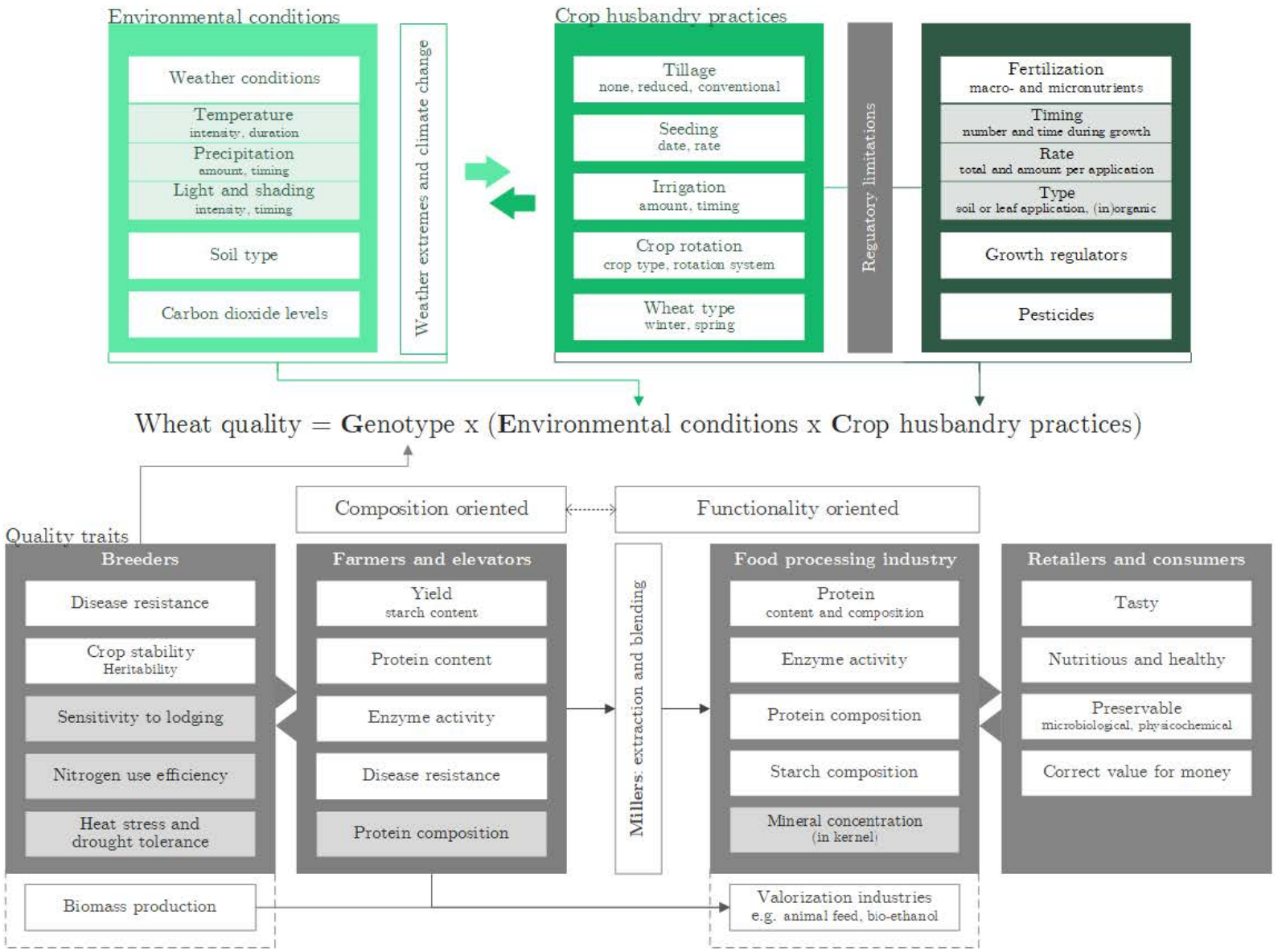

Figure 1.8: Schematic overview of the quality traits for wheat per stakeholder in a general supply chain and how these are influenced by environmental and cultivation practices.

1.5 Crop husbandry practices

Within the domain of crop husbandry practices, a distinction can be made between continuously applicable practices, such as the use of fertilizers, growth regulators or pest control methods (fungicides, insecticides, *etc.*), and single-use measures. The latter will be applied only once during the cropping cycle or will be part of a long-term approach for crop quality improvement. This includes crop rotation, the use of catch crops, tillage and seeding density. Although solely fertilization is investigated in its different aspects, other topics are briefly discussed in the following sections to provide background in the practices that can be used to steer wheat quality.

1.5.1 Crop rotation

Using crop rotation is generally preferred above monoculture since the yield of crops grown in a continuous monoculture declines as a result of an accumulation of soil or stubble-borne diseases specific to the monoculture crop (*e.g. Fusarium*). Moreover, the use of crop rotation can improve soil structure, water and nutrient use efficiency, mycorrhizal associations and can provide better weed control (Riedell et al., 2009; Rahimizadeh et al., 2010; Amossé et al., 2013). These effects can enhance both grain yield and quality traits such as the grain protein content (Riedell et al., 2009).

The advantages of crop rotation and the use of catch crops, either sown every year in a rotation system or during wheat growth (under-sowing or relay intercropping), depend on the type of crop and the post-treatment (mulching, ploughing and residue return). Legume crops will decrease volatilization and leaching of N due to their ability to fix a substantial amount of nitrogen from the air (96–376 kg N ha^{-1} (Pandey et al., 2017)) in symbiosis with rhizobia and their humification and thus N mineralization potential. This results in an improved soil mineral status with an increased N-availability for the subsequent crop. Additionally, as some legumes are deeper rooting than other agricultural plants, leached nutrients can be pumped up and will consequently become partially available for the preceding crop roots. For example, roots of *Lupinus angustifolius* and *L. consentinii* can reach a depth up to 2.2 m compared with 1.3 m for wheat (Hamblin and Hamblin, 1985). Furthermore, some legumes (*e.g. Lupinus albus*) also enhance the mobilization of fixed phosphorus in the soil through the formation of cluster roots. Supplementary, thanks to the decomposed network of in-depth root hairs, the water capillarity of the soil is enhanced resulting in less drought stress for the following crop (Felderer et al., 2015).

Rahimizadeh et al. (2010) illustrated the beneficial effects of crop rotation using potato, silage corn, clover or sugar beet as preceding crop. A yield increase from 2.1 ton ha^{-1} to 3.9 ton ha^{-1} was obtained for the potato-wheat rotation in comparison to the continuous wheat system (wheat-wheat). Concerning the grain protein content, clover as a preceding crop was most effective as it resulted in an absolute increase of 1.11 % in the grain protein content (from 13.01 % to 14.12 %), independent from the applied N-fertilizer rate. These findings are in accordance with research from Doltra et al. (2011) who noticed that specifically including legumes in the catch crop mixture had a positive effect on winter wheat yield. Nevertheless, as environmental conditions such as high degrees of precipitation or elevated temperatures can induce leaching of mineralized N from the cover crop's biomass, the time between the incorporation of the residues and the sowing of the subsequent crop is determining. It was shown that, depending on the

development rate of the cover and main crop and the N availability (intrinsic or through fertilization), under-sowing or intercropping can lead to a competition for N (Carof et al., 2007; Picard et al., 2010). Bergkvist et al. (2011) investigated if the yield of winter wheat was affected by white clover while applying multiple N-fertilization rates. No effect of under-sowing on the yield was noted which was also confirmed by Amossé et al. (2013); Conrad and Fohrer (2009). In their research, wheat grain yield was not significantly disturbed by intercropping whatever the above-ground development of legumes (black medic, alfalfa, red and white clover).

In general, the use of crop rotation systems has been found to have beneficial effects on both the soil conditions and crop yield. Nevertheless, its magnitude is highly dependent on the type of system used, the meteorological conditions (mainly precipitation) and the fertilization regime. In case the cover crop biomass is incorporated before sowing the wheat, it can form an additional source of N. When relay intercropping, under-sowing or crop rotation without ploughing is used, the cover crop aids in fixating the N in the soil, reducing losses due to leaching or volatilization.

1.5.2 Seeding

As winter wheat requires cold (5–10 °C) for vernalisation, it should be sown, depending on the region and prevailing climate, between September and November. The optimal moment during this time span is mainly determined by the timing of precipitation (Ferrise et al., 2010). Moreover, when the wheat is sown beyond the optimum period, the average yield decreases, resulting in a relatively increased protein content (Hossain et al., 2003; Ferrise et al., 2010). In addition, Ehdaie and Waines (2001) observed that early sowing (days to anthesis: 129–151) resulted in a longer vegetative growth period. This, however, did not lead to a higher grain yield in comparison to later sown wheat (119 to 146 days to anthesis). Only straw length, and thus total biomass, increased. In contrary, Baloch et al. (2010) found that earlier planting and a prolonged growth period, results in an enhanced ear development and thus in an increased yield. For spring wheat varieties, comparable effects were obtained by Subedi et al. (2007). A 15 to 45 % yield reduction was observed for the delayed sowing times (10 or 20 days after the first sowing). Besides differences in the optimal timing due to environmental variation, this is also greatly influenced by the genotype.

Gooding et al. (2002) conducted experiments to evaluate the effect of seed rate on wheat yield and quality. It was shown that a lower number of seeds per area unit ($\leq$ 200 seeds m^{-1}) was associated with delayed, and more variable, crop maturation. This asynchronous grain development, which is common for wheat, will be more pronounced in case low seeding rates are used (Jie et al., 2005). In one experiment, grain yield followed a parabolic response to seed rate with apparent reductions in yield at very high seed rates (tested at 50, 100, 200, 350 and 600 seeds m^{-1}). Plants compensated for low plot densities by increased production and survival of tillers and, to a lesser extent, increased grain numbers per ear. Effects of seed rate on grain specific weight and thousand kernel weight were small and inconsistent, possibly due to varying compensation effects. HFN increased (thus, α-amylase activity decreased) linearly with seed rate which was associated with a quicker maturation of the crop. Grain protein concentration was reduced by increasing seed rate from 50 to 100 seeds per square meter, due to competition for nitrogen.

Compensation effects, resulting in more tillers (with a higher number of spiklets per ear) at lower seeding rates, can be indirectly used to gain a maximized yield or an altered protein composition. Li et al. (2016c) found that wheat yield can be increased by decreasing the number of basal and top sterile spiklets and by enhancing (through breeding) the grain weight at the center grain positions. Moreover, a parabolic effect was noted for the number of grains per spiklet in function of its position in the ear. In addition, Jie et al. (2005) also found a parabolic respons within the spiklet for grain weight and protein content. Individual grain protein content also decreased with increasing grain position (base of the ear to top). A variety effect became more pronounced under low Nitrogen input levels.

> Crop rotation, the use of catch crops and seeding density have become conventional practices for improving the wheat yield and the kernel protein content as it enables the uptake of minerals from the soil or fixates them when the main crop does not require them. An optimal balance is however depending on the prevailing environmental conditions and the type of crop.

1.5.3 Fertilization

Although fertilization rate and splitting is studied most often, the number of applications and their timing, as well as the used form of fertilizer is also important. Moreover, as wheat can be cultivated in various regions throughout the world, the soil type and composition (initial fertilizer content, availability, *etc.*) and the prevailing meteorological conditions affect these concentration effects. Besides these environmental variables, genotype influences this as well by the efficiency to translocate and remobilize the components during the different growth stages. As this literature study tries to summarize the various effects, each of the aforementioned influencing factors is discussed separately.

1.5.3.1 Nitrogen

Nitrogen (N) is a major element essential for plant growth and a fundamental component of amino acids, and therefore proteins. Moreover, since N is also part of the enzymes associated with chlorophyll synthesis, its availability impacts all phases throughout crop development, affecting seedling establishment, tillering, canopy development and grain filling. In general, an increased N supply drives the plant towards a higher productivity and a higher grain protein content (Hawkesford, 2014). To optimize fertilization, insight into the availability of N in the soil is detrimental. Factors affecting this availability are both soil type and watering conditions (irrigation or rainfed), as well as information on the depletion should be obtained. Furthermore, the form in which N is administered as well as the times and the distribution across the different fractions (splitting) must be considered. In addition, excessive use of N can also have negative effects, *e.g.* delayed maturity and increased risk of lodging. Moreover, it adversely affects the environment by creating favourable conditions for weeds or algae when N comes into waterways, rivers and oceans (Diacono et al., 2013). To achieve a certain protein concentration and composition, while ensuring a sustainable production, optimized N-fertilization practices are essential.

Fertilization rate and timing The effect of N-fertilization and irrigation on yield and protein content of winter wheat grown on the sandy loam soil in Cambridge (U.K.) was investigated by Pushman and

Bingham (1976). Grain yield increased by both irrigation and N-fertilization, whereas protein content increased by applying additional N, but decreased by 18 % in the irrigated plots compared to the non-irrigated plots. Applying an additional dose of 90 kg N ha^{-1} in Zadoks growth stage (Zadoks G.S.) 32 resulted in a yield increase of 21.1 % and 4.8 %, while the grain protein content grew by 14.1 % and 33.7 %, with and without irrigation respectively. An additional dose of 45 kg N ha^{-1} (applied as an aqueous foliar spray of urea) at anthesis (Zadoks G.S. 60), resulted in additional yield increase of 5.5 % for the irrigated plots and a further increased protein content by 12.4 % for the irrigated plots and by 7.5 % for the non-irrigated plots. Martin (1987) studied the effect of N-fertilization on both winter and spring wheat by applying three fertilizer regimes; (1) 50 kg N ha^{-1} at late tillering (Zadoks G.S. 30), (2) 100 kg N ha^{-1} at late tillering and (3) 50 kg N ha^{-1} at late tillering combined with 50 kg N ha^{-1} at booting (Zadoks G.S. 45). For winter wheat, it was seen that the 50 + 50 kg N ha^{-1} and 100 kg N ha^{-1} treatments gave similar yields (7.9 ton ha^{-1} and 8 ton ha^{-1}), which were about 1 ton ha^{-1} higher than the 50 kg N ha^{-1} treatment. Furthermore, significant differences in grain N content were noticeable between the treatments for winter wheat. The wheat fertilized with 50 + 50 kg N ha^{-1} had a significantly higher N content than in case 100 kg N ha^{-1} was applied (1.82 % N versus 1.70 % N), which in turn was significantly higher than in the 50 kg N ha^{-1} treatment (1.58 % N).

In spring wheat, applying extra N had no significant effect on yield or grain N percentage. In Garrido-Lestache et al. (2004) the effect of N rate and splitting on yield of spring wheat grown in Mediterranean conditions was investigated. For the N rate experiment, 0, 100, 150 or 200 kg N ha^{-1} was applied in equal amounts (1/3rd) at sowing, tillering (Zadoks G.S. 20–25) and stem elongation (Zadoks G.S. 30–35). Yield increased from 3 to 4 ton ha^{-1} when applying 100 kg N ha^{-1} compared to the control without N-fertilization (0 kg N ha^{-1}). However, no significant yield increase was recorded for the higher N rates. For grain protein concentration, a highly significant response to N-fertilizer rate was noted. The grain harvested from the unfertilized control treatment contained 11.2 % protein, whereas 100, 150 and 200 kg N ha^{-1} resulted in grain with a protein concentration of 13.5 %, 14.6 % and 14.8 %, respectively. Only for the two highest concentrations no significant differences were found. Furthermore, the influence of timing was studied by applying 150 kg N ha^{-1} split in various proportions between sowing, tillering and stem elongation (0 + 0 + 0, 150 + 0 + 0, 100 + 50 + 0, 100 + 0 + 50, 75 + 75 + 0, 75 + 0 + 75, 50 + 100 + 0, 50 + 50 + 50, 0 + 150 + 0, and 0 + 75 + 75 kg N ha^{-1}). The best grain yield response was obtained when half or one-third of the total N-fertilizer rate was applied at stem elongation (100 + 0 + 50, 75 + 0 + 75, 0 + 75 + 75). Also the grain protein content was highest for these treatments, or in some cases when N was applied only at tillering. Splitting of the total N rate between sowing and tillering prompted a lower yield and the lowest yields were observed when the total rate of 150 kg N ha^{-1} was applied at sowing. Analogously to the yield reduction, the latter treatment lead to a significant decline in grain protein content. Szentptery et al. (2005) conducted a series of fertilization experiments on winter wheat grown in Hungary, with the following doses: 40, 80, 120, 40 + 80 and 80 + 40 kg N ha^{-1} applied at tillering (Zadoks G.S. 25) and after anthesis (Zadoks G.S. 60). Increasing amounts of fertilizer resulted in a considerably higher baking quality, particularly in case it was applied in two rounds (40 + 80 kg N ha^{-1} or 80 + 40 kg N ha^{-1}). The latter was found to be the most effective treatment since the large first dose provided the wheat with the nutrient boost required for the first phase of its growth. The 40 kg N ha^{-1} applied after anthesis, enabled the genetic potential (given the season) resulting in a maximized baking quality. Abedi et al. (2011) studied the effect of nitrogen rate (0, 120, 240 and 360 kg N ha^{-1}), each applied in three

equal fractions (1) at sowing, tillering and stem elongation, (2) at tillering, stem elongation and grain filling, (3) at sowing, stem elongation and grain filling or (4) at sowing, tillering and grain filling (Zadoks G.S. 70), on winter wheat yield and grain quality grown in Iran. The results indicated that the highest grain yields were obtained at a rate of 240 kg N ha^{-1} when it was applied through the vegetative growth stages (sowing, tillering, and stem elongation). Additionally, application of 240 kg N ha^{-1} resulted in the maximum protein concentration, irrespective of the timing. Although only insignificant effects of N rate on gluten content were noticed, N timing however altered this significantly. Highest gluten contents were obtained in case fertilization was applied at tillering, stem elongation and grain filling (treatment 2). Finally, this study showed that over-application of N (360 kg N ha^{-1}) decreased the protein content. A similar effect for yield was reported by Noureldin et al. (2013) who studied the effect of six nitrogen levels ranging from 0 to 125 kg N ha^{-1}, applied as urea in two equal portions. Adding 75 kg N ha^{-1} resulted in the highest yield (53 % higher compared to the unfertilized control). Both lower and higher N rates adversely affected grain yield. Based on experiments with winter wheat under Mediterranean conditions, Erekul et al. (2012) concluded that grain yield increases up to 210 kg ha^{-1} N without substantial losses in the grain quality. In contrast, Mandic et al. (2015) already noticed a stagnation in winter wheat yield at a N level up to 75 kg N ha^{-1}.

Uptake efficiency From the results obtained by independent researchers, it is clear that the N-fertilization rate is important, but the timing and splitting of the application is critical as well. The application rate influences the grain yield and protein content quantitatively, whereas the timing mainly impacts the protein composition. The appropriate amount of N-fertilizer depends on how much N the soil can supply, the growth rate of the crop and the nitrogen use efficiency (NUE). The latter, which has been subject of a wealth of literature, is defined as the ratio between the amount of N removed from the field by the crop and the amount of N applied as fertilizer. A higher NUE is the result of a better N translocation (portion of N absorbed after anthesis and allocated to the grain) and/or a better N remobilization (N which is recycled from other plant tissues (Cormier et al., 2016)). Accumulation and redistribution of N are important processes determining grain yield and grain quality. In wheat, around 60–95 % of the demand for N during grain filling comes from remobilized N which was stored in vegetative organs (roots, shoots, leaves and stems) before anthesis. If these sources would be depleted, the photosynthetic capacity of the leaves is reduced, resulting in a natural leaf senescence. Through this process, N is recycled following from protein hydrolysis, and is subsequently exported in the form of amino acids to grains. Increasing their senescence, and consequently shortening grain filling duration, will also substantially impact yield (Semenov et al., 2014). A remaining fraction (5–40 %) of grain N comes from the post-flowering N-uptake and translocation to the grain. However, this will only occur if the assimilated N in the leaves is insufficient (Kichey et al., 2007; Gaju et al., 2011). Furthermore, the potential contribution of an organ as a supplier of N depends on the growing conditions. For example: the role of the flag leaf, in comparison to other upper parts of the plant, as a potential supplier of N to grains increases under improved growing conditions. In contrast, the relative importance of the ear and peduncle increases under water stress conditions (Sanchez-Bragado et al., 2016).

The NUE is determined both by the wheat genotype (*e.g.* root size and morphology) and by the environment. Indeed, N-uptake depends upon the N availability and soil moisture along with root related traits. In many climates, the dry conditions associated with the period of crop maturation may limit post-anthesis

N-uptake (Hawkesford, 2014). Furthermore, split- and late season-applications of mineral N-fertilizers are common approaches to improve NUE (Garrido-Lestache et al., 2004). In Brennan et al. (2014) the NUE at 0, 140, 180, 220 and 260 kg N ha^{-1} of winter wheat grown in a cool Atlantic climate (Ireland) was studied. It was concluded that the NUE efficiency ranged from 14.6 kg grain per kg N in case 260 kg N ha^{-1} was applied, to 62.4 kg grain per kg N, in case no additional fertilization was applied. The fact that the NUE declines at high N rates was approved by Mandic et al. (2015). At 75 and 150 kg N ha^{-1}, a NUE of 58.62 and 29.96 kg grain kg^{-1} N was observed for winter wheat grown in Serbia.

Beyond the effect of N rate and timing, the used N form may also influence soil pH and thus the availability of other nutrients, particularly micronutrients (Weber et al., 2008). Urea (UR) is the most produced and used N source in agriculture. However, depending on soil and weather conditions, volatilization can lead to considerable NH_3-N losses when applied on the soil surface. Because of high costs of N-fertilizers, the use of N sources which promote lower NH_3-N losses by volatilization, such as calcium nitrate (CN) or ammonium sulfate (AS), would be a way to increase fertilizer efficiency and maximize wheat yield (Schwenke et al., 2015). To gain insight into the effect of different N-fertilizers, three N rates (40, 80 and 120 kg N ha^{-1}) in the form of UR, CN or AS were applied in top dressing at tillering. For the three N sources, wheat grain yield was highest when 80 kg N ha^{-1} was applied. The N sources only provided significant differences in wheat grain yield when the higher N rates were applied (80 and 120 kg N ha^{-1}). Grain yield was significantly higher with the use of CN and AS than with UR at 80 kg N ha^{-1}. At 120 kg N ha^{-1}, grain yield was higher with the application of CN compared to the use of AS and UR. Garrido-Lestache et al. (2004) also conducted experiments with different N types using 150 kg N ha^{-1}, equally divided over an application at sowing, tillering and stem elongation. N was applied either in the form of UR at sowing and ammonium nitrate (AN) as top-dressing, or as UR at sowing and AS as top-dressing. In addition to the 150 kg N ha^{-1}, one leaf fertilization at ear emergence (control, 25 kg S ha^{-1}, 25 kg N ha^{-1}, 25 kg S ha^{-1} + 25 kg N ha^{-1}, and 50 kg N ha^{-1}) was applied. It was concluded that use of different types of fertilizer to the soil and of N and/or S-fertilizer to the leaf, had no significant effect on the grain yield. However, grain protein content increased when the maximum leaf N rate was applied at ear emergence (50 kg N ha^{-1}).

Method of application Grahmann et al. (2016) studied the effect of fertilizer application method (broadcast, applied in furrows or disk-banded on top of beds) and timing (applied before planting or split between pre-planting and first node (Zadoks G.S. 31)) of 120 kg N ha^{-1} as urea in northwestern Mexico (a hot and arid climate, where spring wheat is cultivated during winter). A pre-planting application of 120 kg N ha^{-1} resulted in lower wheat yields compared to a split application. Furthermore, the results showed a clear advantage of furrow and bed application over broadcast application to increase wheat yield and quality. Highest test weights were obtained with 40 kg N ha^{-1} bed, 80 kg N ha^{-1} furrow or 80 kg N ha^{-1} bed - 40 kg N ha^{-1} furrow. Basal broadcast application resulted in significantly lower protein content than the other fertilized treatments.

As mentioned above, the optimal N-fertilization is highly depending on the initial soil properties, the wheat variety and type (winter or spring). So, no general conclusions concerning the N rate that has to be applied can be drawn. Applying additional N, up to a certain limit, improves grain yield and protein content. However, over N-fertilization has an adverse effect on yield and favours plant logging.

Concerning the timing, it can be concluded that N application before sowing has an effective role on seed germination and plant settlement, whereas the influence on yield and quality is rather limited. According to Abedi et al. (2011), N-fertilization before wheat planting is unnecessary due to two reasons. First of all, soil N is in most cases sufficient for seed germination and early growth and, moreover, in most cases the N applied pre-sowing moves beyond the root zone, especially in irrigated fields or fields with a high amount of precipitation around sowing. Early N, applied at tillering, will be used mainly by the plant for yield attainment, whilst for increasing grain protein N applications should be made between booting and ear emergence. By applying N at anthesis, the grain-filling period can be prolonged, given appropriate weather conditions this can result in the preference for protein build-up over starch synthesis. Furthermore, additional N-fertilization at heading (Zadoks G.S. 50–59) improves loaf volume of wheat flour based breads by increasing the grain protein concentration and altering its composition. N splitting in four fractions enhances the percentages of prolamin (PRO) and glutelin (GLU) as well as certain high molecular weight GLU subunits (HMW-GS), which leads to an overall improved baking quality (Xue et al., 2016). Therefore, it is argued that N splitting is more effective in improving wheat quality than the increase in N rate. This offers the potential to cut down N-fertilization rates in wheat production systems. Furthermore, from the literature review above, it is clear that late season N application improves wheat quality. Nonetheless, late applications (post-anthesis) do not guarantee an increase in bread-making quality (Pushman and Bingham, 1976). However, in an in-depth study of Li et al. (2016b) in which labeled N was used to differentiate between the amounts absorbed pre- and post-anthesis, contradictory observations were made. It was shown that N stored after anthesis mainly contributed to the concentration of the storage proteins (globulin and GLU) in the kernel. Moreover, the authors proposed that optimizing N application during the growth stages post-anthesis could be a feasible approach to regulate the distribution of the protein fractions in the grain for specific end-use.

Currently, the timing of N-fertilization is mostly studied in relation to the growth stage. However, modern non-destructive imaging techniques give the opportunity to asses crop N status and thus more accurately estimate crop N-fertilization requirements. By using spectroradiometers, reflectometers, imagery from satellite sensors and digital cameras, optical properties, such as crop canopy reflectance, leaf transmittance, chlorophyll and polyphenol content, can be measured to estimate N in plant. These technologies allow for highly sensitive plant N status information, and may thus contribute to a better N management (Tremblay et al., 2012; Muoz-Huerta et al., 2013). In practice, these techniques can be applied for precision agriculture allowing farmers to apply the right input, in the right amount, to the right place, at the right time and in the right manner improving agronomic, economic and environmental efficiency (Diacono et al., 2013).

> The use of nitrogen fertilizer remains one of the most common and widely accepted practices for wheat yield enhancement. The total dosage, number of fractions and type of application can however be determining in the magnitude of its effects. At the same time, this is dependent on the soil properties and meteorological conditions and has to be tailored according to the plant development.

1.5.3.2 Sulfur

Besides N, Sulfur (S) is an element essential for plant growth as it is a key element in the formation of chlorophyll. Moreover, it forms a building block of various proteins, including the gluten proteins as they contain more S-rich amino acids such as cysteine and methionine. The S requirement for optimal wheat growth is about 15–20 kg S ha^{-1} (Gallejones et al., 2012). Although findings with regard to the yield gain achieved by applying additional S vary greatly between studies, it is indisputably demonstrated that S deficiency results in a reduced yield. According to Jrvan et al. (2008), for winter wheat grown in a nitrogen background of 75 and 100 kg N ha^{-1}, additional S (10 kg S ha^{-1} divided over two applications) can lead to a yield increase from respectively 7.7 % to 43 %, depending on the prevailing weather conditions and the time point at which the fertilizer is applied. For spring wheat Klikocka et al. (2016) reported a yield increase of 3.58 % with respect to the control without S-fertilization, by applying 50 kg S ha^{-1} (40 kg S ha^{-1} before sowing and 10 kg S ha^{-1} at heading) in combination with 80 kg N ha^{-1} (40 kg N ha^{-1} before sowing and 40 kg N ha^{-1} at stem elongation). Stroud et al. (2010) even reported a yield decrease from 1 to 15 %, depending on the year, when no S-fertilization was applied.

Fertilization rate Besides affecting yield, S affects both total grain protein concentration and the accumulation of different protein groups during grain development (Jrvan et al., 2008; Zhao et al., 1999; Shahsavani and Gholami, 2008; Erekul et al., 2012; Klikocka et al., 2016). However, the effect of S on total protein concentration in the wheat kernel is, in most cases, rather limited (Steinfurth et al., 2012). Additionally, according to Jrvan et al. (2008), protein content can even decrease when crop yield responds to S due to a dilution of nitrogen in the grain. Therefore, the main benefit associated with S-fertilization is considered to be the effect on protein composition rather than on the protein content. Wheat designated for application in bakery products such as bread, requires a correct balance both between the gluten proteins, PRO and GLU, as well as within their respective subgroups (α-, β-, ω- and γ-PRO, and low molecular weight glutelin-subunits (LMW-GS) and high molecular weight glutelin-subunits (HMW-GS)). The latter group mainly contributes to the elastic character of bread dough in contrast to PRO which provides the extensibility. Therefore, an optimal composition aids in the formation of a strong, but flexible gluten network with a high gas retention capacity. Sulfur deficiency leads to the accumulation of S-poor storage proteins such as ω-PRO and HMW-GLU subunits at the expense of S-rich proteins (α-, β-, and γ-PROs and LMW-GS). Therefore, S-deficient wheat is characterized by lower concentrations of S containing compounds (cysteine and methionine). These changes in protein composition are associated with alterations of dough rheology and thus breadmaking quality (Van Bockstaele et al., 2011). Dough made from S-deficient flour is more stiff, has increased mixing requirements, reduced extensibility, and smaller loaf volumes (Jrvan et al., 2008; Zhao et al., 1999). From the experiments with spring wheat of Klikocka et al. (2016), it was concluded that S application significantly increased the gluten content (3.2 %), and the concentration of cysteine (6.0 %) and methionine (16.5 %). The most beneficial effect on the total protein and gluten content was observed with an application rate of 80 kg N ha^{-1} (40 kg N ha^{-1} before sowing and 40 kg N ha^{-1} at stem elongation) and 50 kg S ha^{-1} (40 kg S ha^{-1} before sowing and 10 kg S ha^{-1} at heading). Erekul et al. (2012) confirmed these findings for winter wheat by demonstrating a significant increase in the gluten-index (ratio strong gluten over total gluten) and especially the sedimentation value as a result of S-fertilization.

Furthermore, studies have shown that bread making quality parameters were correlated with the grain S concentration, more than with the N concentration (Singh, 2003; Zhao et al., 1999; Salvagiotti and Miralles, 2008; Naeem, 2008). In addition, a synergistic effect between the applied N and S-fertilizers appears to occur, increasing N and S assimilation in wheat grain, consequently improving bread making quality. Podlesna and Cacak-Pietrzak (2008) noted that the magnitude of response to S varies with the rate of N added. Although all levels of N-fertilization (30, 60, 90, 120, 150 kg N ha^{-1}) resulted in an increased grain yield, the applied S only showed an effect on yield in the middle range of N doses (60 and 90 kg N ha^{-1}). Besides protein properties, Li et al. (2013) demonstrated that combined N and S-fertilization influenced starch properties, such as total starch concentration, amylose and amylopectin concentration and ratio and thus pasting properties, when a critical concentration (230 kg N ha^{-1} and 46 or 76 kg S ha^{-1}) is exceeded.

Timing In analogy of the findings for N application, the timing of S-fertilization is equally important. Wheat appears to be more sensitive to S deficiency during the generative growth stages resulting in reduced yields (Zhao et al., 1999). To enhance baking quality on the other hand, Zrb et al. (2009) found that a late S-fertilization around flag leaf sheet opening (Zadoks G.S. 47), is most suitable. S accumulation itself occurs mainly after flowering (Zadoks G.S. $>$ 69), as is noted by an increased expression of high affinity S transporters in the flag leaves. The latter functions as a sink organ to the kernels. In S-starved plants, their expression increases only after anthesis (Buchner et al., 2010). During this developmental stage, large amounts of S are remobilized from the flag leaf to supply the developing kernels (Steinfurth et al., 2012). Presumably, S only gets remobilized due to senescence when S-fertilization is applied in high dosages and late during the growth (Howarth et al., 2008). Also the results from Monaghan et al. (1999) highlight the importance of S uptake (translocation) after anthesis to the accumulation of S in grain.

> Sulfur fertilization is practically only applied when intensive agricultural practices are applied with the aim of producing high quality wheat. Most often, it is applied later during growth thereby impacting the kernel composition. Due to its role in the formation of S-rich proteins (both monomeric and polymeric), it can greatly alter the functionality of the gluten protein.

1.5.3.3 Potassium and Phosphorus

The influence of potassium (K) and phosphorus (P) is mainly an aggregate of the functions played by nutrients in mitigating negative effects of biotic and abiotic stresses. Plants provided with sufficient amounts of K and P are less vulnerable to water deficiency, low temperatures and pathogen attacks. Potassium aids in maintaining the crop structure and firmness, reducing the risk of lodging, preventing comprised quality as a result of increased enzymatic activity and a lower specific weight. Furthermore, K is an indispensable component during the main stages of protein biosynthesis. Its deficiency can lead to a decrease in the protein concentration. Since this occurs regardless of the N-uptake, a possible accumulation of non-protein nitrogen will occur which fosters fungal infections (Rice, 2007). Furthermore, K deficiency impedes nitrogen uptake which results in the decreased leaf assimilation surface thereby reducing the uptake and transport of nitrates in the plant (Bharti et al., 1996). This can also result in less photosynthesis and therefore, a general slower plant development.

Although phosphorus (P) is the second most important nutrient for wheat (Haileselasse et al., 2014), many agricultural soils in Europe have large P reserves (Sattari et al., 2012). In the Netherlands, France and Germany, recent national P surpluses are on average as high as 25–30 kg ha^{-1} (Smil, 2000; Tunney et al., 2003) while in Sweden, Norway and UK the P surpluses of intensive livestock farms are about 8–20 kg ha^{-1} (Uln et al., 2007). In contrast, agricultural lands in tropical and subtropical areas are suffering from P deficiency which is partially a result of high rainfall (Zhang et al., 2016a). Furthermore, it has to be noted that P is often slowly available to plants within the soil environment. This is mainly due to soil P being adsorbed to the soil reactive clay surfaces, Al and Fe oxides, carbonates and organic matter (Tóth et al., 2014) as well by the high P fixation. The use of rhizosphere bacteria to solubilize fixated P, is a majorly studied subject as this forms a sustainable approach to be able to conform with the increasingly stricter regulation on P fertilization (Bhattacharyya and Jha, 2012). This ecofriendly microbial mediated P management could be a cost effective alternative for regions where soil P reserves are large (Sharma et al., 2013).

Cereal crops require about 20 kg P ha^{-1} for normal growth. Adequate P availability enhances many aspects of plant physiology like photosynthesis, flowering, seed maturity and seed development (Ziadi et al., 2008). It is generally applied pre-plant or at seeding since deficiency during the early growth stages has a much larger impact on yield than P limitations later in the season. Application of P fertilizers positively influences both grain yield and the number of tillers (Damene, 2003). According to Haileselasse et al. (2014), a combined 1:1 to 1:3 P:N-fertilizer is required. The response of spring wheat yield to various levels of P_2O_5 (0, 72, 108, 144, and 180 kg ha^{-1}) in combination with 180 kg N ha^{-1} (applied in three factions) under Chinese conditions was studied by Zhu et al. (2012). The response of wheat grain yield to P fertilizer showed a quadratic response. When P was applied at a moderate level, grain yield dramatically increased with an optimum at 108 kg ha^{-1} P_2O_5. When P was over-applied however, the grain yield did not further increase, it even decreased. Concerning the wheat quality, the results of the study of Gaj et al. (2013) showed that increasing P rates had no direct effect on the protein and gluten content in grain which confirms the results of Cambell et al. (1996) who state that P only slightly affects grain protein composition in winter wheat.

Figure 1.9 shows a comparison between the various agricultural treatments that can be applied to gain an increased grain yield or protein concentration as well as how an enhanced protein composition in winter wheat can be achieved. Nevertheless, besides the proposed practices, meteorological conditions, mutual interactions, and the genotype effect must be kept in mind. Moreover, as is marked by the light and dark blue color, various options for N-fertilization are possible.

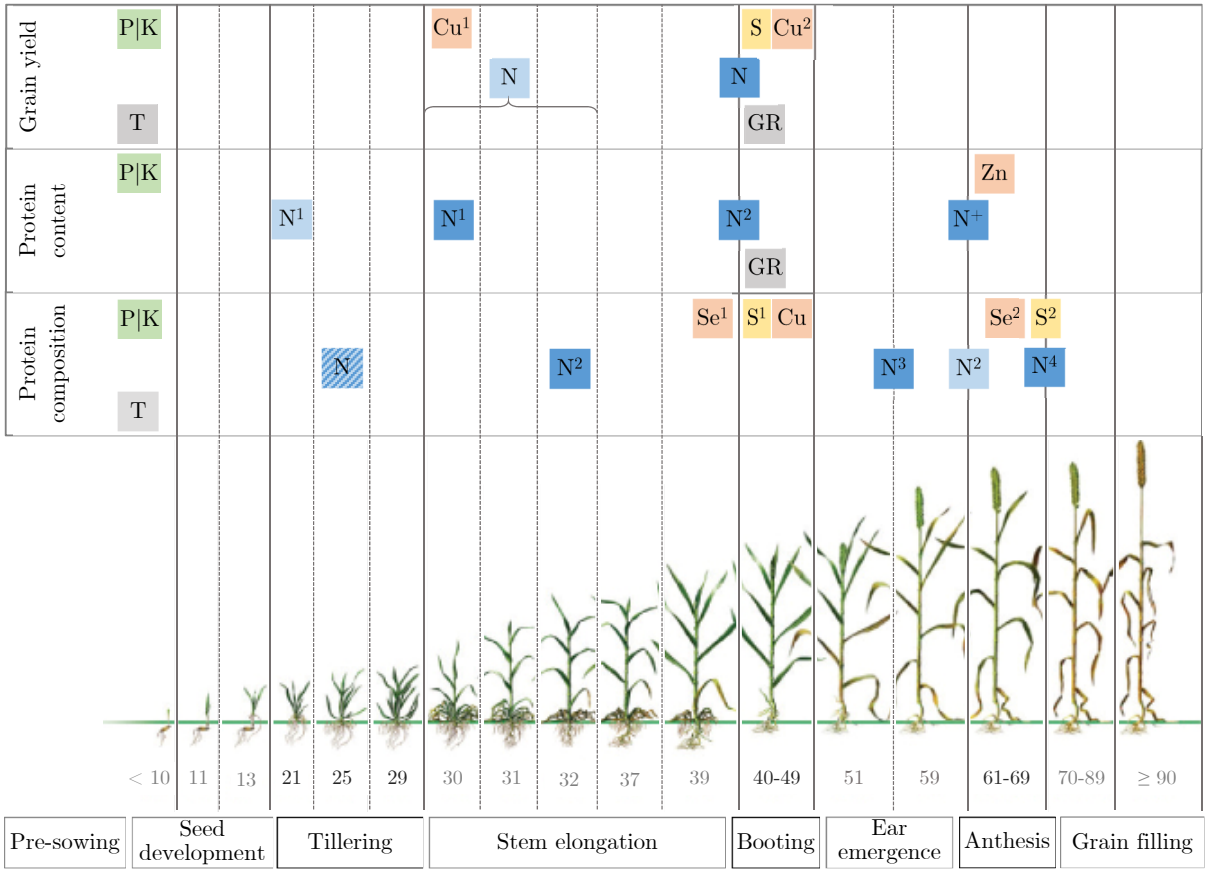

Figure 1.9: Influence of Nitrogen (N), Sulfur (S), Phosphorus (P), Potassium (K) and micronutrient (Cu, Zn, Se) fertilization timing and splitting (*numbers in superscript*), tillage (T) and the application of growth regulators (GR) on yield, protein content and composition.

1.5.4 Growth regulators

One strategy to increase wheat productivity is by optimizing plant architecture (defined by *e.g.* tillering, stature, and leaf and ear morphology) by using plant growth regulators (Gasperini et al., 2012). Culm shortening changes the distribution between vegetative and generative mass. Reducing culm length in wheat prevents lodging and reduces intra-shoot (ear vs. culm) competition for assimilates. Both impacts are beneficial for increasing grain yield (Toyota et al., 2010). Miziniak and Matysiak (2016) evaluated the effect of growth retardants trinexapac-ethyl, chlormequat and prohexadione calcium, applied in mixtures with paraffin oil adjuvant or organosilicone surfactant. When applied on winter wheat at Zadoks G.S. 31 (first node), a varying impact on the protein, starch and gluten content of grains and on the Zeleny sedimentation value was noted. Depending on the year of study and weather conditions, an increased or decreased wheat quality was observed. The objective of study of Ramburan and Greenfield (2007) was to assess the effects of chlormequat chloride and ethephon, applied either individually or in combination with each other at the tillering, stem elongation or the flag leaf stages on agronomic and quality parameters of three winter wheat varieties. This study indicated that the combination of both plant growth regulators was most effective in controlling lodging with applications at the flag leaf stage. In case lodging was successfully controlled, the plant growth regulators improved grain yields. Furthermore, an improved hectoliter weight and grain protein content was obtained after the application

of both plant growth regulators at the flag leaf stage. Additionally, preharvest sprouting tolerance was generally improved. Okon et al. (2015) investigated the effect of the growth regulator furolan on the starch and protein composition of three different winter wheat varieties. Furolan undoubtedly influenced the protein and starch contents, as well as an increased amylose content (0.8–1.2 %) was noted in all furolan treated varieties. Furthermore, the combined application of ethephon and chlormequat offers a degree of protection against O_3 related yield losses (McKee and Long, 2001).

1.5.5 Disease control

Wheat is prone to the several diseases leading to yield and quality losses. Among the economically most important diseases affecting winter wheat yield and quality are obligate parasites (*Blumeria graminis f. sp. Tritici*, *Puccinia graminis f. sp. Tritici*, *Puccinia triticina*, *Puccina striiformis f. sp. Tritici*) and crop residue-borne necrotrophic pathogens (*Pyrenophora tritici-repentis*, *Zymoseptoria tritici*, *Parastagonospora nodorum*, *Cochliobolus sativus*, *Fusarium* species) (Jevti et al., 2017). Globally, potential grain yield losses due to diseases (pathogens and virusses) have been estimated at 18.1 % in the period between 2001 and 2003. Despite the current disease control measures, still 12.6 % of the global acreage actually gets lost (Oerke, 2006).

Septoria tritici Blotch (STB), caused by *Zymoseptoria tritici*, poses a serious and persistent challenge to wheat grown in temperate climates throughout the world. This pathogen secrets enzymes that destroy the plant cell wall to enable them to feed on the glucose within the cell, reducing the plant's grain-filling potential, thus reducing the thousand-grain weight (Farrar and Lewis, 1987). Based on artificially inoculated field trials in Argentina, Castro and Simn (2016) investigated the potential yield and quality losses due to STB. The results revealed that yield reduction fluctuated between 18 % and 49.6 % depending on the variety. Regarding the effect of STB on protein concentration, the results of this work evidenced an increment with the inoculum concentration. This is in accordance with the results of Watson et al. (2010), who observed a relative increase in grain protein concentration of 0.04 % for every 1 % increase in STB severity. Nevertheless, this seemingly positive ability results from a reduced carbohydrate accumulation and thus, a lower kernel yield. Protein yield will in fact be lower when STB is present during growth.

Powdery mildew, caused by *Blumeria graminis*, can affect all above ground plant parts. Yield losses in Canadian winter wheat of up to 20 % were observed by Conner et al. (2003). The protein content of the grain of the moderately resistant varieties was unaffected, but it decreased by 0.7 % in the susceptible varieties. Significant negative correlations between yield and protein content on the one hand and the powdery mildew disease index on the other hand were noted by Cao et al. (2014). The regression coefficients of the models relating disease index at Zadoks G.S. 59 (ear emergence) to protein content indicated a variable protein decrease from 0.06 to 0.12 % for every percent increase in disease index. The fact that powdery mildew reduces grain protein content can be caused by the fact that mildew retains nitrogen in the leaves. Furthermore, mildewed leaves appear to lose both nitrogen as well as ammonia gas. As a result, additional N loss from the plant may be in the production and dispersal of conidia (Dimmock and Gooding, 2002).

Additionally, leaf rust (caused by *Puccinia triticina* Eriks) and stripe rust (caused by *P. striiformis f. sp. Tritici*) affect both grain yield and quality. In the field trial of Sharma et al. (2016) in China, severe

stripe rust infections resulted in a yield reduction up to 36 %. Also grain protein content is often reduced with infection by rust as was confirmed by the findings of Devadas et al. (2014). They observed a reduction in grain protein from 11.7 % to 11.2 % by the presence of stripe rust due to the loss of N from the plant tissue by the pathogen, principally as spores. Furthermore, infections can lead to a reduced uptake of N and a reduced remobilisation from vegetative tissue into the grain after anthesis.

Yield reductions caused by the above described leaf pathogens are mainly due to a loss of photosynthetic capacity (Robert et al., 2015), whereas ear pathogens, *e.g. Fusarium* species, kill or damage spikelets before grain filling. Additionally, kernels in infected heads are shriveled and shrunken resulting in a lower thousand kernel weight. Concerning the effect of Fusarium head blight on quality, it has been reported that proteases from *Fusarium* spp. in infected grains have the ability to degrade gluten proteins (Gartner et al., 2008; Wang et al., 2005). Mosleth et al. (2012) reported severe gluten protein degradation in winter wheat from Norwegian fields in 2011 having extremely low R_{max} (resistance to extension as measured by an extensigraph) values. Furthermore, *Fusarium* infection can lead to the accumulation of mycotoxins, low molecular secondary fungal metabolites which can induce acute and chronic toxic effects. Capouchov et al. (2017) discovered significant negative correlation coefficients between the content of mycotoxins and many technological grain characteristics, for example, between DON content (as an indicator for DON-producing Fusarium species) and Zeleny sedimentation value ($r = -0.60$) and also between DON content and volume weight ($r = 0.63$). Furthermore, *Fusarium* infection worsened rheological quality and had a negative effect on the protein as well as starch part of the Mixolab curves. Detrimental effects on gluten, dough and bread characteristics were found by Schmidt et al. (2017) as a result of strongly increased enzyme concentrations (proteases, amylases, xylanases and lipases) after artificially infecting the grains with *Fusarium culmorum*. In addition, as the deterioration of the bread quality after storage was independent of the level of initial infection, the importance of optimal storage conditions can be emphasized. In general, a reduction for various quality traits was found in previous research as summarized by Ács et al. (2017). The varying results, nevertheless, can be assigned to the variety resistance and the environmental conditions related to *Fusarium* infestation.

Furthermore, the presence of fungal and thermostable alpha-amylases result in an increased enzymatic degradation of starch at, respectively, relatively low (30 °C) or high (100 °C) temperatures which is detrimental for both the dough handling and the end product quality. Gooding et al. (1987); Kettlewell (1997) suggested that fungicides can also lower HFN by retarding the grain drying process. In addition, pre-maturity alpha-amylase activity is stimulated by delayed leaf senescence.

1.6 Environmental conditions

1.6.1 Soil type

Although a loam soil is found to be the best for wheat cultivation, this versatile crop can be grown in various types of soils ranging from sandy to heavy clay. However, most conclusive in the plant's ability to extract water and nutrients is the water holding capacity of the soil and, therefore, its response to rainfall. Limited water availability for the plant and an unfavourable moisture distribution in the soil during the vegetative wheat growth period can, especially on sandy soils, lead to a high variability in yield and

protein concentration with substantial effects on the bread-making quality (Bonfil et al., 2004). The results of the experiment from Erekul and Khn (2006) showed that the influence of soil type on the yield of winter cereals gains its significance if weather conditions become more unfavourable. Low grain yields, mainly as a result of a reduced ear density, and high crude protein concentrations were more pronounced on the poor silty sand soil than on the loamy sand soil in a dry year. Furthermore, principally higher values were found for Zeleny sedimentation value, wet gluten content, gluten index and HFN in a dry year on the loamy sand soil in comparison to the wet year on the silty sand soil. The relation between soil type and wet gluten content was, on the other hand, not coherent.

1.6.2 Weather conditions

Despite improved varieties and advances in cultivation techniques such as irrigation systems, precision agriculture, *etc.*, crop production is highly vulnerable to unfavorable weather conditions. Seasonal and inter-annual weather variations are important issues for farmers. Moreover, it has been shown that climate change is of increased concern to them. From this point of view, there have been numerous attempts to uncover the correlation between quality traits and meteorological variables. Similar to crop husbandry practices, weather conditions influence yield and protein both quantitatively and qualitatively. Furthermore, these adverse effects can occur in a direct or in an indirect way since imposing stress alters the gene expression patterns which on its turn influences for example fertilizer efficiency.

1.6.2.1 Irrigation and precipitation

Drought in the phase of germination and at early tillering can result in a sparse set of plants and a lower intensity of tillering, both leading to a reduction the number of ears per square meter. Moreover, a shortened growth period resulting from an expedite heading and early maturity (seven to ten days prematurely) shortens the growth period. These factors contribute to substantial yield losses. In general, drought stress can form an obstacle during early vegetative growth stages, but it is most sensitive during shooting and booting. Similar detrimental consequences are observed when drought stress arises at the time of head (inflorescence) emergence and at flowering and leads to a shorter ear length. High temperatures and water deficit during the grain filling period shorten the grain filling process and accelerate ripening which, on its turn, reduces the weight of the grain ears and hence, the yield (Bauder, 2001; Mandic et al., 2015).

In contrast to drought stress effects, precipitation prior to grain filling is negatively related to protein concentration. It is thought that the dilution of early N reserves by vegetative proliferation forms the basis of this effect. Although N losses increase due to leaching and other forms of soil nitrogen loss, precipitation augments soil moisture reserves. The latter extends leaf life during grain growth, thus favouring carbohydrate assimilation and translocation exceeding that of nitrogen. The results from literature concerning the effect of rainfall during, instead of prior to, the grain filling period on the protein concentration, are less consistent (Orlandini et al., 2011). According to Lpez-Bellido et al. (2001); Garrido-Lestache et al. (2004) rainfall or irrigation during grain filling is often positively associated with grain protein concentration. Others, however, report that grain protein concentration increases under conditions of drought or low rainfall (Gooding and Davies, 1997). This is in accordance with the results from Ozturk

and Aydin (2004) who found that continuous water stress increased grain protein concentration by 18.1 %, Zeleny sedimentation value by 16.5 %, wet gluten content by 21.9 % and decreased 1000-kernel weight by 7.5 g compared to fully irrigated conditions. Generally, smaller effects could be noticed when water stress was induced later in the growing season (8.3 %, 8.7 %, 10.8 % increase in protein content, Zeleny sedimentation value and wet gluten content and a reduction of 3.8 g in 1000-kernel weight, respectively). Early water stress and rainfed conditions showed similar effects, encompassing an increased Zeleny sedimentation value and wet gluten content, as well as a decreased 1000-kernel weight. However, the effect of late water stress on grain quality was more pronounced than that of early water stress. These results can be explained by the fact that grain protein concentrations are the result of interactions between N and water availability, yield and temperature, whose complexity in many cases hinders their examination (Garrido-Lestache et al., 2004).

To study the effect of drought stress on protein composition, Zrb et al. (2017) created drought stress by withholding rainfall during the whole vegetation period and the plants were entirely grown on the stocked soil water reserves. Control plants were grown next to the shelter with optimal water supply (approximately 800 mm). By analyzing the grain protein fractions, an increase in HMW and some LMW GLU sub-fractions was detected. Under the different environmental scenarios, the GLU fraction in general seemed to be most variable in their gene expression patterns. The drought stress applied in the experiment also led to a significantly decreased yield, ranging between 20 and 55 %. Furthermore, it was demonstrated that the protein concentration increased with drought although this effect was only significant for some varieties.

1.6.2.2 Temperature

Summer temperatures (up to 30 °C) during the grain filling period, negatively correlate with yield, thus positively influencing protein concentration (Wardlaw and Moncur, 1995). Under conditions of heat and drought, the grain-filling period is shorter and grain yield lower, resulting in wheat with a relatively higher protein concentration (Gooding and Davies, 1997; Smith and Gooding, 1999; Gooding et al., 2003). Dupont and Altenbach (2003) report that maximum yields are achieved when temperatures are between 15 and 20 °C as temperatures in this range give the longest duration for the grain filling period. Furthermore, Johansson and Svensson (1998) found that mean day temperature at the end of the growing season was negatively correlated with protein concentration. According to this Swedish research, high temperatures in July (grain filling, sown mid-September) and a low temperature in August (ripening, harvested between 15–25 August) will lead to the highest protein concentration.

Based on the forecasts of global climate change models, heat stress during the reproductive phase (anthesis and grain filling) will be more common due to the predicted increase of 1.8 to 5.8 °C in mean ambient temperatures. As is stated by Farooq et al. (2011), this increase can cause pollen sterility, tissue dehydratation and lower CO_2 assimilation. Moreover, elevated photorespiration can be expected resulting in an overall decline of plant health. Photorespiration is - in contrary to photosynthesis - a seemingly wastefull process, consuming O_2 for the assimilation of nitrate from soil. It is activated at low plant CO_2 levels. Despite the expected increase of CO_2, the elevated ambient temperature will force the plant to close its stomata to prevent moisture loss, thus lowering the leaf CO_2 concentrations (Sage and Kubien,

2007). In addition, RuBisCo, which is the key enzyme in photorespiration, is less able to discriminate between CO_2 and O_2 at higher temperatures.

Some work has already shown that a certain threshold (ca. 30 °C) exists before the positive correlation between grain protein percentage and dough strength is lost. For heat-sensitive varieties, the aforementioned threshold is even lower. The correlation may even become negative as the accumulation of PRO is less reduced by elevated temperatures than that of GLU, resulting in an increased PRO:GLU ratio (Stone and Savin, 2000; Saint Pierre et al., 2008). For spring wheat, the influence of weather conditions on the protein quality seems to be even more pronounced than on its concentration due to the shorter growth period. Protein quality, represented by the mixogram index, showed a correlation with the mean day temperature during May ($r = -2,65$, $p < 0.05$), June ($r = 2,83$, $p < 0.01$) and July (head emergence until late milk stage) ($r = 3,63$, $p < 0.005$). This is a longer period compared to the most important period determining protein concentration (July and August, early dough stage until harvest) (Johansson and Svensson, 1998). Also lower resistance to stretching of the gluten dough (R_{max}) possibly due to higher PRO concentrations was reported when the mean daily temperature dropped below 17–18 °C. Moldestad et al. (2011) found that temperature during grain filling was the most determinative weather parameter since its powerful association with gluten quality.

Alpha-amylase activities are often higher when the weather is relatively cool and wet during grain filling or when a heat shock occurs during this growth stage. A lower HFN arises when harvest is delayed due to unfavorable weather conditions (Mares and Mrva, 2008). According to Garca et al. (2016) post-anthesis warm nights reduced thousand grain weight and yield by ca. 3 and 4 % respectively per degree Celcius the night temperature increased. In the experiment, the night air temperature was artificially increased by ca. 4.1 °C which resulted in an accelerated development, in turn leading to a shorter effective grain filling period and consequently reducing the final grain weight. A similar conclusion was drawn by Alvarez Prado et al. (2017) who observed reductions in mean grain weight due to increases in both the number of hours above 30 °C and mean night temperature.

Dupont et al. (2006) studied the effect of temperature on wheat flour proteins during grain development. It was seen that high temperatures during grain filling increased protein content and altered the relative proportion of S-containing proteins by the increased accumulation of S-poor proteins in comparison to S-rich proteins.

1.6.2.3 Light intensity and shading

Besides temperature and rainfall, also light intensity is an important factor influencing wheat growth and development since it affects both the photosynthesis and respiration rate of crops (Goswami et al., 2006). Li et al. (2010) studied the effects of various levels of shading (92 %, 85 %, 77 % of full radiation) on wheat yield. The results indicated that the effect on grain yield was dependent on the level of shading. Under heavy shading (77 %), the yield loss ranged between 5.9 % and 6.7 %, depending on the variety. Under less shaded conditions (85 %), the yield loss was limited to 2.3 %. Thus, it was concluded that yield losses were much lower than the reduction in radiation, indicating that there must be physiological and morphological compensation effects at both leaf and canopy level to mitigate the adverse effect on grain yield under shading. Additionally, a slight increase in grain yield was observed in case 92 % of

full radiation was applied. These results are in line with the results obtained by Xu et al. (2016) wherein mid and severe shading (67 % and 35 % of full radiation) led to substantial yield losses. Reductions up to 23 % for mid shading and up to 82 % for severe shading were observed. However, under slight shading (88 % of full radiation), leaf senescence was delayed and photosynthesis rate was stimulated resulting in an increase in grain yield up to 8.5 % compared to full radiation. According to results of Hernndez-Barrera and Rodrguez-Puebla (2017) the increase of solar radiation over Spain, due to climate change, could force an overall yield decrease. This can be attributed to the fact that higher solar radiation increases transpiration demand, thus supporting water stress and consequently lowering yields. In addition, more solar radiation implies less cloudiness, less precipitation, lower minimum temperatures and higher maximum temperatures. Based on the findings of Maydup et al. (2010), selection of varieties with higher ear photosynthesis (*e.g.* ears with longer awns) could counteract this possibly unfavorable evolution as grain yield is substantially affected by ear photosynthesis capacity. Results showed that this could contribute for 13 to 33 % under non-stress conditions and for 22–45 % when the plant was defoliated or when a water deficit was applied.

1.6.3 Carbon dioxide levels

Following the start of the industrial revolution in the 19th century the atmospheric levels of carbon dioxide (CO_2) have been steadily rising. In 2013, CO_2 levels surpassed 400 ppm. Though different climate change scenarios predict a wide range of trends, all predict a further increase in CO_2 levels over this century, with some of the scenarios predicting a doubling or even trebling of today's levels (IPCC2003. IPCC Working Group I, 2013). These elevated levels have a profound effect on the growth, physiology and chemistry of plants (Ziska, 2008). Especially in C3 plants, such as wheat, elevated CO_2 levels enhance photosynthesis rate and water use efficiency (due to a reduction in stomatal conductance) which, as a consequence, boost yields. According to Bannayan et al. (2014), yield increase per unit change of CO_2 (1 ppm^{-1}) was considered 0.05 %. The review of Amthor (2001) summarizes results from 50 papers evaluating the effects of elevated CO_2 levels on wheat yield. It was seen that in experiments with superambient CO_2 levels, combined with ample water and nutrients and favorable temperatures, CO_2 levels up to 2000 ppm increased yield, with a maximum yield increase (37 %) at about 890 ppm CO_2. On average, doubling CO_2 levels from 350 to 700 ppm increased yield by approximately 31 %. From the meta-analysis based on 59 papers conducted by Wang et al. (2013), it was also concluded that elevated CO_2 levels (450800 ppm) significantly increased yield (24 %). Furthermore, it was seen that the foliar chlorophyll and soluble protein content declined significantly with 7.5 % and 11 % respectively, and that the N concentration in the whole-shoot was reduced by 23 %. These changes, along with remarkably increased aboveground biomass (28 %), demonstrate that the increased growth rates accompanying elevated CO_2 levels were not matched by increased N acquisition or assimilation, leading to a dilution of shoot N. In contrast to increasing yields, evidence is mounting that ramping CO_2 levels reduce grain quality by decreasing protein content, changing the balance of amino acids and the stoichiometry of several trace elements (Bloom et al., 2014).

The average decrease in grain protein content, estimated in a meta-analysis by Taub et al. (2008), was 10 % to 15 % in case CO_2 levels elevate from the ambient 400 ppm to 540–958 ppm. Another meta-analysis by Broberg et al. (2017) revealed that the response function for the relationship between N concentration and CO_2 showed a quadratic behavior (change in N $= 49.9 - 0.2x + 0.0017x^2$, with x = CO_2 levels). This

equation shows an initial reduction in N concentration with increasing CO_2 levels, reaching a minimum at 600 ppm. Myers et al. (2014) stated that the decrease in protein content, which was found to be 6.3 % lower at CO_2 levels of 546 ppm in comparison to the ambient concentration of 382 ppm, was not solely as a result of a dilution effect. Therefore, according to Wang et al. (2013), key targets for future wheat breeding are to select new genotypes which have higher sink capacity for photosynthetic products and are capable of increasing N-uptake under elevated CO_2. Due to the higher stimulation of net photosynthesis by the ear compared to the flag leaf under high CO_2 atmospheric conditions, selecting varieties with higher ear photosynthesis could increase assimilate availability to fill the grains, thus supporting yield increases (Maydup et al., 2010).

Additionally, the effects of elevated CO_2 levels on Fe, S and Zn are very comparable with the effects on protein content. A reduction of the Zn and Fe content by 9.3 % and 5.1 % respectively, was reported for C3 crops based on meta-analysis data (Myers et al., 2014). Both mineral elements are most strongly negatively affected by the rising CO_2 levels. In contrast, minimal (B, Cu, Mn) or even insignificant (K) effects were observed for other mineral elements (Broberg et al., 2017).

CHAPTER 2

RP-HPLC for screening of wheat protein *quality*

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2.1 Introduction

In recent years, the relevance of the protein *content* in wheat (*Triticum aestivum* L.) grain or flour as indicator for bread quality has been progressively criticized in academic literature. Moreover, some researchers postulate that not solely the protein fraction contributes to the end-product quality of bakery products (Guzman et al., 2016). It was also shown that cultivars with low protein concentrations (PROCs) can be used to obtain a high loaf volume, the main quality attribute for bread. This has been related to an optimal protein *composition* as well as to the presence of other components in the flour such as non-starch polysaccharides, lipids, *etc.* (Gabriel et al., 2017). Underlying complex biochemical mechanisms, which come to expression in functional parameters such as elasticity, extensibility and water absorption (Section 1.3.1.2), are eventually responsible for the baking quality. However, as many of the interactions of the various protein sub-fractions and their effect on these mechanisms are not yet fully understood, a main interest in unraveling these from a compositional level remains existent (Kurtanjek et al., 2013). Various attempts have been made to get an improved understanding of these phenomena using empirical techniques which are also commonly used in industry (AIBI International Association of Plant Bakers, 2017). Unfortunately, predictive models on the basis of protein properties—including both protein *content* as well as easily determinable *compositional* and functional attributes—fail to predict bread quality in a sufficient manner to be broadly applied in industry.

2.1.1 Current techniques for analyzing protein composition

As elaborately described under Section 1.3 (page 22), protein composition is an extremely comprehensive term which can be approached from different angles and acts on different levels. The use of advanced techniques such as sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), high-performance size-exclusion chromatography (HP-SEC) or reversed-phase (RP) high-performance liquid chromatography (HPLC), helps in disclosing the different aspects of the proteins going from presence/absence information to a (basic) molecular weight-distribution and the relative proportion of the sub-fractions (Skylas et al., 2005; Bietz, 1986; Vensel et al., 2014). Additional information can be obtained by applying different extraction methods on the gluten-forming proteins (Hurkman and Tanaka, 2007), thereby distinguishing soluble and insoluble fractions (*e.g.* percentage of unextractable polymeric proteins as discussed in Section 1.2.2.3). In most of the research in which the correlation between protein composition and end-product quality is studied, one to several of these techniques are combined with basic and empirical methods for protein characterization comprising Zeleny sedimentation value (ZEL), Farinograph, and extensibility tests (Thanhaeuser et al., 2014). Together with the relative or absolute contents of the various types of prolamin (PRO) and glutelin (GLU) and the prolamin-to-glutelin-ratio and the ratio of HMW-GS to LMW-GS, a comprehensive dataset can be compiled allowing researchers to investigate the relation with end-product quality.

> Protein content and dough rheological properties are, even when examined in a multivariate way, mediocre predictors for bread quality. As the latter are model-system dependent, they lack the ability

> to forecast end-product quality attributes of other bakery products. Moreover, no insight is obtained in the mechanisms behind phenomena such as water binding, elasticity and extensibility.

In research environments, mass spectrometry can be used to gather an even more detailed insight in the composition of the (digested) protein fractions (Skylas et al., 2005; Mak et al., 2006; Vensel et al., 2014). This exciting and relatively novel 'high-throughput'—in terms of the number of proteins which can be analyzed in parallel—identification technique, results however in complex datasets which require tremendous amounts of time to process and to interpret. With 2D-SDS-PAGE being a cornerstone of the so-called 'proteome analysis', there is also a great dependency on the scientist's skills with a limited throughput. Moreover, specialized equipment is extremely expensive in purchase, operation and maintenance, thereby offering only a limited added value for R&D laboratories of food manufacturers. Moreover, a prerequisite for being able to exploit the potential of the latter technique is the availability of a proteomic database making it possible to identify the otherwise meaningless spectra (Agrawal et al., 2013). This possible hurdle is illustrated by the study of Mak et al. (2006) in which, from the more than six hundred protein spots obtained using matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS), approximately half of the proteins could be identified from protein sequence database interrogation. Of these, only 94 were classified as gluten-forming proteins.

> Costly, difficult to interpret and not routinely applicable techniques will not be adopted by industry although the obtained data would be highly suitable to accurately estimate quality attributes. Additional hurdles towards data processing (no information in databases, requirement of an extensive knowledge of data processing, reduction to lower resolution outcome, *etc.*) leads to the loss of the initial potential of the high-resolution data.

A disadvantage of all former techniques is the fact that these are laboratory based (*i.e.* off-line) and destructive. In the light of '*Industry 4.0*', continuously measuring sample properties on-line may be favorable as this could allow to directly adjust process parameters to the incoming sample. This also contributes to big data acquisition and process automation (Jerome et al., 2019). Near-Infrared Reflectance Spectroscopy and related techniques (*e.g.* transmission spectroscopy, Fourier-transform Infrared Spectroscopy) have already proven to be useful for a rapid screening of basic sample properties (moisture, protein, ash and dietary fiber content) in wheat kernels, flour, dough, *etc.* with great ease (Hell et al., 2016; Jerome et al., 2019). Accuracy is however highly dependent on the robustness of the underlying model present in the equipment. Results from Dowell et al. (2006) have show that the technique is mainly relevant for predicting the grain and flour moisture, protein, gluten content while the accuracy of other parameters is insufficient ($R^2 \leq 0.90$). As discussed before, these parameters are not good predictors for end-product quality attributes of bakery products.

The lack of proper reference samples is a general concern when analyzing wheat proteins due to the large diversity within both fractions and sub-fractions (Hajas et al., 2018). Various attempts to compile a reference set have already been undertaken, however, without a conclusive outcome on which (blend of)

cultivars and extraction methods are needed to fully comprehend all protein fractions of interest (Schalk et al., 2017; Hajas et al., 2018).

> Despite the existence of standard methods to perform some of the techniques (mainly those used for quality control in industry), a company or lab specific execution may result in incomparable outcomes. In addition, references (not to be confused with standards to assess equipment performance) for wheat protein are scarce, do not enclose the present variability within all protein classes and provide limited additional information on composition or classification.

2.1.2 Problem statement

On an industrial level, characterization of raw materials (*i.e.* flour) is done routinely for both quality control and research purposes. The obtained information—which mainly covers protein compositional and functional attributes—is related to end-product quality. Furthermore, to estimate end-product quality, time-consuming and labor-intensive breadmaking trials have to be performed as this is considered to be the 'ultimate' indicator for baking quality. The latter, however, only partially represents the variation seen on industrial lines.

One of the most straight forward approaches to improve predictions, is by including the predictor variable(s) which improve(s) the model by providing *additional* information which can be related to end-product quality. In the search for an ultimate and universal tool for predicting end-product quality, an unceasing development of new analytical techniques has taken place (AIBI International Association of Plant Bakers, 2017). With bread being the most important product in the bakery segment, equipment which directly measures bread dough properties (*e.g.* Mixolab, Extensograph, Alveograph, Farinograph) or related functional attributes (*e.g.* solvent retention capacity, GlutoPeak) is preferred over techniques which provide insight in compositional attributes. However, as is elaborately discussed by Vigni et al. (2013), food producers may experience major benefits from understanding the relationship between gluten quality (*i.e.* protein composition) and dough properties as well as from applying this knowledge for (directly) optimizing the production process.

Although compositional attributes are often more difficult to interpret and provide only a narrow window on the total composition of the sample (*e.g.* a single or a few discrete values), studying dough properties is limited in its usability to predict quality attributes of other bakery products. An asset of the latter type of measurements is that functional properties—including interactions and macromolecular effects—are captured in contrast to composition oriented approaches. Despite techniques used for studying these properties require limited operator experience, they are also labor-intensive and time-consuming. Moreover, empirical techniques are not model-system independent making it important to carefully interpret their outcomes, mainly when used to predict the end-product quality of other bakery products.

> Numerous techniques are available for screening protein composition and functionality, however, they are time- and/or labor-intensive, provide only (semi-)quantitative or very complex outcomes or require various pieces of costly equipment and trained and experienced lab personnel.

Following the statement from Vigni et al. (2013), the obtained parameters—independent from whether they are categorized as (complex) compositional or functional attributes—are considered in a univariate way, thus loosening the effect of the correlation of these properties with flour performance. Hence, the application of more advanced predictive models for preemptive quality assurance or even more, for adjusting process parameters (in real-time), remains a great challenge. Nevertheless, from a modeling point of view, complex traits such as loaf volume require vast amounts of data (samples) and a comprehensive number of variables to be included in the dataset. In this framework, both (quantitative) starch and protein related parameters, preferably from high-resolution techniques, should be included to maximally cover the multidimensional nature of underlying biochemical processes. Although this second approach requires knowledge of mathematical modeling as well as access to high-quality datasets, it can be considered the ideal moment to introduce such an approach in an industrial setting as this is an inherent part of '*Industry 4.0*' together with the emerging field of machine learning techniques for converting volatile datasets to directly attainable output.

The objective of the current study is to establish an approach through which **highly detailed** and **quantitative** information from the **composition** of **all protein fractions** of a **vast number** of **various gluten-protein containing materials** can be obtained. From this **model-system independent** data, it should be possible to **predict** continuous **end-product quality attributes** and to deduct **descriptive** (categorical) properties. To realize this, the chosen analytical technique has to be: (a) easily performable without elaborate practice or prior knowledge, (b) feasible to implement in other laboratories, (c) executable for a single sample to a large set of samples, and (d) compatible with all protein fractions of interest (after sample preparation). Furthermore, an efficient collection of the raw data is required in order to expedite the versatile processing, depending on the prevailing research questions, afterwards. It is however not mandatory for the data to be directly interpretable in its raw form.

2.2 Material and methods

Keeping in mind the constraints and goals, RP-HPLC was assessed to be the most suitable technique. Depending on the resolution of the chromatography and the ability to discriminate specific (sub-)fractions, it allows to obtain quantitative information on the presence of specific units at a relatively low cost and with much higher ease compared to protein fingerprinting using mass spectrometry or quantitative 2D-SDS-PAGE. In light of the overarching research topic, a trade-off between resolution and analysis time per sample exists resulting the use of *ultra* HPLC. In this way, a sufficient level of detail can be obtained in a high-throughput manner after also optimizing the sample extraction procedure. In contrast with most of the available literature, it is chosen to include all protein fractions comprising both gluten-forming proteins (PRO and GLU) as well as the water extractable protein (WEP) (albumin and globulin).

In order to employ the high-resolution data to the fullest extent, advanced data processing techniques are applied subsequently to the automated data collection and preprocessing. The primary focuses are on using the data for predicting bread quality attributes (*i.e.* loaf volume, gas cell distribution and oven spring) and to distinguish between genotypes, cultivation environments (both location and year) and management practices (*i.e.* fertilization treatments). Details on the used data analysis techniques are discussed in Section 2.2.1.3. In addition to the predictive approach, the chromatographic data was also used to calculate the (relative) contents of the protein (sub-)fractions which are included in following chapters.

> **Intermezzo**
> The approach, which is an *inseparable* combination of the analytical technique (including the sample preparation) and the data processing part, was named 'ProFiT' coming from the double meaning 'Protein FIngerprinTing' as well as using *protein* compositional attributes for *fitting* various variables to it. During its development, it has been applied in two small-scale studies, both acting as a proof-of-concept. The first project with an industrial partner clearly showed its potential by perfectly clustering a set of anonymized samples on the basis of origin, wheat type (hard/soft, spring/winter, red/white wheat), supplier and harvest year. For acquiring a similar distinction, variables from eight conventional techniques (comprising both starch and protein as well as compositional and functional analysis) were required. A second project was performed on vital gluten, thereby indicating the versatility of the 'ProFiT' approach with regard to the starting material. By comparing the results for vital gluten samples from three production sites and multiple harvest years, insight was provided in the variability due to the production process and the input material (*i.e.* wheat grains). The project outcome resulted in a follow-up study in which the experimental setup is broadened to pinpoint the main sources of possible variation in the vital gluten quality and how this relates to the end-product quality of the products in which they are applied.

2.2.1 HPLC-analysis

2.2.1.1 Sample preparation

The sample extraction method was based on the principle of Osborne fractionation distinguishing three protein fractions: WEP, PRO and GLU. As described in Mejías et al. (2014), a sequential extraction procedure (Figure 2.1) was used to separate the fractions from non-defatted flour. The sample preparation method was optimized in order to increase throughput while obtaining a sufficient protein recovery and resolution during chromatography.

After weighing of 100.0 $\pm$ 5.0 mg sample in 2.0 ml Eppendorf tubes (noting the exact weight), 1.0 ml of a 0.4 M NaCl solution was added to the sample using a micro pipette. The sample was vortexed (MS2, IKA-Werke, Staufen, Germany) and further suspended under shaking conditions for 20 min at room temperature (25 °C). As shake intensity, temperature and time were found to be critical for obtaining reproducible results, an Eppendorf ThermoMixer C (Eppendorf, Hamburg, Germany) equipped with a 2.0 ml thermoblock (Eppendorf SmartBlock) was used. For all extraction steps, shaking speed was set at 1,600

min^{-1}. After centrifugation at 10,000 × g for 20 min at room temperature (3-18K centrifuge, Sigma), the supernatant was directly transferred to a 1.5 ml screw capped (preslit silicone septum) transparent HPLC-vial (VWR, Pennsylvania, USA). During transfer, the supernatant was filtered through a 0.2 µm (d = 15 mm) reversed cellulose syringe filter (Phenomenex, Torrance, USA) using disposable 5 ml syringes (VWR, Pennsylvania, USA).

Remaining supernatant in the tube was removed by inversion after which 1.0 ml of a 60 % ethanol (absolute, for analysis) solution was added. The pellet was redissolved by manually loosening it using a pipette point and by vigorously vortexing the tube. Suspension and centrifugation steps were done under the same conditions as for the WEP-extraction. Additionally, the supernatant containing the extracted prolamin was again transferred after filtration to a separate HPLC-vial in preparation for the analysis. Analogously with prolamin-extraction, GLU were extracted by the addition of 1.0 ml extraction solution. For stability reasons, this was prepared maximum 48 h in advance by mixing 50 % 1-propanol (C_3H_8O) with 50 mM TRIS-HCl ($NH_2C(CH_2OH)_3 \cdot HCl$) and 2.0 % w/v DTT ($C_4H_{10}O_2S_2$). The pellet was resuspended at 53 °C and subsequently, centrifuged at 14,000 × g (40 °C). Finally, the obtained supernatant was filtered into a separate HPLC-vial.

To calculate the protein recovery, the raw material was dried (AACC International., 2000b) whereas the remaining pellet was assumed to be free of moisture after evaporation of the final extraction solution. Therefore, the Eppendorf tubes were left opened in the fume hood overnight. Protein content was determined by measuring the nitrogen content in the sample (extracted pellet and dried initial sample) using a VarioMax C/N (Elementar Analysesystemen, Langenselbold, Germany). Four hundred milligrams of each was accurately (precision of 0.1 mg) weighed in a crucible and placed in the autosampler.

2.2.1.2 Analytical procedure

After protein extraction, the vials ($n \times 3$, with n being the number of samples times the number of technical repetitions) were placed in the autosampler of the HPLC-system. The analysis was performed using an Agilent 1290 Infinity II series (Agilent Technologies, California, USA) ultra high pressure liquid chromatography system equipped with a quaternary pump (G7104A), a temperature-controlled multisampler (G7167B), a column oven (G7116B) and diode-array detector (DAD, G7117A) equipped with a Max-Light Cell (volume = 1.0 µl) set at 210 nm ± 4.0 nm (bandwidth) and at a sampling rate of 40 Hz (0.13 sec per data point). A Zorbax 300 SB-C8 RRHD column (Agilent, California, USA) of 150 mm (internal diameter = 2.1 mm, particle size = 1.8 µm) was used without guard column. Column and heat exchanger were maintained at 60 °C during all measurements. As eluents, gradient grade acetonitrile (HiPersolv chroma gradient) from VWR (Pennsylvania, USA) and ultrapure (ASTM Type 1, resistivity ≥ 18.0 MΩ cm) water (Milli-Q Plus equipped with QPAK2, Merck-Millipore, Massachusetts, USA) were used. To both eluents, respectively denoted as eluent A and B, 0.1 % v/v HPLC-grade TFA (Sigma-Aldrich, Missouri, USA) was added. To prevent bubbles from entering the HPLC-system, both solutions were sonicated abundantly (≥ 15 min at 35 kHz) after preparation (Transsonic T460/H, Elma Schmidbauer, Singen, Germany).

TFA acts as an ion-exchange reagent thereby increasing peak sharpness, thus significantly increasing chromatographic resolution. Contrastingly, the addition of TFA is known for its negative impact on the

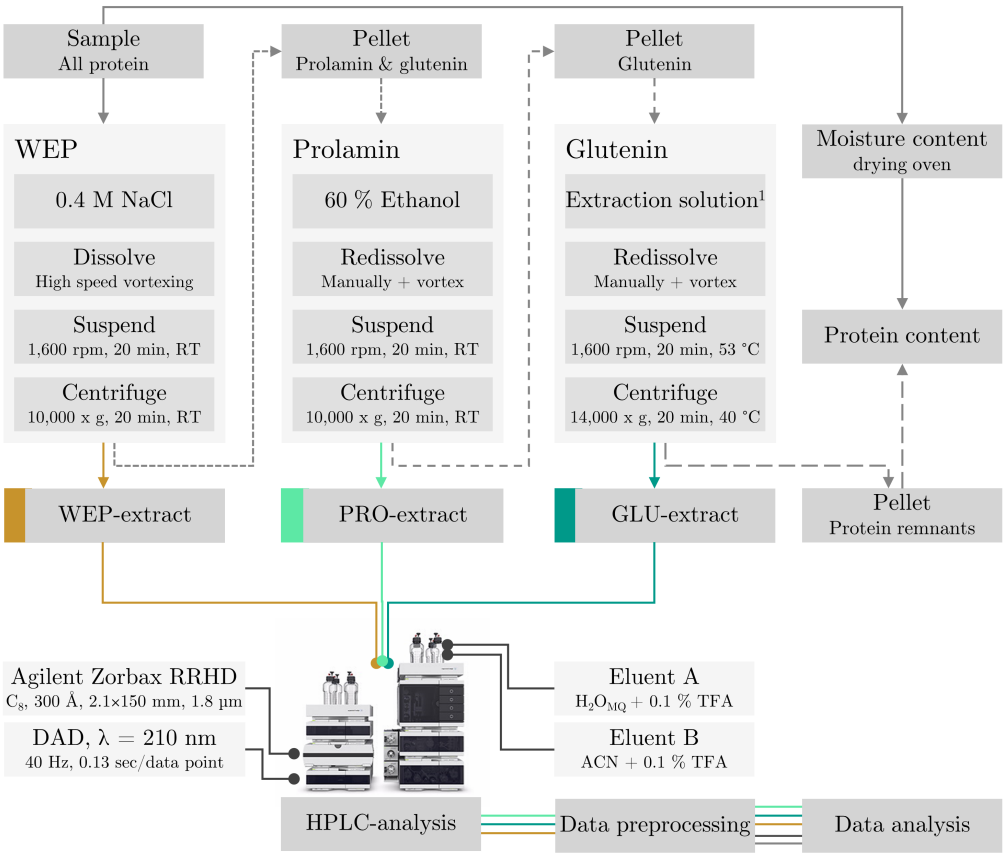

Figure 2.1: Overview of the sample preparation steps for extracting the water extractable protein (WEP, ●), prolamin (PRO, ●) and glutelin (GLUT, ●) from gluten-forming containing materials (*e.g.* wheat flour, vital gluten, *etc.*).
[1] Extraction solution: 50 % 1-propanol, 50 mM TRIS-HCl, 2.0 % *w*/*v* DTT.
Room temperature (RT); ultrapure water (H_2O_{MQ}); acetonitrile (ACN, C_2H_3N); trifluoroacetic acid (TFA, $C_2HF_3O_2$).

stability of the baseline and the potential decrease of column lifetime. To obtain a sufficiently stable separation, the column had to be preconditioned for at least 12 hours using a mixture of 50 % eluent A and 50 % eluent B at a flow rate of 0.2 ml h^{-1}. Moreover, it was found that to prevent an irreversible loss of column resolution, the flow rate had to be maintained at 0.2 ml h^{-1} between sequences. Column temperature, however, was gradually lowered to room temperature.

Prior to starting a sequence, the system was conditioning for 20 min using the starting conditions of the first method (75 % eluent A and 25 % eluent B) until a stable column pressure was obtained. A checkout sample (containing 8 phenones) was injected three times before each series (PRO, GLU or WEP) to check column performance an to let the system equilibrate further. Additionally, the first sample was injected twice removing the first injection from the data analysis. Each analysis was terminated by a short column wash at 10:90 A:B (PRO and GLU methods) or 90:10 A:B (WEP) for 3 to 4 column volumes to remove possibly remaining proteins. Finally, the blending ratio was restored to the initial conditions for 2 minutes before commencing the next analysis.

Flow rates, injection volumes and gradient conditions differed slightly per method (single method per protein fraction). For WEP and PRO, a flow rate of 0.7 ml h^{-1} was used whereas GLU was analyzed using a flow rate of 0.6 ml h^{-1}. Injection volumes were respectively 6, 4 and 5 µl. These optimized

settings were obtained on the basis of preliminary trials. Figure 2.2 shows the used gradient conditions for the various methods.

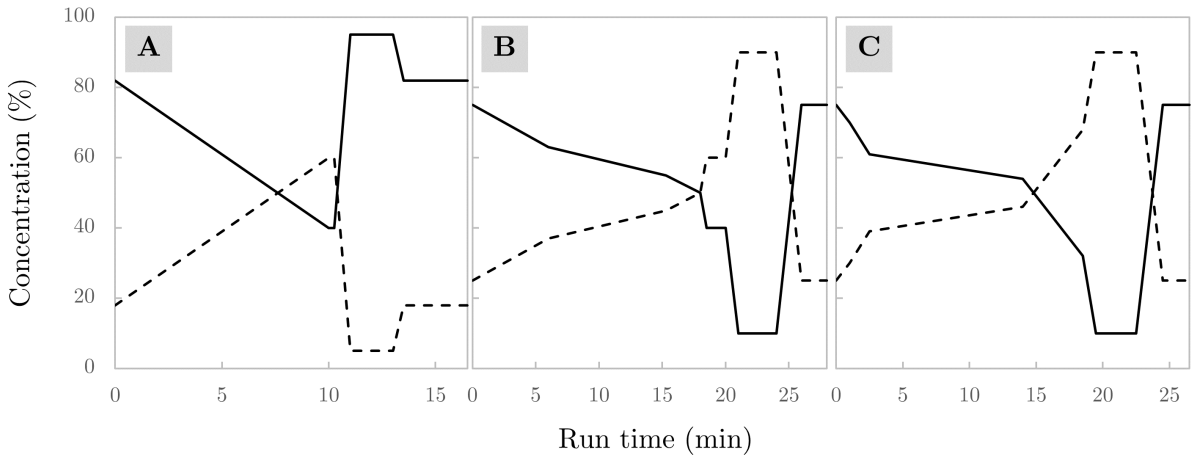

Figure 2.2: Overview of the gradient conditions for the analysis of **A** water extractable protein (WEP), **B** prolamin (PRO) and **C** glutelin (GLU).
Eluent A (H_2O_{MQ} + 0.1 % TFA) —, eluent B (ACN + 0.1 % TFA) - -.

After the analysis was completed, the raw signal information was exported as a .csv-file and recompiled with other analysis results and the descriptive sample information (harvest year, origin, treatment, phenotypic information, *etc.*) prior to commencing data analysis.

2.2.1.3 Data processing

Data processing was performed in R (version 3.4.3, R Core Team (2018)) using an in-house developed script and consisted out of two major parts: firstly data management and cleaning (preprocessing) and secondly data analysis. For spectral data, preprocessing is an important but often underestimated step in which undesirable variation in the data (noise and redundant data) is removed. As shown in Figure 2.4, preprocessing can be split up in multiple steps beginning with the selection of the desired samples from a set and removing the spectral data outside the region of interest. This includes the injection peak and the area corresponding with the column wash and equilibration time at the end of each analysis.

Baseline correction After transforming the data matrix to a spectra-object, the *ChemoSpec* package (Hanson, 2019) was used to perform the baseline correction by applying the '*fillPeaks*'-algorithm as described by Liland (2015). In general, the algorithm uses Whittaker smoothing followed by subsampling (for improved efficiency) and iterative suppression of the baseline. The final stage of the algorithm consists of interpolating the estimated baseline back to full spectrum length resulting in a smooth baseline.

Peak alignment Another crucial step in raw data preparation is peak alignment. The *CluPASpectra* algorithm was used to determine both the number of peaks (depending on the baseline threshold set) and the optimal shift which has to be corrected. After converting the spectra to peaks (discrete x-values at maximum y-values), values are automatically grouped using hierarchical Cluster-based Peak Alignment (CluPA) using the *speaq* package (Vu et al., 2011). Minor modifications to enhance data handling

were made to the wrapper function. Before continuing, the aligned spectra were visually inspected by comparing them with the original data.

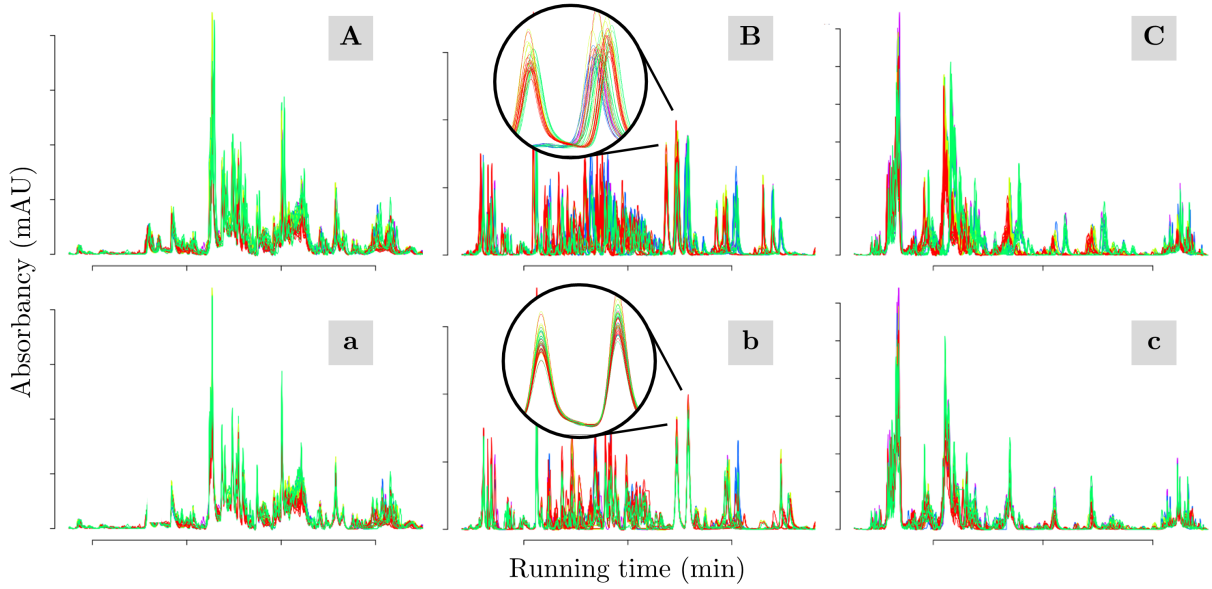

Figure 2.3: Plots of the raw, baseline corrected, unaligned (capitals) and aligned (small letters) chromatograms of the set of 52 cultivars. **A, a** water extractable protein (WEP), **B, b** prolamin (PRO), and **C, c** glutelin (GLU).

Due to unavoidable column degradation (resulting in large shifts) and minor fluctuations in retention times (due to system running and equilibration time), shifts in the chromatograms occurred. When sample sets were analyzed in a single run (maximum of 24 samples) and no systematic shifts were observed visually, alignment was not performed as the complexity of the chromatogram resulted in interfering corrective actions of the automated function. However, as is shown in Figure 2.3, alignment had to be applied for sample sets which were obtained over multiple runs.

Curve-to-area conversion Earlier empirical tests showed the benefits of using area data (*i.e.* area under curve (AUC)) instead of all single data points of the curve. In this way, undesirable variability introduced by the analytical procedure was further decreased. Prior to area calculation, each spectrum was split in y windows. The number of windows was calculated by dividing the total length of the region of interest (number of data points or time) by the total number of peaks. To include all peaks, peak detection was performed again using a lower baseline threshold, despite the computational cost. To obtain a single number of windows per protein fraction (for the studied sample set), the average number of peaks per fraction was used to calculate the width of the windows. The starting and ending point of each window was used to set the integration limits. Through an iterative process of fitting a spline function to the curve in each window (by cubic spline interpolation) and integrating this between the aforementioned boundaries, a matrix of AUC-values was constructed.

Area normalization and data recombination Normalization was performed to remove the effect of possible variation in the protein content of the initial sample or variability due to extraction efficiency. Per spectrum, the AUC of each separate window was divided by the total area under the chromatogram (sum of all separate AUCs). The obtained areas were stored per spectrum and per fraction in a separate

matrix. For further analysis, a combination of the values obtained for PRO and GLU (gluten-forming protein), as well as for all protein fractions, was made. These combined sets are denoted in Figure 2.4 by the additional dark gray and light gray lines going form 'data recombination' to 'data analysis'. Prior to normalization, the prolamin-to-glutelin-ratio (PGr) and the ratio of HMW-GS to LMW-GS (HMW:LMWr) were calculated as the total absolute AUCs of the respective fractions. Area limits for high molecular weight glutelin-subunits (HMW-GS) and low molecular weight glutelin-subunits (LMW-GS) were chosen on the basis of literature.

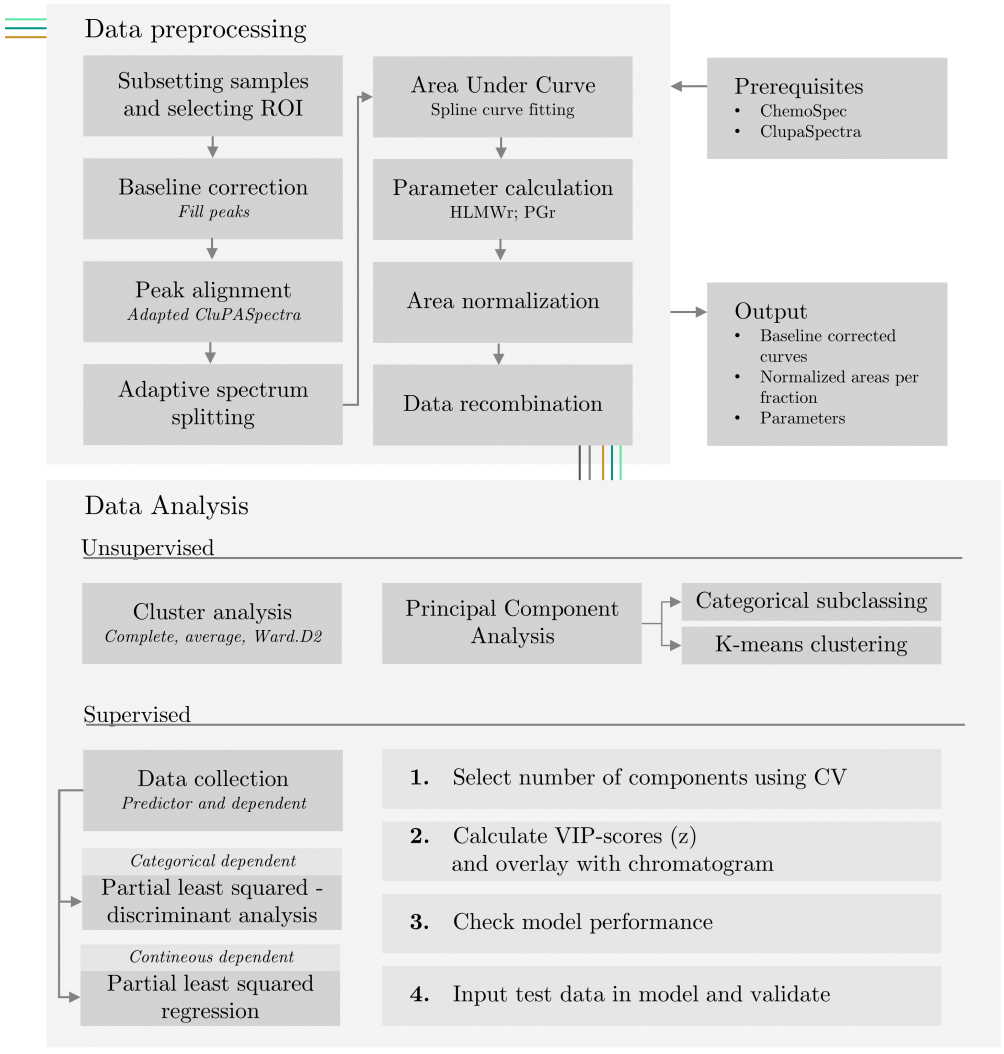

Figure 2.4: Overview of the data preprocessing and analysis steps.
Region of interest (ROI); ratio of HMW-GS to LMW-GS (HMW:LMWr); prolamin-to-glutelin-ratio (PGr); cross-validation (CV); variable importance in projection (VIP).

Unsupervised and supervised learning Having removed the undesirable variation to a maximum extent, statistical techniques were applied to transform the preprocessed data into an interpretable outcome. To achieve this, two groups of machine learning techniques were been used: unsupervised and supervised learning techniques. Two main methods in the former group are clustering and dimensionality reduction (using principal component analysis (PCA)) which both aided in uncovering previously unknown patterns in data sets without pre-existing labels. Clustering was performed using the Ward.D2 algorithm ('*stats*'-

package) which minimizes the total within-cluster variance by merging the pair of clusters with a minimum between-cluster distance. Euclidean distances were calculated and merged in a bottom-up approach using agglomerative hierarchical clustering. Within the PCA, data points were classified using categorical variables.

The secondary class are supervised learning techniques whereby a function is learned which maps input variables (typically a vector) to an output variable (the supervisory signal). By using training data—a set of example input-output pairs—a predictive model can be developed which can afterwards be used for mapping new examples. Partial least squares modeling techniques were used as it is found to be most suitable to deal with the problem of a small number of objects (*i.e.* samples) and large number of input variables and multicollinearity (Fu et al., 2016). To predict categorical variables (*e.g.* growth location, quality class, *etc.*), partial least squares (PLS) Discriminant Analysis was used whereas traditional PLS regression was used for continuous variables. As all studied classification problems concerned multiple classes (≥ 3), the more complex PLS2 algorithm was used for calculating the indicator matrix. Scores ranging between -infinity and +infinity indicate the sample's relation to the dependent. For prediction, the class with the largest score is the assigned output class (Fu et al., 2016; Daisey and Brown, 2017).

Independent from the applied supervised learning technique (PLS discriminant analysis or regression), an optimal number of components (ncomp) had to be specified. To prevent overfitting, the first value for ncomp with the lowest value for the used performance indicator (BER or root mean squared error of prediction (RMSEP) for categorical or continuous predictions respectively) was used. This could only be obtained by performing cross-validation (CV) which is further split up in leave-one-out cross-validation (LOO-CV) and m-fold cross-validation (m-fold CV), depending on the amount of samples per group in the training data. If possible, m-fold CV was used to obtain a more reliable estimation of the optimal ncomp for the model. In practice, levels for which at least three or more samples were available were retained and tested using m-fold CV. The number of folds (m) was calculated by taking 75 % of the number of samples of the smallest group, thereby preventing that an entire group was excluded from the training set. When only two samples remained, LOO-CV was chosen as alternative although this is less robust and prohibits the full exclusion of main sources of variability (genotypes and environments) from the training set, thereby probably resulting in an overestimation of the model performance for unknown samples (Minasny, 2009).

To select the input variables (*i.e.* windows in the chromatogram) which are most determining for predicting the supervisory signal, variable importance in projection (VIP) was used. By selecting the i % of most-contributing windows, additional insight in the part of the chromatogram related to the parameters of interest could be obtained.

2.2.2 SDS-PAGE

Proteins were extracted from 20 mg of wholemeal flour and PRO and GLU were separated using the method of Singh et al. (1991) with minor modifications. In short, PRO were separated from the GLU using 50 % v/v propanol and dried. The GLU in the remaining pellet were redissolved in 50 % propanol and consecutively reduced using a 1.5 % v/v DTT-solution. Alkylation was done to improve the resolution of the LMW-GS by the addition of a 1.4 % v/v 4-vinylpyridine. Prior to loading the samples on the

gel, buffers (pH 8.0 and 6.8 for PRO and GLU respectively) containing Coomassie Brilliant Blue were added to the dried extracts. The concentrations of arcylamide and cross-linker used to prepare the linear separation gel were 15.0 % and 1.3 % respectively. The pH for the separation gel was pH 8.5 and the current of the running gel was 12.5 mA for 20 h at 15 °C. After staining and washing the gels, a scan was made using a ChemiDoc Gel Imaging System (Bio-Rad Laboratories, California, USA).

For labeling the gels, the nomenclature of Payne et al. (1987) was used for the HMW-GS. Resolution of the GLU gels allowed to differentiate between all HMW-GS while samples with a known composition were included as a standard (Figure 2.5). The nomenclature of Gupta and Shepherd (1990), Jackson et al. (1996), Branlard et al. (2003) and Appelbee et al. (2009) were used for both LMW-GS and ω-prolamins. As PRO can be screened more readily than LMW-GS, the former were used as indicators based for LMW-GS based on their linkage.

2.2.3 Sample sets

The protein composition of two distinct sample sets (CIMMyT sample set (CIMMyTss) and cross-year cross-location sample set (CY-CLss)) was analyzed with the HPLC-technique. The method was validated by comparison with SDS-PAGE using the CIMMyTss as this includes a broad variability in the protein composition (all common sub-fractions of both PRO and GLU). Secondly, the feasibility to predict bread quality attributes or other compositional and functional features was investigated. Apart from the primary research objectives, the CY-CLss was used to qualitatively assess the discriminative power of the technique.

2.2.3.1 CIMMyT-sample set

Sixteen cultivars were selected on the basis of their protein composition from a master set composed by CIMMyT (Liang et al., 2010). Samples were obtained from the CIMMyT seed bank and were propagated under greenhouse conditions. Entire kernels were milled to wholemeal flour using an analytical ball mill (IKA-Werke, Staufen, Germany).

Within each class (LMW-GS and HMW-GS, A, B and D), the most common subunits were represented in multiple samples (Table 2.1). As some lines contained unclassified HMW-GS, no Glu-1 score (Payne score) could be calculated. At the time the scoring system was developed, the LMW-GS fraction was not taken into account as its contribution to baking quality was considered to be limited. Upon today, their contribution to dough mixing properties and bread making are still not well understood (Pistón et al., 2011; Oury et al., 2010; Dhaka and Khatkar, 2015).

2.2.3.2 Cross-year cross-location sample set

The CY-CLss comprises *flour* samples from 15 genotypes cultivated in the area of Ghent between 2016 and 2018 (3 harvest years), and in Koksijde and Tongeren (single harvest year, 2018). In this way, 5 different environments were obtained. However, not all genotypes were present at all environments

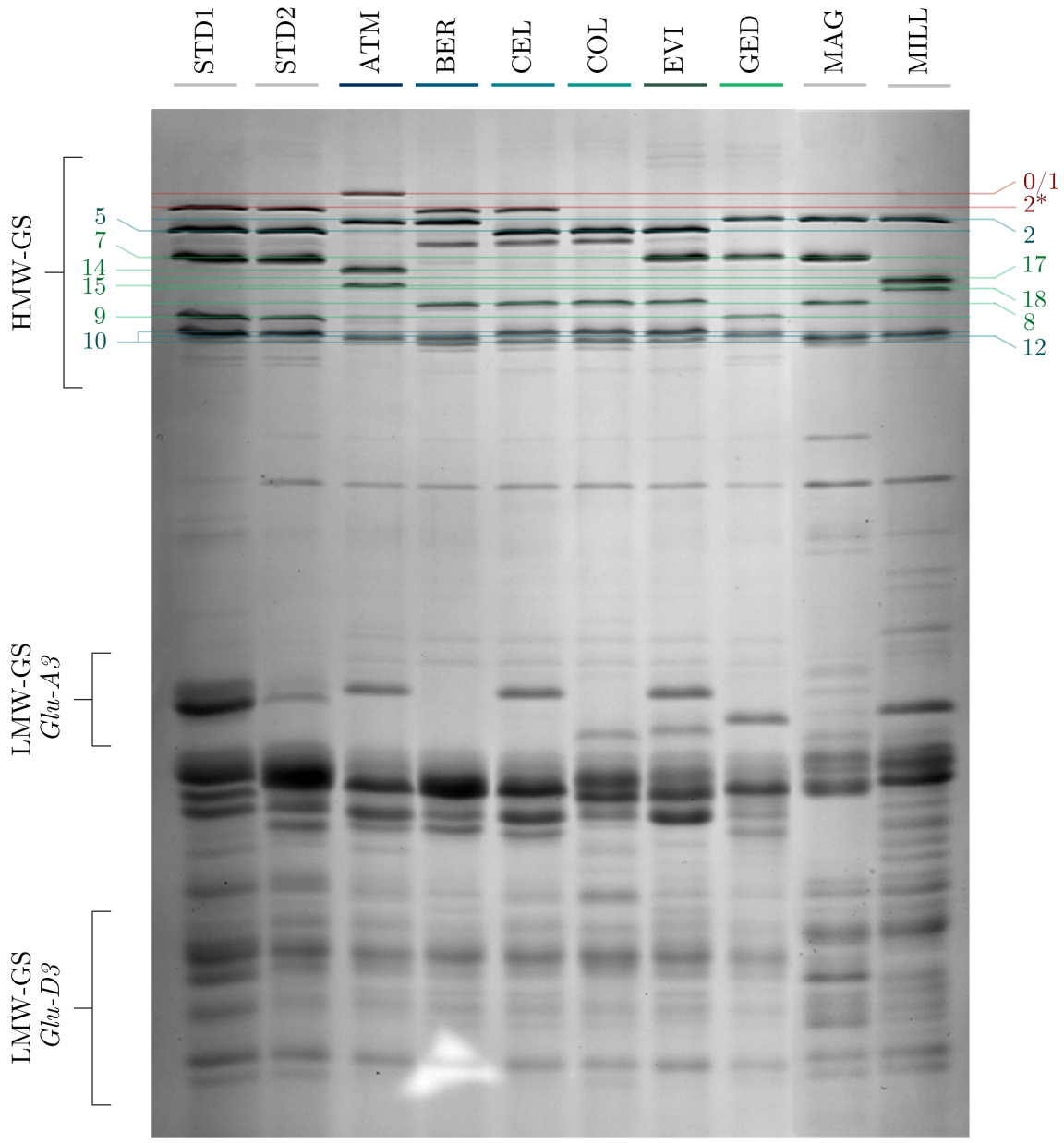

Figure 2.5: Illustration of the glutelin gel including two standards (STD1 and STD2) and two reference samples (MAG and MILL) with a known composition (based on prior research by Branlard et al. (2003)). Samples in the center lanes have an unknown protein composition.
Glu-A1 subunits, ●; Glu-B1, ●; Glu-D1, ●. LMW-GS subunits are not specified in this gel as the classification has to be verified using the prolamin gel.

(marked by a '-' in Table 2.2) resulting in a total of 55 samples. A detailed description of the cultivation, cleaning and milling conditions the samples can be found in Section 3.2.2 (page 99).

The narrow genetic diversity in the sample set in terms of the composition of the gluten proteins was reflected by the limited variability for all screened sub-fractions. Mainly within the *Glu-B3* and *Glu-D3* fractions, minor to no differences were observed. Within the *Glu-D1* fraction, two groups can be distinguished, 5+10 and 2+12. For *Glu-A1*, only two cultivars ('Bergamo' and 'Cellule') contain the 2^* subunit whereas the *Glu-B1* fraction shows to enclose the largest diversity. Mainly 6+8, 7+8 and 7+9 were represented in the latter group whereas 'Atomic' and 'Mentor' showed bands 14+15 and 17+18.

Table 2.1: Results from sodium dodecyl sulphate polyacrylamide gel electrophoresis analysis for the CIMMyT sample set.

			HMW-GS			LMW-GS			Glu-1 score[1]
No.	**Code**	**Cultivar**	*Glu-A1*	*Glu-B1*	*Glu-D1*	*Glu-B3*	*Glu-D3*	*1BL/1RS*	
1	ACV	AC Vista	1	7+9	5+10	j	b	+	6
2	APO	Apollo	2*	7+9	5+10	b	c	-	9
3	BSK	BlueSky	2*	7_{oe}+8	5+10	c	c	-	
4	CS	Chinese Spring	null	7+8	2+12	a	a	-	6
5	ERN	Ernest	2*	7+9	2.1+10	g	f,g	-	8
6	GLEN	Glenlea	2*	7_{oe}+8	5+10	g	c	-	
7	HAL	Halberd	1	20	5+10	c'	c	-	
8	KAT	Katepwa	2*	7+9	5+10	h	c	-	9
9	MAG	Magali-Blondeau	null	7+8	2+12	g	a[2]	-	6
10	MAR	Marquis	1	7+9	5+10	b'	a	-	9
11	MILL	Millewa	null	17+18	2+12	g	b	-	6
12	NEEP	Neepawa	1	7+9	5+10	h	c	-	9
13	OPA	Opata	2*	13+16	2+12	i	a	-	
14	PAST	Pasteur[3]	1	14+15	5+10	g	e	-	
15	PAV	Pavon	1	7_{oe}+8	2+12	i	c	-	
16	Z41S	Zhongyu 415	null	20	4+12	d	c	-	

[1] Calculated according to Payne et al. (1987) with a correction factor for those varieties containing the 1BL/1RS chromosome. Due to the presence of non-classified HMW-GS in some cultivars, no score could be calculated.
[2] For this cultivar, a contamination (classified as 'b', $\approx 20\,\%$) was observed.
[3] Pasteur is originally not part of the CIMMyT master set but was added as is contains an 'e' at the *Glu-D3*.

Table 2.2: Samples included in the cross-year cross-location sample set and the according sodium dodecyl sulphate polyacrylamide gel electrophoresis results.

							SDS-PAGE					
		Environment					*HMW-GS*			*LMW-GS*		
No.	**Code**	*G.16*	*G.17*	*G.18*	*T.18*	*K.18*	*Glu-A1*	*Glu-B1*	*Glu-D1*	*Glu-A3*	*Glu-B3*	*Glu-D3*
1	ATM	+	+	+	-	-	1	14+15	2+12	d	g	c
2	BER	+	+	+	-	+	2*	6+8	2+12	f	g	c
3	CEL	+	+	+	-	-	2*	6+8	5+10	d	g	c
4	COL	+	+	+	-	-	null	6+8	5+10	a	g	c
5	EVI	+	+	+	-	-	null	7+8	5+10	d	g	c
6	GED	+	+	+	+	+	null	7+9	2+12	c	g	c
7	GRA	+	+	+	+	+	1	6+8	5+10	c	g	c
8	OZN	+	+	+	-	-	1	7+9	5+10	c	g	c
9	MEN	+	+	+	+	+	1	17+18	2+12	d	g	c
11	MND	+	+	+	-	-	null	6+8	5+10	c	b	c
12	REF	+	+	+	+	+	1	7+9	2+12	d	g	c
13	SCR	+	+	+	+	+	null	7+8	5+10	c	g	c
14	RUB	+	+	+	-	-	null	7+8	2+12	d	g	c
15	TER	+	+	+	-	-	null	7+9	2+12	c	g	c
16	TOB	+	-	+	-	-	null	7+9	2+12	c	g	c

Coding for the genotypes can be found on page xii.

2.3 Results and discussion

Figure 2.6 shows the variation in the chromatographic output of the three protein fractions for the CIMMyT sample set (CIMMyTss). Within the water extractable protein (WEP), a high amount of peaks, as well as several peak groups, can be distinguished. However, as this fraction is seldom analyzed, no further separation can be made on the basis of literature. Water extractable proteins contain the non-storage proteins which enclose enzymes and may therefore be interesting to estimate quality attributes related to enzymatic activity such as the starch breakdown (measured by Hagberg falling number (HFN)) by α-amylase or other glycosidic enzymes or the dough softening as the result of protease activity.

Compared to the WEP-fraction, a greater diversity is noticed for the prolamin (PRO), mainly for the peak group with a retention time between 3 and 5 min. The inverse curve underneath the chromatogram provides insight in the inter-quartile range (difference between 75^{th} and 25^{th} quartile) which is markedly higher. Wieser and Seilmeier (1998) stated that—as proteins eluted according to their different hydrophobicity in the series ω5-, ω1,2-, α- and γ-type—this fraction probably contains the ω-type PRO. From minute 11 to 16, peaks are considered to represent γ-type PRO (Pistón et al., 2011).

A more accurate distinction can be made for the chromatogram illustrating the variability within the glutelin (GLU) fraction as peak groups are more clearly separated. The group representing the high molecular weight glutelin-subunits (HMW-GS) was however limitedly separated. This might result in a poor correlation with results from the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Nonetheless, compensation mechanisms as reported by Gil-Humanes et al. (2011) might promote the presence of specific subunits in other, better separated fractions.

In general, it should also be mentioned that a considerable proportion of proteins (up to 20 % according to Wieser and Seilmeier (1998)) which are theoretically part of the GLU-fraction, are extracted with aqueous ethanol (as used during the extraction of PRO). In literature, these are designated as aggregated PRO or ethanol-soluble GLU. PRO containing an odd number of cystein residues may be bound with GLU and will therefore be extracted together with the HMW-GS and low molecular weight glutelin-subunits (LMW-GS).

2.3.1 Comparison with SDS-PAGE

Table 2.1 provides an overview of the gluten composition following the Payne classification (Payne et al., 1987). As the set was compiled to include all possible bands for the various groups (*Glu-A1*, *-B1* and *-D1* and *Glu-B3* and *-D3*), some levels were only represented by a single sample. This limits the possibility to construct robust models with the correct number of components (ncomp) as no cross-validation (CV) can be performed.

2.3.1.1 High molecular weight glutenin-subunits

Glu-A1 subunits could be partially distinguished on the basis of the PRO data with ‘Pavon’, ‘Katepwa’, ‘Apollo’ and ‘Zhongyu 415’ being misclassified (ncomp = 1). A model on the basis of the GLU fraction performed better with all samples carrying the *null* allele being classified correctly whereas ‘BlueSky’

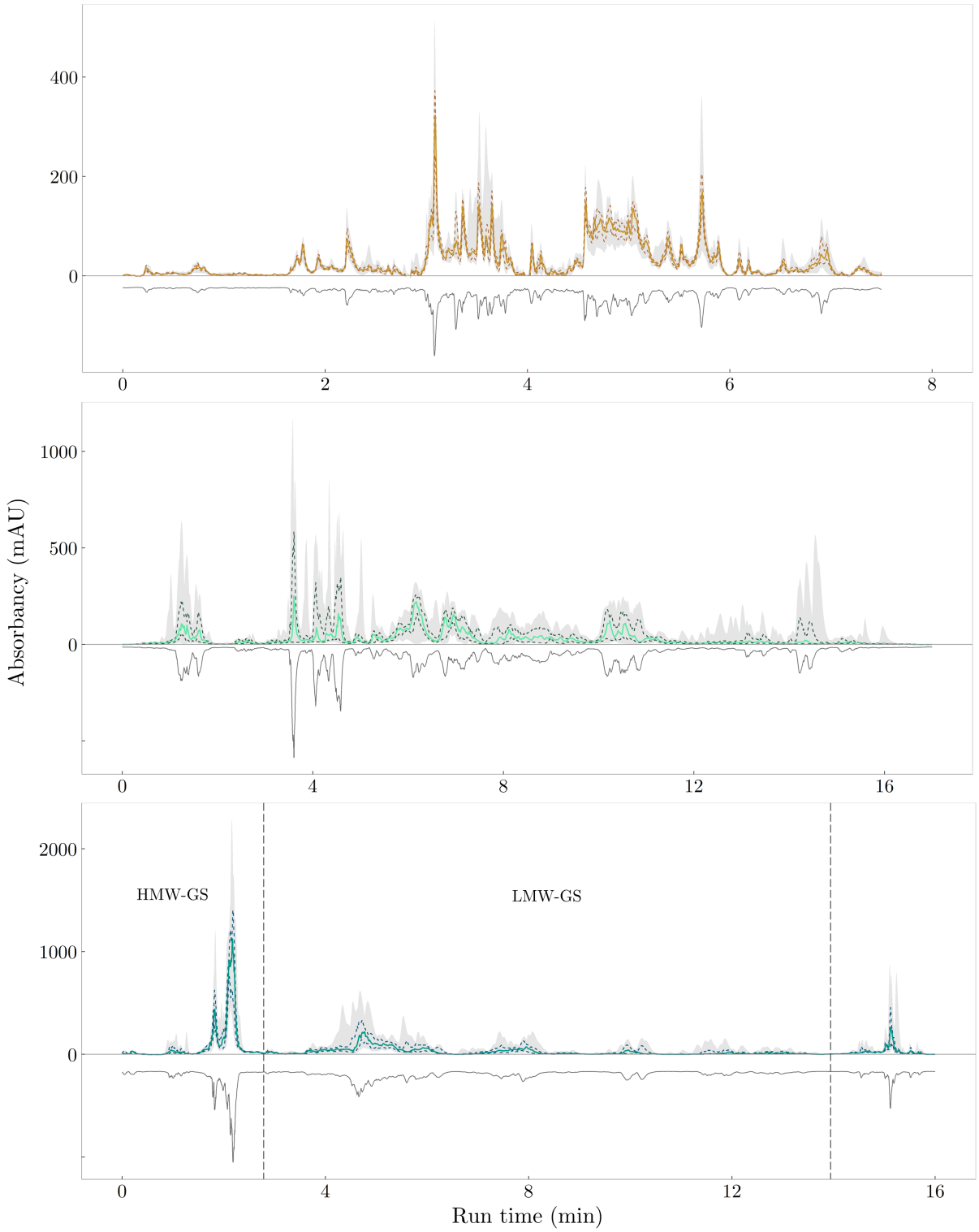

Figure 2.6: Plots of the median spectrum (—), including the 25th and 75th percentiles (- -) and the minimum and maximum (shaded area) for the water extractable protein (WEP, ●), prolamin (PRO, ●) and glutelin (GLU, ●) of the samples in the CIMMyT sample set. The inverted absolute interquartile range is displayed underneath the spectrum as an indication of the regions where spectra varied most.

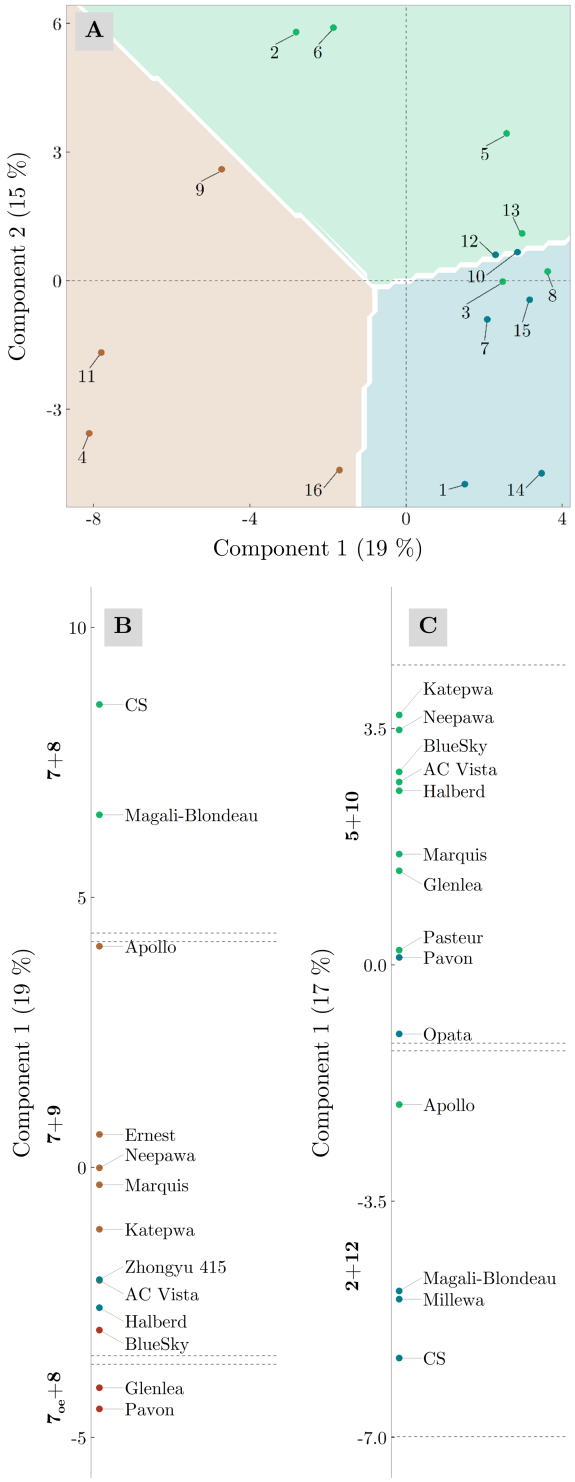

Figure 2.7: **A** two-component model on the basis of GLU-data showing distinction between samples (the number according with the cultivar can be found in Table 2.1) containing the *null* (●), *1* (●), or *2** (●) alleles.
B prediction of *Glu-B1* alleles (7_{oe}+8, ●; 7+8, ●; 7+9, ●; and 20, ●) using a single component model on the basis of prolamin data. **C** *Glu-D1* alleles (2+12, ●; and 5+10, ●) classified using a single component model of prolamin data.
Background colors (2D) and dashed lines (1D) indicate the group boundaries.

and 'Katepwa' were incorrectly classified as *1* (original *2**). 'Neepawa' and 'Marquis' were positioned on the line between *1* and *2** (Figure 2.7**A**). Model accuracies (on the basis of the AUC of the binary ROC-curves) were 0.88 and 0.93 respectively while *null* had a value of 1.00.

For groups with alleles 7_{oe}+8, 7+8, 7+9 and 20, two or more samples were available. Using leave-one-out cross-validation (LOO-CV), the lowest balanced error rate (BER) was obtained at 1 component for both models on the basis of PRO (Figure 2.7**B**) or GLU. The former model is able to discriminate all samples except for those carrying allele 20 (clustered together with 7+9). Also 'BlueSky' is misclassified

as 7+9 instead of 7_{oe}+8. No accurate model could be obtained for discriminating the four selected *Glu-B1* fractions with a nearly random classification of samples having the 7_{oe}+8 or 7+9 alleles.

Within the *Glu-D1* subunits, combinations 2+12 and 5+10 are most important as these have been linked with baking quality multiple times, although findings are contradictory (Liang et al., 2010; León et al., 2010). Using LOO-CV, a single component model using the prolamin data (Figure 2.7**C**) and a four component model for GLU was obtained. Visually, a two component model would be able to fully discriminate both groups while, with a single component, cultivars 'Opata' and 'Pavon' were misclassified as 5+10. 'Apollo' was also allocated to the incorrect group (2+12). A comparison of the four and two component models for GLU did not show any advantage of the two additional components which may indicate overfitting.

2.3.1.2 Low molecular weight glutenin-subunits

The three remaining, most common types of *Glu-B3* alleles (*g*, *h* and *i)* can be fully distinguished on the basis of a two component model using data from the prolamin fraction. An equal discriminative power was obtained when constructing a model using the GLU data. In addition, Figure 2.8 shows that the main areas contributing to the model are positioned in the part qualified as LMW-GS. Both findings support the idea that the fraction assigned as LMW-GS using SDS-PAGE has a relationship with the GLU as separated during the extraction method for high-performance liquid chromatography (HPLC). According to Ram et al. (2011), a close link exists between the *Glu-3* loci and loci controlling the prolamin synthesis in wheat.

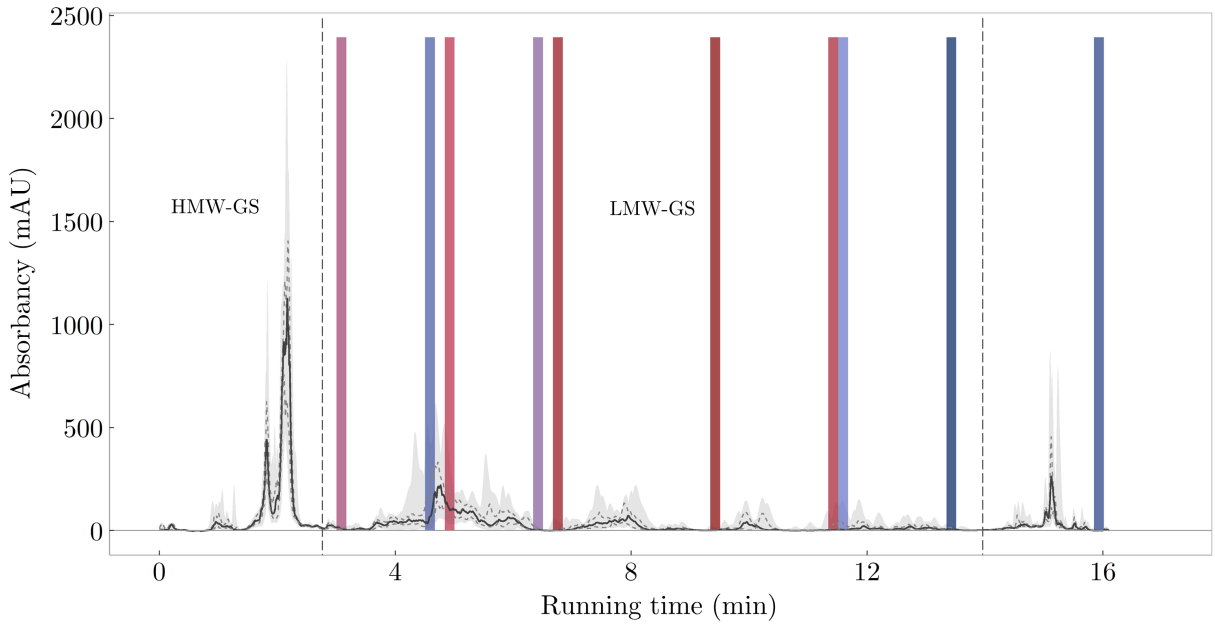

Figure 2.8: Median spectrum (—), including the 25th and 75th percentiles (- -) and the minimum and maximum (shaded area) for the glutelin fraction with an indication of the windows (*i.e.* areas) contributing most to the partial least squares discriminant analysis model for predicting *Glu-B3* alleles *g*, *h* and *i*. Colors indicate the relative contribution (from low (blue) to high (red)).

The *Glu-D3* fraction comprises samples with alleles *a*, *b* and *c* after removing levels containing only a single sample. No decrease in the error rate is observed at an increasing ncomp using the prolamin data,

implying its inability to develop a proper model. Accuracy was very low with no possibility to distinguish *b*-alleles from *a*- or *c*-alleles. On the contrary, error rates for a GLU-based model were lowest at 1, 3 and 4 components with a steep increase when 2 components were used. A three component model was able to perfectly distinguish samples without any overlap between the three groups. As is shown in Figure 2.9, two components appear to be enough to obtain complete separation. However, ellipsoids displaying the 95 % confidence interval are overlapping which may indicate that a third components is required to further separate both groups. It is however uncertain if the Gaussian approximation for the ellipsoids can be used here. Analogously to the findings for *Glu-B3* alleles, fractions contributing most were situated in the LMW-GS-region of the chromatograms with mainly the peak group between minutes 13–16 contributing to the model. Additionally, a single zone in the area denoted as HMW-GS was highlighted.

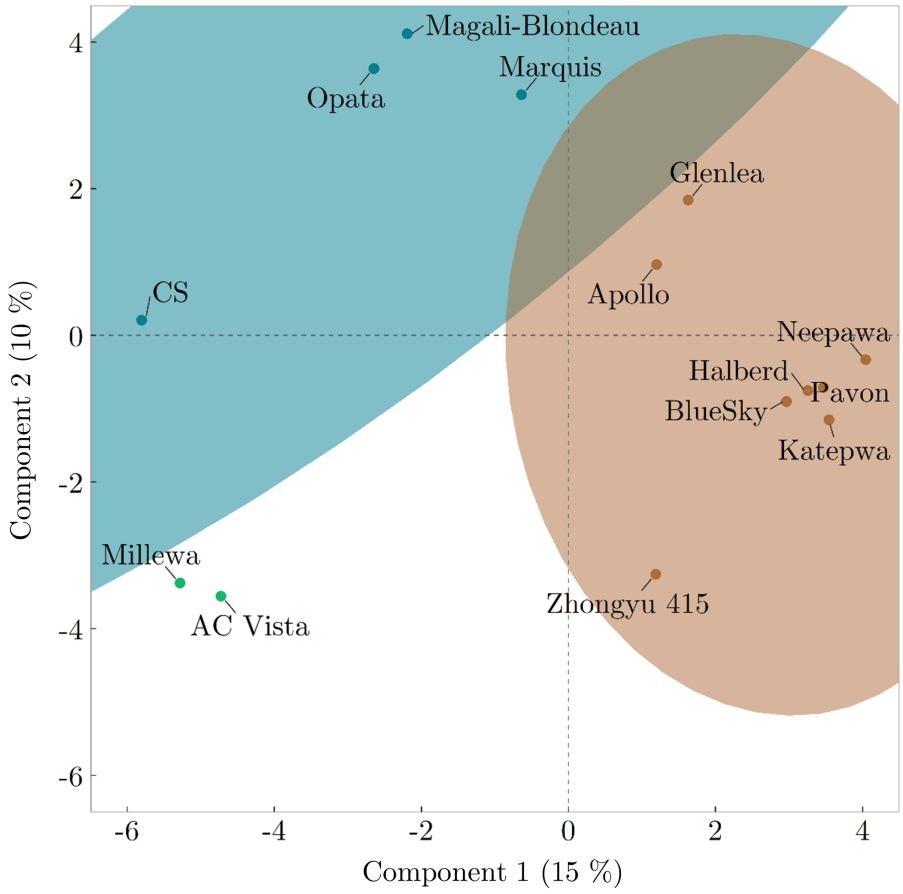

Figure 2.9: First two components of the model for predicting *Glu-D3* alleles *a*, *b* and *c* using chromatographic data (*i.e.* areas) from the glutelin fraction. Ellipsoids show the confidence interval (assuming a normal distribution) of 95 %.

> Leave-one-out cross-validation (CV) provides the possibility to select models with a higher number of components (ncomp) in a statistically responsible manner (compared to no cross-validation), although there is an increased chance of overfitting compared to m-fold cross-validation (m-fold CV). However, the frequently observed increase in the classification error rate at an increasing ncomp may also imply the general lack of data (number of observations).

2.3.1.3 *m*-fold cross-validation

Despite the sample set does not allow the use of m-fold cross-validation for all dependents, its added value is tested for *Glu-A1* (*null*, *1*, *2**) and *Glu-D1* (5+10 and 2+12) alleles. Both are considered to be mainly impacting bread quality. In this way, a better estimation of the correct ncomp to construct the model is obtained. This is then used to determine whether the HPLC-technique can be used to classify samples in accordance with SDS-PAGE.

As only four samples carry the *null* allele, a fold of 3 (m = 3) was selected to perform the CV for a model with 1 to 9 components. For both PRO and GLU, the optimal ncomp remained at one resulting in the same outcome as the results mentioned earlier. In contrast, partial least squares (PLS) discriminant analysis for the *Glu-D1* alleles 5+10 and 2+12 showed a decreasing BER for GLU when using m-fold cross-validation (m-fold CV) (m = 4). A significantly lower BER was obtained with ncomp = 2 while, using LOO-CV, a four component model had the lowest classification error rate (Figure 2.10**A**). On the basis of this two component model, all samples could be classified correctly (Figure 2.10**B**) in a not random way (AUC of 1 in the ROC-curve).

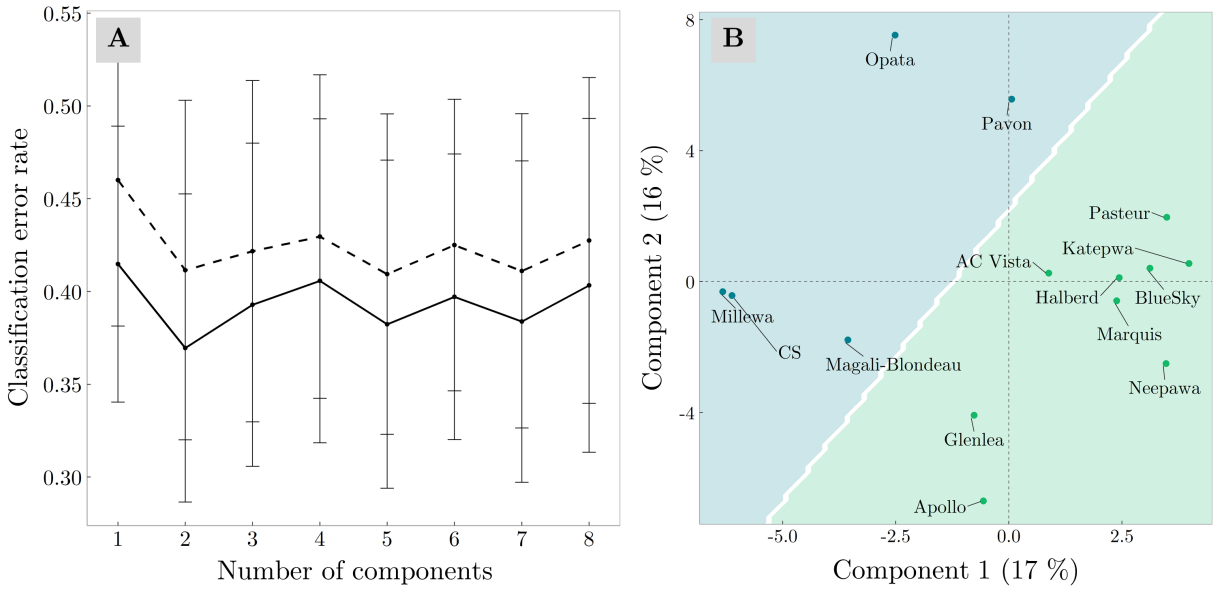

Figure 2.10: **A** Evolution of classification error rate (balanced - - and overall —) for a partial least squares discriminant analysis model using m-fold cross-validation (m = 4) for predicting the *Glu-D1* alleles 5+10 and 2+12 on the basis of glutelin data. **B** First two components of the model for predicting *Glu-D1* alleles using chromatographic data (*i.e.* areas) from the glutelin fraction. The background shows the prediction boundaries of both classes (2+12, ●; 5+10, ●).

The further investigation of the development of a model for predicting the *Glu-D1* alleles required a more comprehensive sample set including both *biological* repetitions —preferably with variation in other regions of the chromatogram by alterations in the protein composition—as well as variation in the alleles of interest. The cross-year cross-location sample set (CY-CLss), which is more elaborately discussed in Chapter 3 (page 95), meets these conditions allowing the development of a multi-fold cross-validated model for alleles 5+10 and 2+12. By comparing the outcome, an improved understanding of the robustness of the previous model can be obtained.

The fifty-two chromatograms of the GLU fractions were used during m-fold CV (m = 18, ncomp = 29). As 28 and 24 samples respectively carried the 2+12 or 5+10 allele, each fold contained at least 6 samples of the other group. Upon plotting the classification error rate, BER decreased steeply from approximately 20 % at 1 component to 3 % at 9 components whereas overfitting was observed for 10 or more components. The lowest ncomp which had a significantly lower BER compared to a higher ncomp was set at 8. The constructed model was able to separate all samples with 100 % accuracy. Figure 2.11 illustrates how an already good separation was obtained using the first 2 components (error rate of ≈ 10 %).

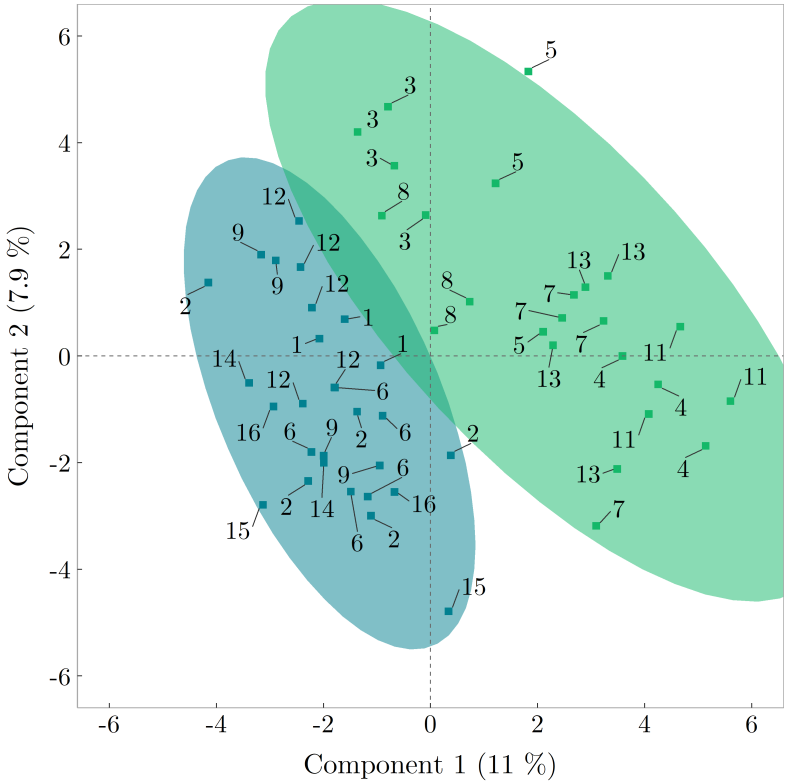

Figure 2.11: First two components of the m-fold cross-validated PLS-DA model for predicting *Glu-D1* alleles 2+12 (●) and 5+10 (●) in the CYCL-sample set. Ellipsoids illustrate the 95 % confidence intervals (assuming a normal distribution).
The number corresponding with the cultivar can be found in Table 2.2 or on page xii.

The figure also shows how samples are sometimes clustered by genotype (*e.g.* cultivars 'Atomic' (1), 'Cellule' (3), 'Gedser' (6), and 'Mentor' (10)) whereas other cultivars are markedly more widely distributed in this 2D presentation. Although this is not within the focus of the current research, this diversity might imply a difference in genetic stability to environmental effects influencing the composition of the GLU fraction.

By calculating the variable importance in projection (VIP)-scores for both models (m-fold CV and LOO-CV) and overlaying these with the chromatograms, insight was obtained provided in the windows (*i.e.* peaks) which contributed most to the prediction. As shown in Figure 2.12, both models (**A** CIMMyTss and **B** CY-CLss) use different peaks to make the prediction. Two reasons might lie at the basis of this discordance: (a) different peaks are related to the proteins denoted as alleles 5+10 and 2+12, or (b) the low sample-to-variable ratio of the CIMMyTss allowed to construct a meaningless model. Additionally, different methods for calculating the variable importance may yield various outcomes. Nevertheless, when comparing the overlays for both models, a certain resemblance can be observed with a region in

the LMW-GS-part of the chromatograms as primary contributors. For the more robust model based on the CY-CLss, a single peak in the HMW-GS-part is selected as well as two apparent non-informative windows —regions in the chromatogram which show no to very limited variation—between minutes 12 and 14. Independent from the model, the main contributing areas are positioned in the LMW-GS-region which is in contrast with expectations as peaks correlating with alleles 5+10 and 2+12 are assumed to be located in the first peak group (HMW-GS).

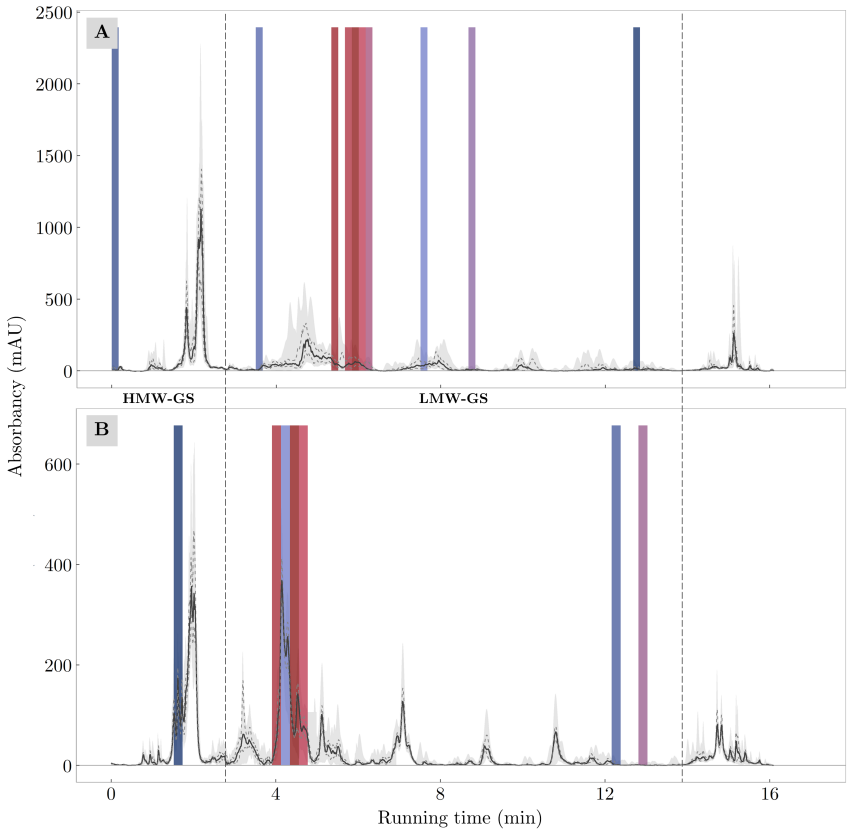

Figure 2.12: Median spectra (—), including the 25th and 75th percentiles (- -) and the minimum and maximum (shaded area) for the glutelin fractions of **A** the CIMMyT sample set and **B** the cross-year cross-location sample set. Colored windows indicate the areas which contribute most to the partial least squares discriminant analysis models (ncomp=4 and 2 resp.) for predicting *Glu-D1* alleles 2+12 and 5+10. Colors indicate the relative contribution (from low (blue) to high (red)).

Although models based on both prolamin and GLU data show to be adequate to predict the gluten composition as determined by SDS-PAGE, the tested sample set contains too much variability and a limited number of repetitions per class to properly derive the regions (*i.e.* peaks) related to these conformations. Moreover, as m-fold CV can be applied for only a selection of cases, not enough evidence can be provided that the same information can be derived from the HPLC-data as from SDS-PAGE. However, alleles 5+10 and 2+12 —which are most frequently used for quality classification—can be successfully discriminated showing the potential of the data.

2.3.2 Categorical discrimination

2.3.2.1 Unsupervised learning

Apart from the method validation, a cluster analysis was performed on the CY-CLss to check for the influence of main effects (*i.e.* environment and genotype). The resulting dendrogram based on the data of both gluten-forming protein fractions (PRO and GLU) can be found on page 82 (Figure 2.13). From this simplified one-dimensional representation of the data, multiple general conclusions can be drawn.

Firstly, all samples (Ghent, 2016–2018 and Koksijde and Tongeren 2018) from cultivars 3 and 12 (resp. 'Cellule' and 'RGT Reform') are clustered together implying a higher stability in the protein composition of these cultivars. A similar effect was noted for 'Bergamo' and 'Mentor' (2 and 9) which are each time clustered together although they are spread out over two super-clusters on the B-level (horizontal cuts in the dendrogram). For 'Graham' and 'RGT Sacramento' (7 and 13) as well as for 'Gedser' and 'Terroir' (6 and 15) are clustered, although the combinations were varying due to interaction with environmental effects. Clustering on harvest year is most pronounced for 2016 (loosely dotted) for which cultivars 5 ('Evina'), 7 ('Graham'), 11 ('RGT Mondio') and 13 ('RGT Sacramento') show most resemblance. In addition, samples from 2018 (solid) are more frequently clustered with the same cultivar harvested in 2016 compared to samples harvested in 2017. Analogously, samples from Koksijde (circles) and Tongeren (diamonds) are clustered together in 50 % of the cases as is also denoted in the figure.

None of the clusters at the A-, B- or C-level could be related with a specific descriptive variable implying the presence of interaction-effects between genotype and environment. Moreover, samples are expected to be closely related to each other as cultivation sites and environmental conditions over the three harvest years were relatively stable. Also, the genetic background of the tested cultivars is narrow compared to the global diversity in (winter) wheat genotypes and breeders are all positioned in Europe. As example, 'Bergamo' and 'Mentor', which showed to be very closely related on the basis of the dendrogram, are from the same breeder (Philips Seeds).

> Despite the high similarity between the tested samples on genotype level, clear genotypic and, to a lesser extent, environmental effects come to expression in the current dataset. Distinction between groups is however not unambiguously and prone to the interpretation of the researcher.

Through the high resolution of the HPLC-technique, it is suggested that environmental influences affect protein composition although this is genotype dependent. Current results also emphasize the need for more complex and *supervised* machine learning techniques to develop models which can subtract descriptive parameters from the data thereby omitting the interaction effects. Additionally, removing noise from the data by focusing on the regions in the chromatogram most affected by environment and genotype, could aid in elucidating sample similarity.

It is stated by various researchers that highly advanced proteomic techniques such as MALDI-TOF MS are required to rapidly and accurately identify different genotypes as LMW-GS and some wheat PRO are not completely resolved by reversed-phase (RP) HPLC (Mak et al., 2006; Vensel et al., 2014). Moreover, it is assumed that these methods do not provide detailed information about the identities of specific proteins

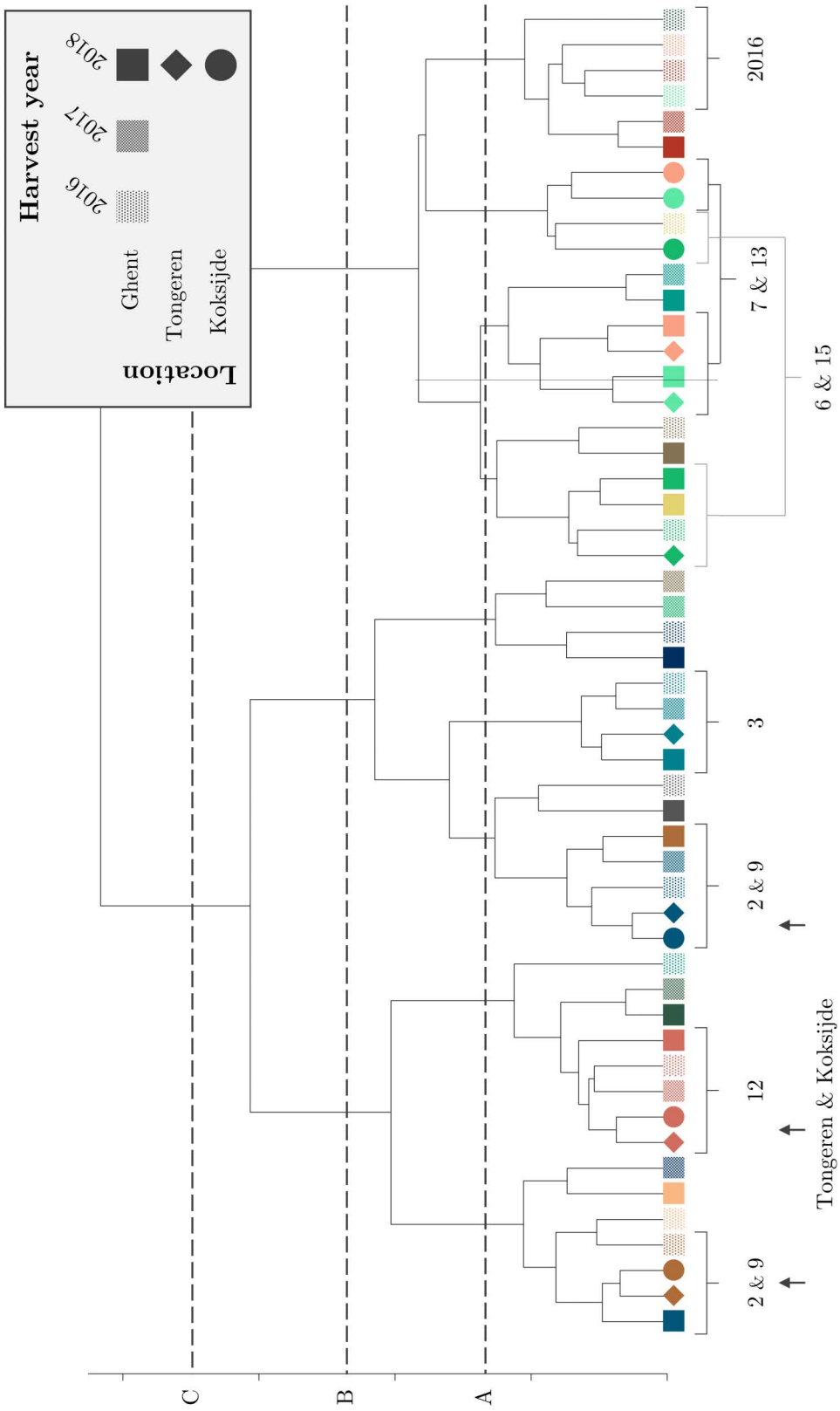

Figure 2.13: Dendrogram obtained through hierarchical agglomerative clustering on the basis of all areas of the prolamin- and glutelin-fractions using the Ward.D2 clustering algorithm for minimal within-cluster variance. Supplementary to the presented legend for harvest year and location, coding for the genotypes can be found on page xii.

present. As alternative, quantitative 2D-SDS-PAGE is proposed as this technique offers a more precise way to calculate the ratio of HMW-GS to LMW-GS (HMW:LMWr) and, when coupled with tandem-mass spectrometry (MS/MS), specific proteins fractions can be fully examined. However, screening the protein composition on such a detailed level might not be required to obtain the aforementioned goals. A proper separation is needed to discriminate high- and low-molecular-weight glutelin subunits whereas combining the fingerprint of the entire protein composition with advanced data modeling techniques may provide a powerful tool for genotype identification.

> A considerable interaction effect (genotype $\times$ environment) appears to be present in the data. The resulting dendrogram is difficult to interpret and no unambiguous distinction can be made between the branches on the basis of the main effects (harvest year, location, genotype). However, this is majorly attributable to the single dimensional representation and not to a result of a too low resolution in the data.

2.3.2.2 Supervised learning

The feasibility to predict the genotype using the current approach was assessed using the complete CY-CLss. As 'Tobak' was present only twice in the dataset, *m* was set at 2 for m-fold CV of a model with 1 to 21 components. Although a high-dimensional model (ncomp = 15) was required to accurately predict the cultivar, a nearly perfect model could already be constructed using 8 components. For the latter, the classification accuracy (on the basis of the AUC of the ROC) of 'Bergamo', 'Graham', 'Mentor', 'RGT Reform' and 'RGT Sacramento' was below 1.000 ($\geq$ 0.9271). Moreover, using a three-component model, 'Gedser' and 'RGT Mondio' could be discriminated with 100 % accuracy while all other cultivars had an AUC ranging between 0.7800–0.9844. A slight reduction in the discriminative power was observed when data of the WEP was excluded, showing the added value of analyzing this protein fraction and including this in the modeling process.

Besides genotype, environment (*i.e.* growth period and location) may influence the protein composition of wheat. Within the current framework, it can be considered valuable if insight is obtained in the regions (and thus, proteins or protein fractions) which are mainly influenced by the growing conditions. In case a model that predicts the environment of a sample can be attained, these areas can be derived on the basis of the VIP-scores which rank the variables following their contribution to the model.

A significant decrease in the BER is observed for models with 1 to 13 components after which the classification error rate slightly increases. As it is preferred to use the lowest ncomp, the model with ncomp 9 was chosen as no significant decrease in the BER was noticed for higher ncomps (Figure 2.14**A**). Although samples can be classified according to their environment at ncomp = 9, a significant overlap between all samples from 2018, mainly between Koksijde and Tongeren, can be observed (Figure 2.14**B**). Nevertheless, the accuracies (AUCs in ROC-curves) for both the two- or three-component models are equally high compared to the model for genotype identification. Samples grown in 2016 and 2017 (Ghent) were fully distinguishable from other environments at 3 components.

On the basis of the VIP-scores, it was noticed that the PRO fraction contributed less to the prediction. Therefore, it was studied if the exclusion of PRO data would reduced noise, thereby improving the

predictive power. A steeper increase in the BER was noticed when the model was based only on the GLU data. However, the optimal ncomp remained at 9. Upon comparing the model accuracy, less groups could be discriminated fully at the optimal ncomp which was also reflected by the high BER of 55.8 %. It has to be noted that data removal will automatically increase model performance due a reduced number of variables and a lower interference of variables who contribute less to the latent variables. Also when including the chromatographic data of only the WEP, a slight increase in the predictive power was obtained (ncomp = 8) compared to a model comprising solely the gluten-forming proteins (balanced classification error rate of 38.6 % and 41.0 % respectively).

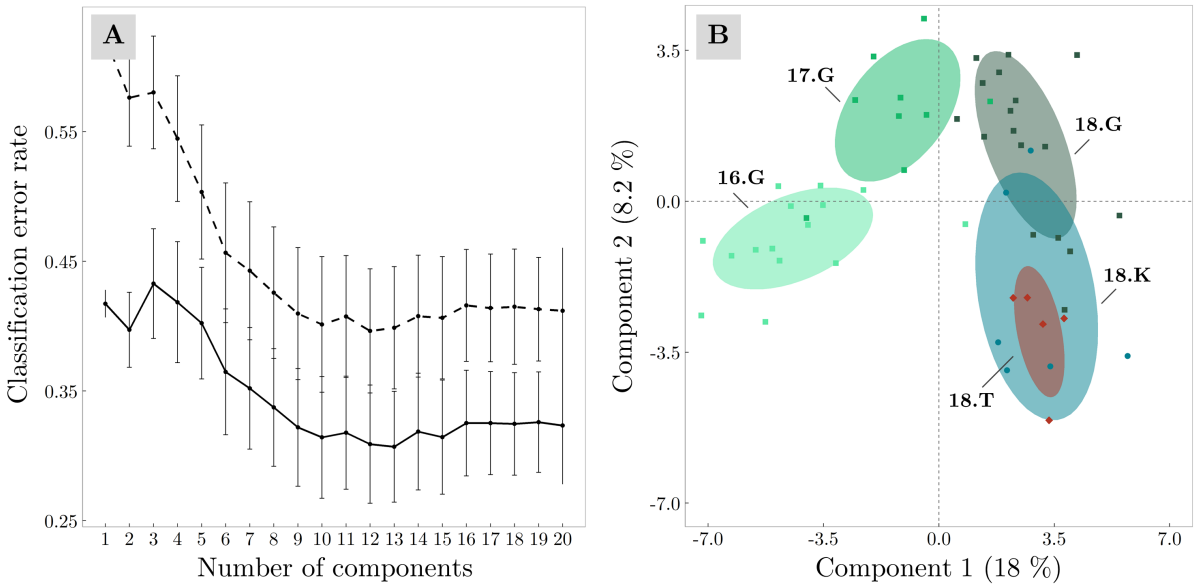

Figure 2.14: **A** Evolution of classification error rate (balanced - - and overall —) for a partial least squares discriminant analysis model using m-fold cross-validation (m = 4) for predicting the environment on the basis of the data from all protein fractions. **B** First two components of the model for predicting environment using chromatographic data (*i.e.* areas) from all protein fractions.
The ellipsoids show the 50 % confidence interval for the various classes (location: G, Ghent; K, Koksijde; T, Tongeren and harvest year: 2016, 2017 or 2018).

> The chromatographic data allows to accurately predict the genotype (genotype identification) using all protein fractions. This implies the added value of screening the protein composition of the water-extractable proteins. Including the latter also reduces the classification error when predicting the environment although error rates were relatively high with a main overlap between samples from the same harvest year. The findings suggest that protein composition mainly varies between genotypes and, to a lesser extent, between environments.

2.3.3 Prediction of continuous variables

PLS can also be applied for predicting continuous variables such as quality attributes of the end-product of interest or functional properties of the wheat flour which are related to the protein composition. In an analogous fashion as described in the previous section, this may also enhance understanding of the protein fractions which contribute to the attribute of interest. In industry, a main focus goes out to the prediction of the loaf volume as the main indicator for the baking quality of the wheat flour. From this point of view,

it was studied whether the chromatographic data can be used to construct a model for predicting the loaf volume (per kilogram flour) (VOLfc) and how this would perform when new data is added. In addition, the feasibility to develop a model for other functional attributes such as the water absorption at 500 BU (WA_{500}) and the Alveograph configuration ratio (AL-PL) was also studied as a proof-of-concept. A full description of the methods used to determine these parameters can be found in Sections 3.2.5 and 3.2.6.2 (pages 109 and 112, respectively).

Using the package '*pls*' (Mevik et al., 2019), PLS regression was performed for all protein fractions separately as well as the combinations of gluten-forming protein and all protein fractions. Areas were both scaled and centered in beforehand in accordance with research outcomes from Seasholtz and Kowalski (1992). The VOLfc, (average of 4 pan loaves, Chapter 3.2.6.1), was used as dependent. Besides m-fold CV, genotype-cross-validation ($CV_{\mathbb{G}}$) was performed to gain insight in the robustness of the model for predicting loaf volumes of unknown genotypes. In the latter situation, a specific genotype is removed from the training set and is used as test data after which the predicted value is compared with the measured value to calculate the model performance indicator. For random m-fold CV, the dataset was split up in nine segments (5–6 samples per segment) which were serially used as test set whereas the remaining samples were used as the training set.

By performing cross-validation, the optimal ncomp could be evaluated by means of the root mean squared error of prediction (RMSEP). The ncomp at which the lowest value was observed, was selected as the preferred number. Model performance (using the optimal ncomp) was assessed through the R^2 values and by visually comparing the observed *versus* predicted values.

As displayed in Figure 2.15, a primary decrease in the RMSEP could be observed for all models when using m-fold CV. After reaching a minimum at ncomp varying from 1–4, a steep increase in the RMSEP occurred indicating overfitting behavior. A similar curve shape and optimal number of latent variables was seen when $CV_{\mathbb{G}}$ was applied (Table 2.3).

VOLfc could be predicted best on the basis of all protein fractions or the combination of the gluten protein as the highest R^2 values were found for these fractions (resp. 0.823 and 0.808). For both types of CV, GLU data showed to be less suitable for forecasting the VOLfc. On the contrary, a PRO-based model was also suitable for making accurate predictions although low values ($\leq$ 4250 ml kg^{-1} flour) were markedly overestimated. When visually comparing the overall prediction quality (as illustrated in Figure 2.16), a similar range (25^{th} to 75^{th} percentile) was observed for predictions on the basis of the all-protein model with a median equal compared to the actual value (4512 ml kg^{-1} flour). From the scatter plot, it could be seen that the majority of the samples are located close to the ideal prediction line (observed (x-axis) *versus* predicted (y-axis) values). However, six samples were largely over- or underestimated ($\geq |13.5|$ %) as denoted by the light gray parallel lines in the plots. Despite this observation could not be linked with specific cultivars or environments, predictions for 'Gedser' (6) and 'KWS Ozon' (8)—those showing largest difference in gluten functionality—were most diverging from the observed values. This may support the hypothesis that functionality largely contributes to end-product quality (more specific, loaf volume development), and that this is not fully captured by this composition oriented technique. However, other samples from the same cultivars (other environments) were predicted with high accuracy ($\leq |1.7|$ % deviation compared to the observed values).

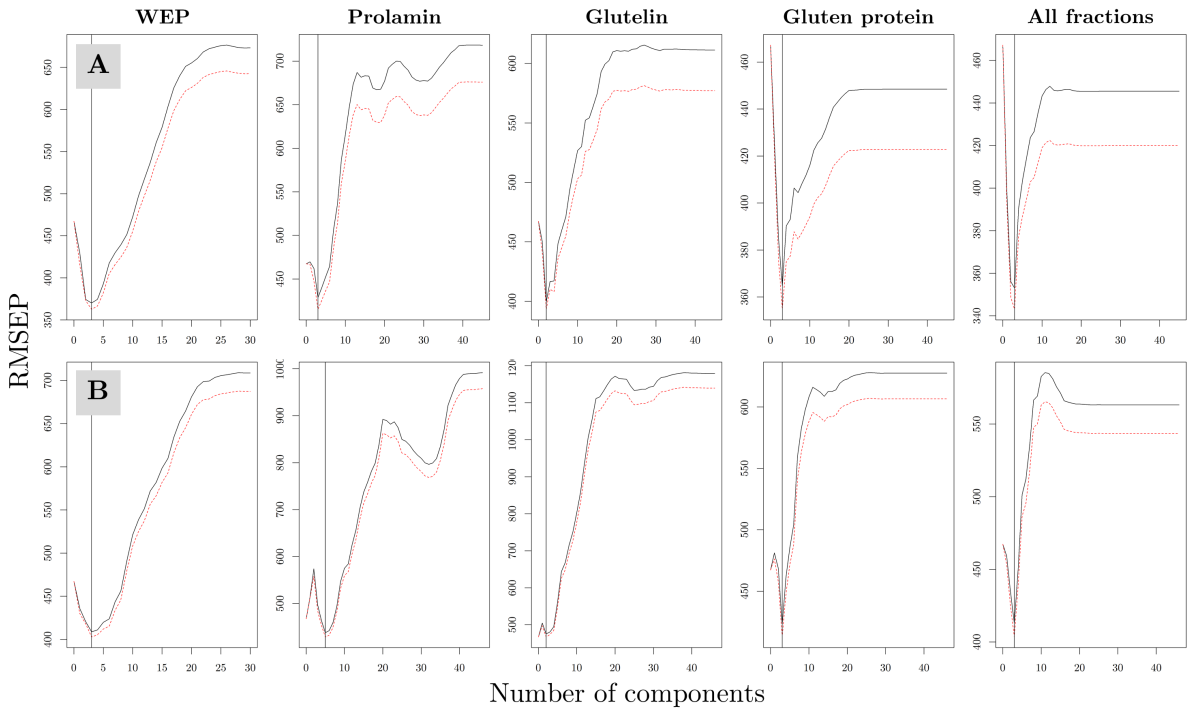

Figure 2.15: Change in the root mean squared error of prediction (RMSEP) per protein fraction for an increasing number of components (ncomp) for **A** m-fold cross-validation and **B** genotype-cross-validation.

WA_{500} and AL-PL are two main dough rheological parameters which provide insight in the quality of the gluten and the gluten network formation. Hence, both supplementary properties are used to evaluate flour baking quality on a dough level. A possible hurdle of these techniques is that both require a large amount of sample (50 or 300 g per test) and are time-consuming (approximately 2 h in total). As a preliminary trial, it is investigated if the quality attributes otherwise obtained through dough rheology can be predicted using the HPLC-data. The first model shows promising results for estimating the water absorption with a very high power for all models ($R^2 \geq 0.804$). Even when validating the model robustness for unknown genotypes, the predictive power remains relatively high with a low number of latent variables (ncomp $=$ 2). Again, GLU alone appears less suitable for modeling ($R^2 = 0.457$) whereas the combination with the PRO data or both other protein fractions greatly increases the power of the model ($R^2 = 0.766$ and 0.854 respectively). This implies the supplementary character of both protein fractions and how their interaction influences their functional properties.

Also for the AL-PL-ratio, a decrease in the ncomp and the predictive power is noted when $CV_{\mathbb{G}}$ is used. Models based on the PRO or GLU are characterized by a low R^2-value (0.401 and 0.412 respectively) whereas solely WEP or the combination of protein fractions resulted in more accurate models. When comparing this with values obtained through m-fold CV, a relatively limited increase in R^2-values is noticed when combining protein fractions. Although further research is required to elucidate this hypothesis, the current result indicates that only in some genotypes the different proteins (*i.e.* proteins from different fractions) interact resulting in functional differences while other genotypes do not show this effect. Moreover, previous research already showed the relation between the AL-PL on one hand and the HMW:LMWr and prolamin-to-glutelin-ratio (PGr) on the other hand, thereby supporting this finding. Hence, this emphasizes the need to select appropriate validation techniques when developing a model.

When looking at the prediction itself, it can be noticed that some extremes are present in the data (ratios of 4 to 8). These are not predicted well using the 12-component model comprising both gluten-protein fractions. While predictions for high measured values are mainly too low, ratios below 2 are predicted to be higher than the actual values. In addition, two of the predicted values were negative.

Table 2.3: Overview of the model properties and performance for predicting compositional, functional and end-product quality attributes on the basis of the cross-year cross-location sample set (CY-CLss) per protein fraction or combinations of fractions.

Variable	CV	PI	WEP	PRO	GLU	Gluten protein	All fractions
Loaf volume (flour corrected)	*m-fold*	ncomp	4	3	2	3	3
		RMSEP	396	378	420	366	361
		R^2	75.1	74.7	71.4	80.8	82.3
	GT-CV	ncomp	3	5	2	3	3
		RMSEP	403	429	467	413	403
		R^2	71.4	82.5	59.6	80.8	82.3
Farinograph water absorption	*m-fold*	ncomp	4	9	8	8	9
		RMSEP	2	2	2	2	1
		R^2	80.4	93.6	90.9	98.1	99.4
	GT-CV	ncomp	2	2	1	2	2
		RMSEP	2	3	3	3	2
		R^2	62.7	67.7	45.7	76.6	85.4
Alveograph P/L	*m-fold*	ncomp	2	6	4	12	8
		RMSEP	1	1	1	1	1
		R^2	68.1	88.6	79.8	99.8	99.1
	GT-CV	ncomp	2	1	1	2	3
		RMSEP	1	1	1	1	1
		R^2	68.1	40.1	41.2	77.4	89.3
Hagberg falling number	*m-fold*	ncomp	2	5	2	5	5
		RMSEP	41	42	38	34	32
		R^2	72.7	87.5	72.2	95.3	96.6
	GT-CV	ncomp	1	2	2	2	2
		RMSEP	41	47	42	38	35
		R^2	62.4	68.6	72.2	81.4	85.5

Performance indicator (PI).

Besides protein functionality, the presence (and possibly activity) of enzymes can be related to the protein fingerprint. The only measure available on the tested sample set is the HFN which is an indirect measure of the α-amylase activity in the flour. Despite not having a practical advantage in predicting this parameter through the current approach, it provides a valuable insight in the potential of the technique through viscosity. Interestingly, a robust and accurate model was obtained for the HFN using data from all protein fractions (independent from the applied type of CV). It was expected that proteins related to enzymes were only present in the WEP-fraction. Therefore, it was investigated if the applied data modeling technique did not overfit the abundant variables to a dependent, thereby obtaining useless models. However, no models could be obtained for a mainly starch related parameter (starch concentration) as well as for several sets

of randomly generated data. Only one-component models with R^2-values lower than 25 were obtained showing that previously obtained models were meaningful and could form the basis for future research.

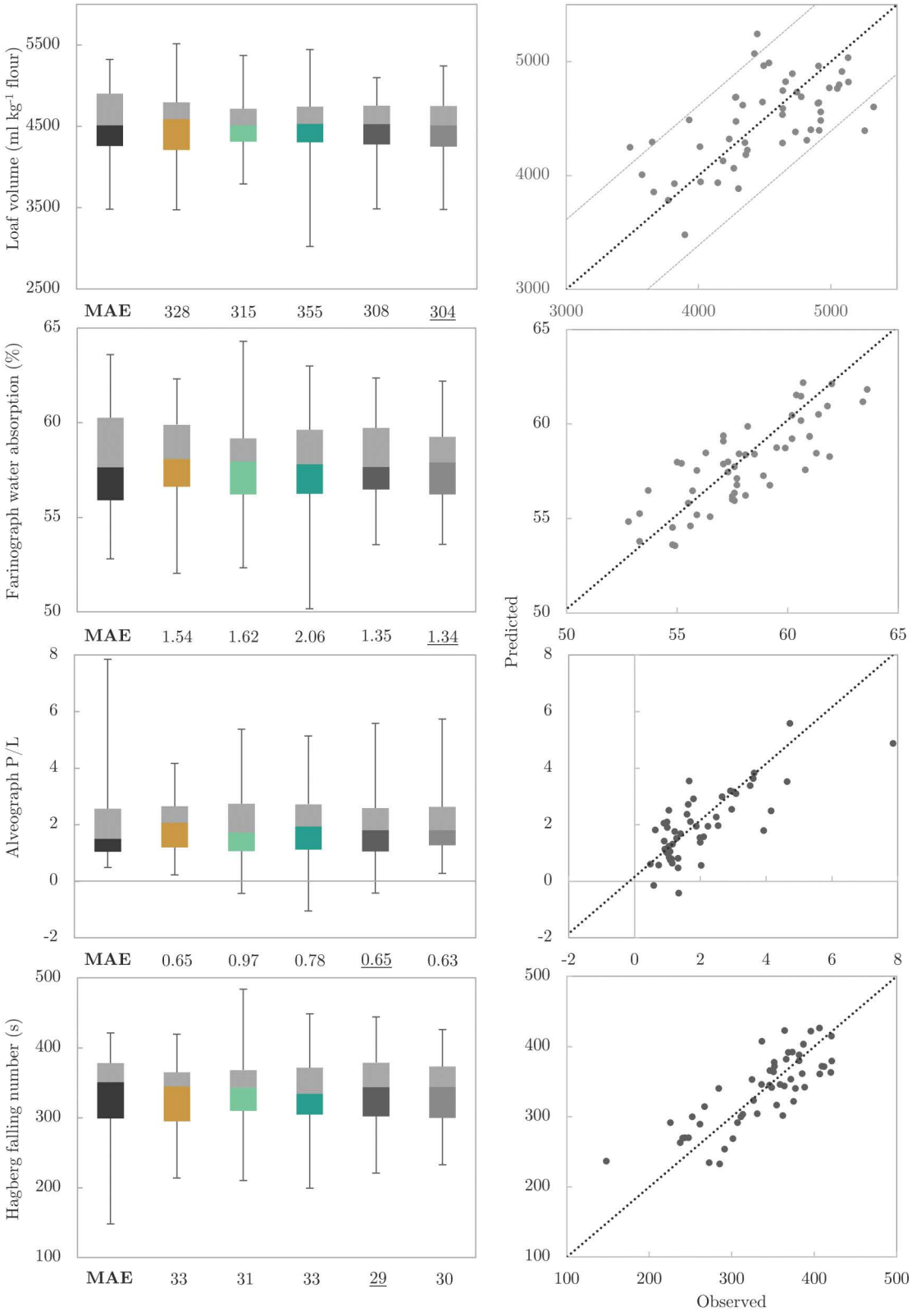

Figure 2.16: Output of the predicted values per model (water extractable protein ●; prolamin ●; glutelin ●; gluten-protein ●; all protein fractions ●) versus the observed values (●). The dashed line in the scatter plot represents ideal prediction (1:1).

2.4 Conclusion

The combination of RP-HPLC analysis with advanced data analysis techniques has shown to be of high value to predict both categorical parameters (*e.g.* origin, harvest year, *etc.*) as well as compositional attributes or even end-product characteristics. Despite the ability to develop a cross-validated model for the prediction of the loaf volume, it requires further investigation to optimize the accuracy. This can be achieved by combining the HPLC-data with additional parameters such as starch related properties. However, the current approach can already provide a primary tool for quality assessment as an alternative for conventional quality screening techniques which are currently used on an industrial scale.

Depending on the used equipment, the approach can be applied easily on an extensive sample set and, through further automation of both the analytical and data processing part, it can form the basis for a novel and innovative quality-assessment-framework. This should allow accurate end-product quality prediction for the trained variables in a fundamental (*i.e.* model-system independent) way. Thereby, it can promote the selection of cultivars during breeding practices as it allows a rapid and extensive quality screening.

Different hurdles remain as the approach is still under development and has only been used in a research setting. One of the main hindrances for its application in industry may be the time required to analyze a single sample. Currently, sample extraction, HPLC-analysis and data processing and interpretation requires approximately 5 hours. However, the more samples that are analyzed in one run, the more efficient the method becomes as the time needed for sample extraction and data processing remains equal. Nevertheless, upon sample reception in a mill, the 'quality' has to be screened within 10–30 minutes. Secondly, data interpretation is highly dependent on the proposed research question and on the constructed models (*i.e.* the information captured in the training set). Although clustering can already provide additional information, the prediction of a class or a continuous value requires a robust model for this specific parameter. Moreover, the data processing is currently done in a tailor-made system and will require a transformation to a software like platform before it can be exploited to its full extent. In the current light, the analysis and data processing has to remain centralized. Other elements which have to be looked in to are (a) the complete and automated alignment of chromatograms, and/or (b) the methods to reduce shifts resulting from analytical conditions, (c) the fluctuations in the extraction rate from different matrices, and (d) the ability of VIP-scores to provide insight in which protein fractions are related to end-product quality.

Highlights

- Main alleles (*e.g.* 5+10 and 2+12) could be accurately predicted using the chromatograms indicating the presence of this information in the data.
- Further validation with regard to reproducibility (mainly extraction recovery and alignment) remains crucial to establish a robust model which can be used over a long term.
- Back-translation to the chromatograms was found to be differing between models and did not always correspond with the area from which peaks were expected to be selected. This may be related to the method of variable selection or may come forth from the limited sample size for the first model.
- Non-supervised learning methods provide a preliminary insight in the similarity of samples. However, interpretation is subjective and interaction effects complicate the translation to concrete outcomes.
- Prediction of genotypes can be done with high accuracy, even when using genotype cross-validation, whereas environments are more difficult to distinguish.
- A selection of functional parameters (Alveograph P/L-ratio and Farinograph water absorption) could be predicted on the basis of compositional data (using all protein fractions).
- An adequate prediction of the loaf volume using solely compositional data was possible despite a larger variation was noted compared to functional properties implying the need to include functional properties or compositional features of other components now excluded.

PART I

CONTROLLABLE AND UNCONTROLLABLE VARIATION IN WHEAT COMPOSITION AND QUALITY

"The story the data tells us is often the one we'd like to hear, and we usually make sure that it has a happy ending."
NATE SILVER, The signal and the noise: why so many predictions fail but some don't

CHAPTER 3

Wheat quality attributes for predicting bread quality

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Acknowledgments:

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3.1 Introduction

For food producing companies and the related stakeholders, the market value of wheat grain is mainly determined by its protein concentration (Oury and Godin, 2007). In addition, the Zeleny sedimentation value, which is often used as an overall quality indicator, and the α-amylase activity (indirectly determined by the Hagberg falling number (HFN)) are measures to differentiate between wheat suitable for bread making purposes or for less demanding bakery applications. In addition, Xue et al. (2016) concluded that a variety's suitability for bread making conceivably improved when prolamin (PRO) and glutelin (GLU) are present in the correct quantity and ratio, thereby giving wheat based dough its unique rheological behavior. Besides protein composition, kernel hardness, (dough) rheological properties, flour water absorption, presence of essential amino acids, dietary fibers and quality-impairing substances (*e.g.* mycotoxins) are important characteristics influencing the functional, technological and nutritional properties of wheat both directly or indirectly (Amjid et al., 2013; Goesaert et al., 2005; Nuttall et al., 2017; Ormoli et al., 2015). Remarkably, the majority of the studies start from the assumption that starch properties do not (or to a limited extent) contribute to baking quality. Thus, starch and protein related attributes (molecular and macromolecular level) are generally not used together for quality prediction.

The contribution of single compositional and functional attributes to bread quality has already received significant research interest. However, the specific research question "which (combination of) parameters and analytical methods reflect(s) baking quality best" is less frequently considered. A large scale European study (AIBI International Association of Plant Bakers, 2017) found that numerous analytical parameters currently used in industry for wheat quality screening were strongly correlated, making them provide no supplementary information. Moreover, non of the parameters alone could provide a satisfactory prediction of bread quality. Also Stojceska and Butler (2012) observed that a large variation in correlations (from insignificant correlation to r-values of 0.92) between rheological properties of wheat dough and baking performance exists. As also concluded by these researchers, such techniques are limited to a narrow range of end-products (*i.e.* only leavened white bread) as they mimic a specific formulation and processing (Chin and Martin, 2014; Salimi Khorshidi et al., 2018).

3.1.1 Research goal

In the current chapter, it is studied how bread quality can be defined and how this varies in the context of a genotype ($\mathbb{G}$) $\times$ environment ($\mathbb{E}$)-study. In a second part, insight in the quality attributes of wheat related to bread quality is provided. This main research question can however be split up in three subquestions which each correspond to a '*degree of diversification*' as proposed in the introduction (page xx) of this dissertation. Although not the main priority, it is attempted to translate the outcomes to recommendations for similar applications on an industrial level.

Primarily, the possible added value of measurements on the entire kernel (as a whole or as wholemeal) is studied as these may provide additional information compared to compositional and functional properties of the flour. Moreover, variables which can be determined earlier in the wheat-to-bread chain may also enable an improved diversification. On the other end, the necessity of including functional properties will be investigated as information not captured by the sole use of compositional attributes may be covered in

this way. Overall, by having compiled a comprehensive set of starch- *and* protein-related compositional and functional attributes, the contribution of each of these components can be investigated.

3.2 Material and methods

The current section provides a comprehensive overview of the methods, both conventional and novel, applied for screening the quality of the kernels, the wholemeal and the flour. As some of these techniques are frequently used in an industrial setting, standard methods apply. An overview can be found in Table 3.3. Following sections mainly focus on the latter methods or elaborate on changes in the execution of the conventional technique. Moreover, data processing and sample preparation are also discussed extensively.

3.2.1 Field trials

In the current chapter, results from a genotype ($\mathbb{G}$) $\times$ environment ($\mathbb{E}$) study are elaborately discussed. The therefore used samples comprise a set of cultivars grown during three consecutive cultivation periods at three locations in Flanders (denoted as cross-year cross-location sample set (CY-CLss) in Chapter 2). The latter are chosen from the locations included in the LCG network (Landbouwcentrum Granen Vlaanderen, Agricultural Center for Cereal Crops Flanders) which is part of the annual variety testing program for winter wheat. The field in Tongeren (Limburg) was managed by vzw PIBO Campus and the Provincial Institute for Biotechnological Education. In Koksijde (West-Flanders), field trials were managed by Inagro vzw. The experimental farm in Bottelare (East-Flanders), part of Ghent University (UGent) and University College Ghent (HoGent), provided the third selected location as part of this network. Locations Koksijde (K) and Tongeren (T) are positioned approximately 200 km apart from each other, respectively in the West and East of Flanders. Ghent (G) lays on the line between both locations at 75 km from Koksijde. Per location, the fields trials were performed within a radius of 10 km from each other on a soil type characteristic for the location (K: cambisol, G: retisol and T: luvisol)

Within the variety testing program, field trials are carried out following standard practices with regard to fertilization practices and disease control. For the former, the fertilization regime suggested by the National soil service of Belgium (Bodemkundige dienst van België) (BDB) is followed, whereas, for the application of pesticides and insecticides, the prevailing disease pressure is used. Before the field is harvested, plots are approved objectively on the basis of their homogeneity and variety purity. An overview of the main cultivation conditions for the different environments can be found in Table 3.1. As the cultivars included in the trials are chosen on the basis of input from breeders and seed companies, changes in its composition throughout the years could not be prevented. Moreover, each location can choose the included cultivars according to their capacity. To partially overcome this issue, a selection of 16 cultivars made on the basis of results from te first crop cycle was maintained only in Ghent for the two consecutive growth periods (2016-2017 and 2017-2018). This approach allowed to study a broader diversity in wheat genotypes currently relevant for industrial use, while including environmental influences to the most feasible level. An overview of the coding used for the environments and cultivars can be found on page xii.

For making the selection, presumed quality grades (as reported by the seed companies) and information on the yielding parameters was used. This was supplemented with basic compositional attributes. Cultivars were chosen from all quality classes (according to the French and German grading system) ranging from wheat for non-bread applications to elite types. This automatically resulted in a desirable variation within the sample set. Apart from quality property related motives, the priority was given to cultivars which were already present in the list at least two consecutive years.

Apart from the cultivation conditions, the monthly precipitation and the average monthly temperature were investigated as this may be indicative for differences on a phenotype level. Weather data was collected from the 'Mety' weather station network. Weather stations were positioned in a radius of 10 km from the field locations. Although not presented in this research, weather data indicated clear differences between environments (locations and years) in terms of both precipitation and temperature. Over the three cropping cycles between November 2015 to July 2018, markedly less precipitation was recorded for Tongeren and Koksijde compared to Ghent. Mainly during the winter and spring period of 2016, Ghent received considerably more rain resulting in an overall increase of 26 % compared to the other two locations. For the consecutive years, differences were smaller although Ghent always received a higher rainfall (7 and 10 % respectively). Also between the growing periods (years), significant differences were observed. During the first cropping season (November 2015 to mid August 2016), on average 660 ml precipitation was recorded with a minimum value of 534 ml for Koksijde compared to 797 ml for Ghent. With only 433 ml, the second cropping season (November 2016 to July 2017) was considerably dryer with Koksijde receiving again the lowest amount of precipitation. During the third cropping season (November 2017 to July 2018), on average 560 ml rainfall was measured.

A similar trend was observed for the average temperature with Ghent having a systematically higher (4–10 %) temperature compared to the other locations. In Tongeren, autumns and winters were colder while temperatures in Koksijde were lower temperatures during summer.

Resulting from the various differences in terms of both controllable and uncontrollable environmental variables, factors year and location are considered together as environments. In this way, nine environments are studied for wholemeal samples while flour samples were represented by five environments.

3.2.2 Sample reception

After harvest, uncleaned samples (per plot) were stored in woven plastic bags in a dry, cold and dark environment until further processing. Prior to milling the wheat, samples were cleaned using a two-step cleaning installation. Firstly, kernels were discriminated on size using a shaking sieve (constant intensity), discarding kernels with a width larger than 4.0 mm and smaller than 2.0 mm. Recovery, measured by comparing ingoing and outgoing streams, ranged between 92–99 %. Secondly, dust and remaining plant components were removed by aspiration. All samples in this research were processed within 3 months after collection from the field.

Table 3.1: Overview of the basic cultivation conditions per environment.

	2015-2016			2016-2017			2017-2018		
	Koksijde	*Ghent*	*Tongeren*	*Koksijde*	*Ghent*	*Tongeren*	*Koksijde*	*Ghent*	*Tongeren*
Sowing date	26/10/2015	31/10/2015	24/10/2015	27/10/2016	27/10/2016	03/11/2016	25/10/2017	01/11/2017	30/10/2017
Harvest date	14/08/2016	08/08/2016	16/08/2016	01/08/2017	25/07/2017	31/07/2017	26/07/2018	18/07/2018	20/07/2018
Growth duration (days)	293	282	297	278	271	270	274	259	263
Forefruit	Sugar beet	Maize	Chicory	Potato	Maize	Sugar beet	Potato	Potato	Sugar beet
Soil type	Clay	Sandy loam	Loam	Clay	Sandy loam	Light loam	Clay	Light loam	Light loam
Sowing density (kernels m^{-2})	400	350	350	400	350	350	400	350	350
Advice[1] ($kg\ N\ ha^{-1}$)	223 (93–63–67)	204 (83–62–59)	225 (100–100–25)	226 (82–59–85)	186 (82–58–46)	194 (80–51–63)	207 (83–56–68)	197 (87–50–60)	168 (76–38–54)
Applied ($kg\ N\ ha^{-1}$)	227 (98–88–41)	215 (60–70–85)	200 (100–100)	235 (101–80–54)	188 (72–58–58)	200 (100–100)	225 (98–86–41)	185 (85–45–55)	170 (80–40–50)
No. of cultivars	7	16	8	10	16	10	6	15	7
Flour extraction	No	Yes	No	No	Yes	No	Yes	Yes	Yes
Tot. precipitation (ml)	534	797	648	390	455	455	541	597	540
Mean monthly temperature (°C)	10.8	11.6	11.0	9.7	10.1	9.5	9.4	10.5	10.1

[1] Advice is based on the Nitrogen-index as determined by the National soil service of Belgium (Bodemkundige dienst van België).

3.2.2.1 Kernel properties

Prior to milling, kernel characteristics including the moisture content (MC), yielding parameters (thousand kernel weight (TKW) and test weight (TW)) and the kernel morphology were determined on cleaned samples per replicate (plot). A Dickey-John apparatus was used for simultaneously measuring the MC and TW (triplicate). Three hundred kernels were weighed after automated counting using a Pfeuffer Contador seed counter (Pfeuffer, Kitzingen, Germany) to calculate the TKW.

Dimensional parameters (area size (AS), length-to-width-ratio (LtWr) and circularity (CIRC)) of the kernels were determined using the SmartGrain image analysis software, version 1.2 (Tanabata et al., 2012). Approximately 300 kernels were distributed on the scanner bed and scanned against a blue background using an Epson Perfection 2580 flatbed scanner (Seiko Epson, Nagano, Japan) at 300 DPI (resolution of 2550×3510 pix). After loading the files in the software, five random background areas and kernel areas were selected for automated color thresholding. The analysis output was visually checked before it was used in data analysis.

3.2.2.2 Milling

Small amounts of wholemeal were produced from the cleaned samples per biological replicate for all environments (Ghent, Koksijde and Tongeren, harvest years 2016–2018) whereas a single parallel was selected for flour extraction. The latter was a prerequisite to be able to perform the vast number of analysis on the obtained flour. However, assuming a larger variance for biological repetitions compared to technical repetitions, the outcome of the statistical analysis has to be interpreted carefully. Selection of the parallel was done using the moisture corrected yield data as this allowed to verify for possible field inconsistencies.

Wholemeal was obtained using a Perten 3100 laboratory mill (Perten Instruments, Hägersten, Sweden) for samples harvested in 2016 or a FOSS Hammertec mill (FOSS, Hilleroed, Denmark) for all remaining samples. Both hammer mills were equipped with a 0.8 mm sieve and were certified by AACC, ICC and ISO to be used as sample mill for HFN analysis. Moreover, no significant differences in compositional attributes were found (protein concentration (PROC), HFN and pasting behavior) as part of preliminary trials.

Samples from Ghent (harvest years 2016, 2017 and 2018) and Koksijde and Tongeren (only for harvest year 2018) were milled to flour using a Bühler MLU-202 laboratory mill. Approximately 24 hours before milling, cleaned wheat samples were tempered to 15.5 % moisture using their initial moisture content. Tap water was added to plastic buckets in which 5–7 kg wheat were weighed in advance. To ensure a uniform hydration of all kernels, the buckets were vigorously mixed multiple times throughout the tempering period. All samples were milled under the same conditions although the feed rate was adapted in case accumulation on the reduction rolls or on the sieves occurred. After leaving the mill run empty for at least 5 minutes, fractions from the three breaker rolls (B_{1-3}) and the three reduction rolls (C_{1-3}) were weighed separately and were combined. Bran and shorts were cleaned respectively once and twice using a bran finisher. The cleaned bran and shorts were weighed as well as the finisher flour (flour collected through cleaning) after which the latter was added to the other flour fractions. The total (sum of all flour weights) was used to calculate the overall extraction rate (ER) following Equation 3.1. Relative contributions of the

separate fractions and the breaker and reducing rolls were calculated by dividing the absolute weights by the total flour weight.

$$ER = \frac{[B_1 + B_2 + B_3] + [C_1 + C_2 + C_3] + FF}{total\ kernel\ weight_{tempered}} \times 100 \tag{3.1}$$

After milling, wholemeal and flour samples were stored in high-density polyethylene containers until further analysis. Prior to compositional and functional analysis, a minimal resting time of 1 week was set. MC was however determined earlier following AACC method 44-15.02 (AACC International., 2000b). Despite no values for the moisture content of wholemeal or flour are presented in this dissertation, the parameter was used for correcting the sample weight required for several analysis. Moreover, all compositional attributes are expressed on dry matter for which the MC is a prerequisite.

3.2.3 Protein attributes

3.2.3.1 Protein content

A VarioMax C/N (Elementar Analysesystemen, Langenselbold, Germany) was used to determine the Nitrogen (N)-content of wholemeal or flour. In both cases, 400.0 $\pm$ 5.0 mg (as is) was accurately weighed in a tin crucible and placed in the autosampler after automatically recording the weight. After flash combustion of the sample, nitrogen oxides (NOx) are reduced to elementary nitrogen (N_2) in the reduction tube whereas water is absorbed in the drying columns. The remaining gases are separated using a temperature programmed desorption column allowing the N_2 to flow along a thermal conductivity detector. An electrical signal proportional to the concentration of N is produced allowing the conversion to a absolute concentration using a calibration curve. A conversion factor of 5.7 was used to calculate the protein content. Using the raw materials MC, the concentration was converted to the concentration on dry mass.

In accordance with the laboratory standards, a separate calibration curve for low N-concentrations is used. Measurements were performed only a single time per sample as the accuracy and reproducibility was monitored through a validation sheet. Therefore, each sequence was preceded by a series of samples for equilibrating and calibrating the equipment. As daily calibration standard, glutamic acid ($C_5H_9NO_4$) is used whereas a control sample (aspartic acid, $C_4H_7NO_4$) was placed after every fifteenth sample and at the end of each sequence. The analytical performance was checked by means of the aspartic acid and two reference samples (flour and plant material).

3.2.3.2 Glutomatic analysis

A quantitative and semi-qualitative determination of the gluten properties was obtained through the Glutomatic analysis comprising the Glutomatic 2200, the gluten index centrifuge 2015 and the Glutork 2020 (Perten Instruments, Hägersten, Sweden). The analysis was performed on both wholemeal and flour according to standard method AACC 38-12.02. The 2 % w/v salt solution (NaCl) was prepared using household salt and demineralized water and was replaced on a daily basis.

For biological replicates (*i.e.* wholemeal samples obtained from different parallels in the field), at least two results were obtained whereas technical repetitions (*i.e.* repeated analysis on a flour sample obtained from wheat harvested from single plot in the field) were always done in quadruplicate. A RSD of 10 % was tolerated for wholemeal whereas for flour, a maximum was set at 5 %.

3.2.3.3 Zeleny sedimentation

This technique for measuring the protein quality (*i.e.* relative gluten strength) in wheat flour was included in the standard analytical toolbox as it is widely use in industry. Samples were measured in duplicate according to standard method AACC 56-61.02.

3.2.3.4 Gluten composition through RP-HPLC

As described in Chapter 2, both chromatographic data (peak areas) and quantitative variables were obtained from the reversed-phase (RP) high-performance liquid chromatography (HPLC) analysis performed on flour. In short, water extractable protein (WEP), PRO and GLU were separately extracted from 100.0 mg flour and were analyzed using a RP C8-column. After data preprocessing (removal of abundant data, baseline correction and spectrum alignment), peak areas were determined and relative ratios were calculated. Besides area information of the PRO and GLU fractions, their absolute amount was added as variables as well as the prolamin-to-glutelin-ratio (PGr) and the ratio of HMW-GS to LMW-GS (HMW:LMWr).

3.2.4 Starch attributes

3.2.4.1 Total starch content

In accordance to standard method AACC 76-13.01, the total starch content of flour and wholemeal samples was determined at least in triplicate using the colorimetric method (K-TSTA) developed by Megazyme (Wicklow, Ireland). The method for starch containing no resistant starch or D-glucose was used. Briefly, starch granules are degraded to maltodextrins after thermal and enzymatic treatment after which a further degradation to D-glucose is obtained using amyloglucosidase. Through the glucose oxidase-peroxidase system, glucose is quantitatively transformed to a quinoneimine dye which was measured at 510 nm using a DU 730 UV-VIS spectrophotometer (Beckman Coulter, California, USA). Starch concentrations (as is) were calculated following the formula in the enclosed manual. By the inclusion of a a standard containing 98 % starch, the correct execution of the method was checked.

3.2.4.2 Starch extraction

For the measurement of the granule size distribution of the starch, the amylose (AM) content and the amylopectin (AMP) fine structure, starch extracts were prepared from both the wholemeal and flour samples according to the method described in Demeke et al. (1999) with minor modifications.

Approximately 0.5 to 1 g of flour or wholemeal was weighed in a 15 ml plastic tube together with 10 ml demineralized water and was vortexed vigorously. The suspension was filtered over a nylon cloth (300 µm) using an additional 5 ml demineralized water, thereby removing coarse particles from the suspension. The obtained suspension was centrifuged at 3000 × g for 10 min after which the supernatant was decanted. The layer of protein on top of the starch was carefully removed using a spatula and the remaining starch pellet was resuspended in 0.5 ml demineralized water using a micropipette. This suspension was transferred to a 2.0 ml Eppendorf tube, pre-filled with 2 ml 80 % w/v CsCl. Tubes were centrifuged at 16000 × g for 15 min allowing the starch granules to migrate through the CsCl-layer. After again decanting the supernatant, the remaining starch pallet was purified using ultrapure water (1 ml), washing buffer (Tris-HCl, SDS, glycerol and ultrapure water, 1 ml), and again ultrapure water (2 times 1 ml) by each time discarding the supernatant, resuspending the pellet and centrifuging (16000 × g, 10 min) the suspension. Finally, 1 ml acetone (analytical grade) was added for removing free lipids. After discarding the acetone, the remaining starch pellets were dried to air at room temperature by placing them overnight in a fume hood.

3.2.4.3 Amylose content

The molecular size distribution of the starch was characterized with high-performance size-exclusion chromatography (HP-SEC) according to Kasemsuwan et al. (1995) with modifications (Patindol and Wang, 2002). For debranched starch, 10 mg of starch was mixed with 3.2 ml of Millipore water, boiled for 30 min, cooled down and added to 0.4 ml of acetate buffer (pH 3.5). Afterwards, it was incubated with 10 µl of isoamylase at 45 °C for 2 h and boiled for 15 min to terminate the debranching process. The buffer was removed with exchange resin IONAC NM-60 H^+/OH^--form, Type I (16-50 Mesh). The HP-SEC-system (Waters, Milford, MA) consisted of a 515 HPLC pump with a 200 µl sample loop, an in-line degasser, a 2410 refractive index detector maintained at 40 °C, and a series Shodex OHpak columns (KB-802 and KB-804) maintained at 55 °C. The eluent is 0.1 M $NaNO_3$ and 0.02% NaN_3 at an elution rate of 0.5 ml min^{-1}.

Calculation of parameters AMP and AM contents determined by HP-SEC are generally obtained by dividing the peak areas (area under curve (AUC)) for the corresponding peaks. These AUCs are obtained by summation of the peak heights over *n* one-second increments or by integrating over a region. Initially, the former method was applied for determining the content of both starch components (illustrated in Figure 3.1**A**). Commercial wheat and corn starch samples were used as references (Figure 3.1**R**) and indicated the suitability of this approach to determine the contents. However, for all wheat flour extracts, unstable baselines and remarkably low AM contents were obtained (9.3 ± 2.7 %). Upon comparison with the reference samples (20.8 and 18.1 % AM respectively), the effect could be attributed to a difference in the AM peak as no significant increase in the AUC for the AMP peak could be noted. This implies that the shift only occurred for AM although apparent shifts towards both lower and higher retention times (higher and lower molecular weight, respectively) were seen. Different approaches were investigated in an attempt to improve the calculation method for the AM concentration by interpreting the shifts observed in the chromatograms. Method characteristics are presented in Table 3.2 together with Figure 3.2 presenting the obtained values.

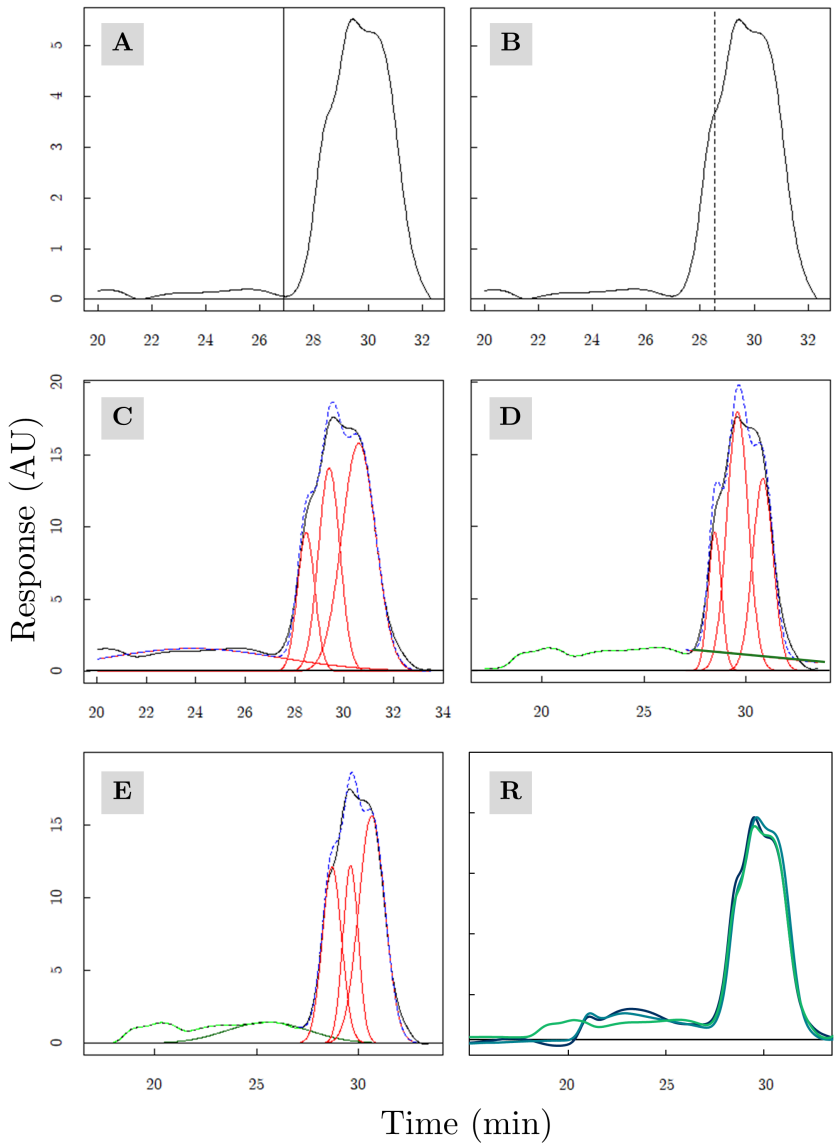

Figure 3.1: Plots **A**–**E**: illustration of the raw and processed high-performance size-exclusion chromatography curves of a single wheat flour starch extract with an indication of the cutoff or integrated areas for parameter calculation. Plot **R** shows the resulting raw chromatogram from a commercial wheat — and corn — starch sample and a wheat flour starch extract —.

For the AMP peak (retention time $\geq$ 27 min), an apparent increase in the width of the shoulder at retention time $\approx$ 28 min indicated a partial breakdown of AM molecules (enzymatic or mechanical) thereby decreasing the chain length and thus, the molecular weight (and hydrodynamic volume) of starch components (Figure 3.1**B**). In this way, shifts towards the right (higher retention times) would be observed, thereby interfering with the AMP peak (Cave et al., 2009). As contents are expressed relatively with a straight cut between both components, a shift of AM towards the right would lead to a double negative effect on AM contents, thereby explaining the remarkably low contents. When the peak heights of the first shoulder in the AMP peak were added to the AM fraction, a significant increase in the mean AM content (22.3 %) was observed with even decreasing standard deviations (2.1 *vs.* 2.7 %). However, with AMP being the preferred substrate for enzymatic degradation—due to the multiple attack action of amylolytic enzymes on AMP before debranching(Bijttebier et al., 2010)—considerable shifts in the AMP peak would

be expected as well. Moreover, this hypothesis does not explain the elution of components with a higher hydrodynamic volume at retention time $\leq$ 20 min. Therefore, it was investigated if the use of peak areas, taking into account the overlap between the AM and AMP peaks, gave more representative values.

Table 3.2: Output of the amylose concentrations using different calculation methods.

Method *Parameter*	**A** (Initial)	**B**	**C**	**D**	**Ea**	**Eb**
Integration limits	20–33.5 min	20–33.5 min	20–33.5 min	dynamic	dynamic	dynamic
Baseline shift correction	yes	yes	no	no	yes	yes
Integration	Peak height	Peak height	AUC	AUC	AUC	AUC
Mean	9.3	22.3	20.5	21.9	19.1	14.8
STDEV	2.7	2.1	7.0	8.2	4.5	4.3
Min	2.9	18.2	5.6	4.4	8.6	3.9
P_{25}	7.2	20.7	15.6	16.3	15.9	11.7
Median	9.1	22.3	21.1	22.0	18.7	14.8
P_{75}	11.6	24.0	26.3	28.2	23.1	18.1
Max	15.0	28.5	34.9	37.2	28.3	24.3
mean STDEV (per sample)	0.6	0.6	5.2	6.2	2.9	2.6
mean RSD (per sample)	6.8	1.6	28.1	32.3	15.3	18.3

Approaches **C** and **D**, in which peak areas instead of peak heights were used and which assumed an overlap between peak areas (*i.e.* overlapping chain-length distribution (CLD)), showed a considerable variation in the shape of the AM peak and hinted at the presence of a fraction of molecules with a higher molecular weight. To compensate for the latter, the start of the AM peak was determined dynamically in **method D**. Additionally, only the part overlapping with the AMP-area was estimated using a Gaussian approximation as a high absolute error for the AUC was obtained upon fitting a Gaussian curve to the entire AM peak. The AUC of the peak before the AMP peak was determined using spline integration. After summation of both areas, the AM content could be calculated which resulted in a comparable low concentration as calculation method **B**, however, with markedly higher standard deviations. The large variation between technical repetitions might be attributed to the incorrect assumption that the entire AM peak follows a Gaussian distribution resulting in broad peaks (high σ-values). This would also imply the existence of AM chains with a degree of polymerization (DP) similar to the short A-chains of AMP (DP $\leq$ 12) as the distribution spreads over the entire AMP peak. To overcome this technical problem, only the first sub-peak to the left of the conventional cutoff between AM and AMP was used to fit a Gaussian curve to. This is illustrated in Figure 3.1**E** by the dark green curve.

During curve fitting, using the '*nlslm*' package (Elzhov et al., 2016), constraints for σ were set to 0 and 2.5 resulting in proper fits to this peaks for all samples after automated parameter optimization using the Levenberg-Marquardt algorithm. In addition to the optimized Gaussian fitting process (method **E**), baseline shifts were corrected by subtracting the lowest value in the AM peak (generally the first value) from the entire cure in order to improve the reproducibility. When using the integrated areas, including the overlap between both peaks (method **E***a*), a mean AM content of 19.1 $\pm$ 4.5 % was obtained. Contrastingly, when again using the crude cutoff between both AM and AMP (method **E***b*), mean contents decreased while maintaining a similar standard deviation of 4.3 %. In this way, the RSD for the latter

method (crude cutoff, method E**a**) was higher (18.3 %) compared to **method E***a* in which overlap was taken into account (RSD = 15.3 %).

Although these (relative) standard deviations are markedly higher, it is considered incorrect that components eluding at lower retention times may be excluded for the calculation. A potential explanation for the presence of this fraction may be the formation of amylose-lipid complexes as only a short rinsing step was performed during extraction. Alternatively, the starch extraction method using CsCl will yield more B-type starch granules in the extracts compared to the conventional dough method. As this granule type contains less AM, the relatively higher proportion in the starch extracts may result in lower values compared to those reported in literature (He et al., 2019). Current results also indicated the added value of applying curve fitting using a nonlinear least-squares approach in combination with Gaussian curve fitting in order to more accurately determine the overlap between both fractions compared to using a crude cutoff between AM and AMP. Moreover, it is required to use this approach for calculating the relative concentration of short, medium and long AMP-chains as major overlaps between the three sub-peaks within the AMP peak are observed. In a more general situation, it can however be questioned if a Gaussian distribution can be fitted to the AM peak as peak shapes are largely differing. Considering the theoretical improvements made to the calculation method and the correspondence with values reported in literature, it was chosen to continue with the values obtained through calculation **method E***a*.

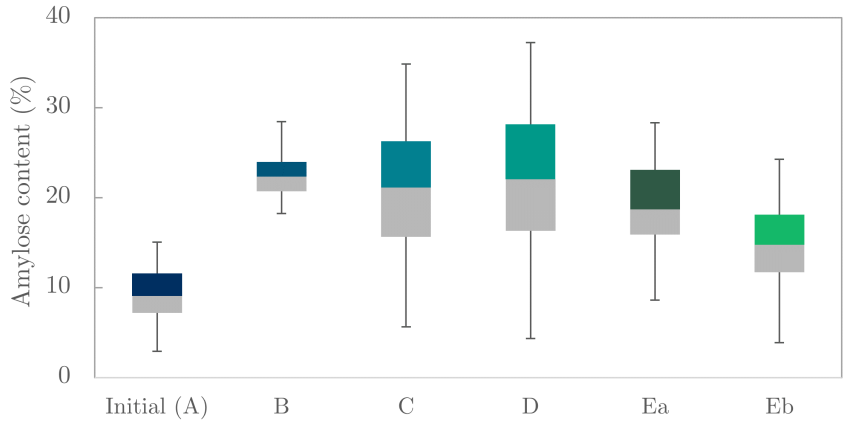

Figure 3.2: Boxplots indicating the variation in the amylose contents obtained through various calculation methods.

3.2.4.4 Amylopectin fine structure

Amylopectin CLD was characterized by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). A 10 mg defatted starch sample was mixed with 3.2 ml of deionized water, heated in a boiling water bath for 30 min, cooled to room temperature, and the pH adjusted with 0.4 ml of 0.1 M acetate buffer (pH 3.5). Ten microliter (10 U) of isoamylase (Pseudomonas isoamylase, Megazyme International, Wicklow, Ireland) was added and the mixture was incubated in a water bath shaker at 45 °C with stirring at 150 min^{-1} for 2 h. The pH of the mixture was neutralized by adding 0.21 ml of 0.2 M NaOH, heated in a boiling water bath for 15 min, and allowed to cool at room temperature for 5 min. A 1.5 ml aliquot was centrifuged at 5000 × g for 5 min in a non-stick Eppendorf tube to remove insoluble materials. A 0.6 ml aliquot of the supernatant was used for the HPAEC-PAD analysis. A Dionex ICS-3000 ion chromatography system (Dionex Corporation, Sunnyvale,

CA), equipped with an AS40 automated sampler, a 50-mm CarboPac PA1 guard column, and a 250-mm CarboPac PA1 analytical column was used. Two eluent systems (150 mM NaOH and 500 mM $NaNO_3$ in 150 mM NaOH) were used to separate the branch-chain fractions by gradient elution. Sugars with DP 1 to 7 were used to identify the chromatographic peaks. The assignment for the chromatographic peaks with DP higher than 7 was based on the assumption that each successive peak represented a saccharide that was 1 DP longer than that of the previous peak. Duplicate measurements were performed for each starch sample.

3.2.4.5 Granule and particle size distribution

From the extracted starch samples (Section 3.2.4.2), granule size distribution was determined in aqueous conditions using a Mastersizer 2000S (Malvern Panalytica, Malvern, UK) equipped with a Hydro 2000S module. Directly after initiating the measurement, the starch extract (12–18 mg) was suspended in 1 ml ultrapure water using a micropipette. The suspension was added to the dispersant (water) tank until an obscuration between 11 and 16 % was obtained. During the 75 s measuring cycle, three snapshots (technical repetitions) were taken. A general purpose analysis model was used with the particle refractive index and an absorption index of 1.53 and 0.1 respectively, while the refractive index of the dispersant was 1.33.

For the starch extracts, the relative concentration of A-, B- and C-type granules was calculated according to the distinction proposed by Zhang et al. (2010a). In an analogous manner, the *particle* size distribution of the wheat flour samples was determined. However, classes on the basis of the observed peaks in the volume distributions were distinguished.

3.2.4.6 Crystallinity

Starch relative crystallinity was determined by means of wide-angle X-ray scattering using a Rigaku Ultima IV X-Ray diffractometer (Texas, USA) with Cu K_α radiation (wavelength 0.1542 nm) operating at 40 kV and 44 mA. The scattering angle (2θ) covered the range from 4° to 40° with a step size of 0.02°. Samples were prepared by loading an excessive amount of the flour or wholemeal on the sample holder (approximately 1 g), compacting it using a glass coverslip and removing the redundant sample while ensuring a flat top layer. The sample holders were carefully placed in the 10-position autosampler after which the sequence was commenced.

The raw data from the recorded spectra was imported in R, version 3.4.3 (R Core Team, 2018), and processed in a similar fashion as described in Frost et al. (2009). In short, spectra were smoothed to reduce noise using an 11-point Savizky-Golay moving filter after which a baseline correction using the '*fillPeaks*' algorithm (Hanson, 2019) was applied. The area under the obtained baseline was used to calculate the amorphous area whereas Gaussian peaks were fitted to the baseline corrected spectra using the 2θ values reported by Lopez-Rubio et al. (2008) for A-type crystallinity (as expected for wheat). From the total area under the thirteen crystalline peaks, the relative crystallinity was calculated. V-type crystallinity, which is related to the presence of amylose-lipid complexes, was quantified by expressing the AUC of the peak at $2\theta \approx 20°$ to the amorphous region. This kind of crystallinity is however also characterized by two other

reflections at $2\theta \approx 7°$ and $2\theta \approx 13°$. Due to a decreased signal-to-noise ratio—resulting from performing the measurements on (wholemeal) flour instead of extracted starch—only the intensity of the first peak was sufficient.

3.2.5 Functional attributes

3.2.5.1 Pasting behavior

From both flour and wholemeal samples, the pasting behavior was recorded using an Anton Paar MCR 102 stress-controlled rheometer (Anton Paar, Ostfildern, Austria), equipped with a starch pasting cell and six-blade vane. A 14 % w/v suspension was prepared by weighing the raw material (2.8 g on a 14 % moisture basis) directly in the aluminum measuring cup and subsequently adding 20.0 ml demineralized water using a dispenser. For measurements with α-amylase inhibition, (denoted by '$_{EI}$'), 300 µl 0.1 M silver nitrate ($AgNO_3$, analytical grade, VWR, Pennsylvania, USA) was added to the measuring cup using a micropipette.

Prior to starting a measurement, the measuring cup was maintained at 20 °C. The pasting cycle was preceded by an automated mixing phase during which the suspension was homogenized. A pre-shear phase (960 rpm) was used to heat the sample to the desired starting temperature of 50 °C. Plastic lids were carefully placed on top of the cup to prevent evaporation during the measurement. The pasting cycle comprised a holding phase of 1 min at 50 °C, a linear temperature increase to 95 °C at 5 °C min^{-1} (9 min), a second holding phase at 95 °C for 5 min and a cooling phase opposite to the heating phase. During the third and final holding phase, the starch paste was maintained at 50 °C for 2 min.

From the obtained time-viscosity curves, multiple parameters are derived. Final viscosity (FV) was calculated by taking the average of the last 10 measuring points (20 s). Two pasting temperatures (Tg1 and pasting temperature 2 (Tg2)) were defined as pasting curves showed a two step viscosity increase for all wheat samples. Furthermore, peak properties (viscosity, time and temperature) and the holding strength (HS) ('hot paste viscosity') were calculated as well as the absolute differences between peak viscosity (PV), HS and FV (breakdown (BD) ($BD = PV - HS$), setback from peak (SBp) ($SBp = FV - PV$), and setback from trough (SBt) ($SBp = FV - HS$)). In an analogous fashion to Mixolab parameters, three directional coefficients, pasting rate (α), retrogradation/gelations rate (β) and gel stability (γ), were determined. Analysis of the curves was done using R, version 3.4.3 (R Core Team, 2018).

3.2.5.2 Farinograph

For measuring dough consistency, Farinograph measurements were performed on flour using the ICC standard method 115/1 with a constant flour weight, corrected for moisture content. A 300 gram mixing bowl was used for all measurements in this research.

3.2.5.3 Alveograph

In the standard Alveograph test, a dough is obtained by mixing flour (moisture corrected) with a fixed amount of salt solution (50 %). After extruding five dough pieces, these were inflated to a bubble until rupture to evaluate dough extensibility and elasticity. For measurements from samples harvested in 2017 and 2018, values from the five technical repetitions were used whereas single values (average of five curves) per sample were used for 2016.

Table 3.3: Overview of the applied techniques, the used international standards and the resulting variables.

Class	Description	Technique/method	Equipment	Output variables	Preferred matrix	Moisture correction	International standard
Kernel	Yielding parameters	Gravimetric	Dickey-John	Moisture content, test weight	Cleaned kernels	15 %	-
		Gravimetric	Pfeuffer contador	Thousand kernel weight	Cleaned kernels	15 %	-
	Kernel morphology	Image analysis	SmartGrain software	Area size, perimeter, length, width, length-to-width-ratio, circularity, distance to gravimetric center	Cleaned kernels	-	-
General	Extraction rates	Gravimetric	-	Overall extraction rate, extraction rate of (separate) breaker and reduction rolls	Flour	-	-
	α-amylase activity	Viscosity	Perten Falling Number	Hagberg Falling Number	Flour, wholemeal	14 %	ICC 107/1 AACC 56-81.04
	Particle size distribution	Laser diffraction (aqueous)	MasterSizer 2000S	$D[3,2]$, $D[4,3]$, D_{10}, D_{50}, D_{90}	Flour	-	AACC 55-40.01
		Laser diffraction (powder)	Beckman Coulter LS13 320	D_{10}, D_{50}, D_{90}, small (2–40 µm) and large (40–400 µm) particles	Flour	-	
Protein	Protein content	Dumas	Variomax CN	Protein content	Flour, wholemeal	dm	-
	Gluten quality	Solubility and water hydration	Glutomatic	Strong gluten, weak gluten, wet gluten content, dry gluten, gluten index, water binding to wet gluten	Flour, wholemeal	-	ICC 155 AACC 38-12.02
		Hydration	Zeleny sedimentation	Zeleny sedimentation value	Flour	-	ICC 116/1 AACC 56-60.01
	Protein composition	RP-HPLC	Chapter 2	Area of total prolamin, area of total GLU, prolamin:GLU-ratio, high-to-low molecular weight-ratio, peak areas	Flour, wholemeal	-	-
Starch	Starch content	Enzymatic conversion	K-TSTA Megazyme	Total starch content	Flour, wholemeal	dm	ICC 168 AACC 76-13.01
	Granule size distribution	Laser diffraction (aqueous)	MasterSizer 2000S	$D[3,2]$, $D[4,3]$, D_{10}, D_{50}, D_{90}, relative proportion of A-, B-, and C-type granules	Starch extracts	-	-
	Crystallinity	WAXS	Rigaku Ultima IV X-Ray diffractometer	Crystallinity, percetage V-type crystalls	Flour, wholemeal	-	-
	Amylose content	SEC	HPLC-system with SEC-columns	Amylose concentration, concentration short, medium and long amylopectin chains	Debranched starch extracts	-	-
	Amylopectin fine structure	HPAEC-PAD	Dionex ICS-3000 ion chromatography system	Relative proportions of A, B_1, B_2, B_3^+ branch chains, average chain length, short:long-ratio	Debranched starch extracts	-	-
	Damaged starch	Conductivity in presence of excess I^2	SD-matic	Damaged starch content	Flour	dm	ICC 172 AACC 76-33.01
Functionality	Pasting behavior (with enzyme inhibition)	Viscosity in function of temperature and time	Rheometer with starch pasting cell	Initial, peak, hot paste (holding strength), and final viscosity, setback from peak and through, breakdown, peak time and temperature, pasting rate, retrogradation rate, gel stability	Flour, wholemeal	14 %	AACC 76-21.02 (*adapted*)
	Dough consistency	Torque-measuring recording dough mixer	Farinograph	Dough development time, water absorption at 500 BU or 14 % moisture, degree of softening, stability, quality number	Flour	14 %	ICC 115/1 AACC 54-21.02
	Dough visco-elasticity	Pressure and time	Alveograph	Maximum required pressure (P), extensibility (L), P/L-ratio, deformation energy (W), index of swelling (G), elasticity index (Ie)	Flour	14 %	ICC 121 AACC 54-30.02

3.2.6 Breadmaking tests

As the ultimate tool for quality screening, breadmaking tests were performed for all flour samples. This large scale breadmaking test for evaluating the baking performance is a straight dough baking method based on the ICC standard method 131 with modifications (Vanneste and De Leyn, 2004). For enabling the correct measurement of all desired bread quality attributes, various types of bread were included. This comprises pan breads (conventional rectangular pan), closed pan breads (conventional pans with fixed lid) and plate breads (U-shaped baking tin). To distinguish the biological repetitions (different breads from same dough), breads were labeled and were assigned a 'bread id'. In this way, the oven spring (OvnS) could be determined per bead and correlations between quality attributes could be examined more accurately.

3.2.6.1 Formulation and bread baking

The bread formula contained wheat flour (≈ 2.5 kg, 14 %), dry instant yeast (1.00 % *Saccharomyces cerevisiae*, obtained from Algist Buggeman, Ghent, Belgium), salt (1.5 %), ascorbic acid (vitamin C, 0.0025 %, 25 ppm), tap water and malt flour. Water was added according to the percentage required to obtain a consistency of 500 BU as determined by the Farinograph measurement. The amount of malt flour was adapted in order to adjust the HFN of the flour to 250 s. Flour samples having a HFN lower than 250 s were used without malt addition.

Salt and ascorbic acid were dissolved in the water just before dough preparation, while malt flour and yeast were blended with the flour. The dough was prepared using a single speed spiral mixer with rotating bowl (De Danieli IS.06). After mixing for 7 minutes, dough temperature was checked. Water temperature was adjusted to control the dough temperature in subsequent tests. It was aimed to reach a dough temperature between 26.5–27.5 °C. After mixing, the dough was placed in the fermentation cabinet (30 °C, 85 % relative humidity) for 10 minutes (dough rest). Afterwards, the dough was divided in eight dough pieces of 400 g and three pieces of 175 g. Dough pieces were rounded with a Brabender rounder and placed in the fermentation cabinet for an additional 30 min. Following this first fermentation period, the dough pieces were molded and placed in baking pans (pre-greased with Olix, Puratos, Groot-Bijgaarde, Belgium). Prior to the final fermentation period (65 min), four baking tins were covered using a lid to restrict the doughs to a maximum of 1150 ml during the 2^{nd} fermentation.

The loaves were baked in the oven for 30 min with steam injection at the start of the baking program (240 ml in 2 min). The temperature program during baking consisted of 3 consecutive phases: 14 min at 230 °C, 13 min at 200 °C and 3 min at 190 °C during which the steam valve was opened. Prior to measuring the quality attributes (discussed below), breads were left to cool to room temperature.

3.2.6.2 Bread quality screening

Despite the conventional use of loaf volume as sole quality indicator for bread in industry, additional characteristics determined on the crumb or the bread in its whole can provide a broadened insight in quality differences and mechanisms contributing to volume development. As breadmaking trials from

samples harvested in 2016 were conducted outside the scope of this research, no full screening was performed with no usable information on the OvnS and crust color.

Dimensional attributes Loaf volumes of all breads were determined using a Volscan (Stable Microsystems, Godalming, United Kingdom) with a step size of 5.0 mm at rotation speed 'fast'. From the conventional pan loaves, the loaf volume (per kilogram flour) (VOLfc) was calculated through Equation 3.2 using the actual ingredient weights, thereby neglecting possible differences in the dough weight (tolerated deviation was ± 1 g).

$$\begin{aligned} flour:dough\text{-}ratio &= \frac{[flour\ weight]}{[flour + water + yeast + malt\ flour + salt + ascorbic\ acid]} \\ VOLfc &= \left(\frac{[Volume/400]}{flour:dough\text{-}ratio} \right) \times 1000 \end{aligned} \tag{3.2}$$

In addition to VOLfc, the OvnS was calculated from the height difference before placing the breads in the oven (after the second fermentation period) and just after taking them out. Both heights were determined using a height gauge with an accuracy of 0.01 mm. Also the height and width of the plate breads was measured (in the center) in a similar fashion, allowing to calculate the height-to-width-ratio (HtWr) as an indicator of dough rheological behavior during baking.

Crumb color Crumb color was measured in the CIE-LAB color space using a portable spectrophotometer (CM-700, Konica Minolta, Tokyo, Japan). After vertically cutting the plate bread in half, the center of the crumb was used for the measurement. This was done in quadruplicate using both sides of two of the three plate breads. Values are expressed as L (lightness) ranging from 0–100, a^* (green–red) and b^* (blue–yellow), both ranging from -100 to 100.

Crumb texture Crumb texture was determined on the closed pan breads (CP) in order to exclude potential effects of volume. By performing measurements on day one and four after baking, effects related to retrogradation can be recorded. Therefore, the 4 CP-breads were stored at a controlled temperature of 21 °C in hermetically sealed plastic bags until the measurement. Prior to commencing the analysis, two loaves were cut into 9.0 mm thick slices using an automated bread slicer after which 8 stacks (4 per bread) of each time 3 slices (excluding the outer two slices) were taken out. These were positioned on the measuring platform with the side originally closest to the center of the bread upwards.

A texture profile analysis was performed using a TX.XTplus Texture Analyzer (Stable Microsystems, Godalming, United Kingdom), mounted with a 5 kg load-cell and aluminum circular probe (P/36R, D = 36 mm). From the obtained force-time curves, crumb hardness (HARD), crumb springiness (SPRING), crumb cohesiveness (COH) and crumb resilience (RES) were calculated. By comparing the averages (8 measurements per day) of both time points, absolute and relative differences (denoted with 'Δ') could be calculated. The difference observed for HARD was used as an indicator for the bread staling.

Crumb structure Crumb structure was analyzed using a Python-script named 'BreadAnalyzer' (developed by prof. dr. ir. Jan Verwaeren). A 600 by 600 pixels cutout from the center of the crumb was

generated for each scanned slice and was thresholded resulting in a binary image. From this image, the number of gas cells and their corresponding sizes are calculated after which the data is transformed to a distribution with class borders of 0, 20, 40, 60, 100, 200, 500, 1000, 5000, 10^4 and 10^6 pix^2. By dividing the number of cells in each size range by the total number of detected cells in the image, a relative distribution pattern was be obtained. Additional parameters such as the total number of cells (NoC), the absolute number of cells larger than 100 pix^2 (aGC100$^+$), and the relative gas cell area (rGCarea) were calculated from the acquired data.

In practice, at least 8 slices were selected across the entire loaf (excluding the two outer slices) of two pan bread. These were positioned on the scanner bed (Epson Perfection 2580, Seiko Epson, Nagano, Japan) using a blue background to enhance automated image processing. Although the number of holes in the entire slices was simultaneously determined in a similar fashion, it was chosen to exclude the obtained information as unreliable data was obtained.

3.2.7 Data processing

Data preprocessing and analysis was performed using R (version 3.4.3, R Core Team (2018)). Prior to statistical analysis, outliers—defined as values outside the range of the 25$^{\text{th}}$ or 75$^{\text{th}}$ quartile $\pm$ 1.5$\times$ the inter quartile range—were removed in case data from three or more technical repetitions was available.

For the comparison of means, parametric tests were used thereby assuming that the values are distributed normally. Secondly, is was adopted that the condition of homogeneity of variances was fulfilled or that an equalization would not negatively impact interpretation. When for all samples in the analysis two or more technical repetitions were performed, two-way analysis of variance (ANOVA) was used with type III sum of squares as the dataset was unbalanced (Hector et al., 2010). In case no significant interaction effect was observed, one-way ANOVA was used to further investigate the influence of single factors on the group means. In case only a single value per sample was available, solely one-way ANOVA was used removing the factor genotype. In the latter case, the Tukey HSD post-hoc test was used to differentiate between multiple groups.

Broad-sense heritability (H^2), defined as the relative variance explained by the genetic factor (*i.e.* genotype), was calculated according to the formula proposed in Visscher et al. (2008). Mean squares were obtained from the ANOVA table in case interaction effects could be estimated. Equation 3.3 was used to calculate the H^2 in which both factors harvest year and location were combined into a single factor environment ($\mathbb{E}$). $\mathbb{R}$ is the total sum of squares that is not explained by the model.

$$H^2 = \frac{\sigma^2_{\mathbb{G}}}{\sigma^2_{\mathbb{G}} + \sigma^2_{\mathbb{E}} + \sigma^2_{\mathbb{G}*\mathbb{E}} + \sigma^2_{\mathbb{R}}} \times 100 \tag{3.3}$$

Correlation analysis was done using the average values (calculated after removing outliers) per sample for a selection of the variables. One-to-one correlations between continuous variables were investigated using the Spearman's rank correlation coefficient (r) which was calculated using the '*corrplot*' package (Wei and Simko, 2017). Except stated otherwise, insignificant correlations ($p > 0.05$) were excluded as well as relations for which an R^2-value lower than 0.40 was observed. For deleting missing values from

the analysis, pairwise complete observation selection was used. As such, a the correlation was computed using all complete pairs of observations on those variables.

Prior to applying dimension reduction techniques, a subset of the total dataset (using a selection of variables) was screened for gaps. Firstly, in case the number of missing values per sample exceeded a threshold (0.25 $\times$ the number of variables), these single samples were excluded for analysis. When parameters measured on the flour were included, only the 52 samples from which flour was milled were selected. To fill remaining gaps in the possibly reduced dataset, data imputation as described by Josse and Husson (2016) was used. Variables for which, after reaching the maximum number of iterations, no imputed output could be generated (and *i.e.* no reliable value could be estimated) were also excluded from analysis. Data imputation was also not used in case the data of all samples representing an environment were missing.

Principal component analysis (PCA) was performed in order to obtain insight in the variance present in a dataset using the '*FactoMineR*' package (Lê et al., 2008). Depending on the total explained variance, the number of principal components (2 or 3) was selected. No higher number was preferred as dimension reduction was performed to obtain an enhanced insight in the correlation between variables and the possible grouping of the samples. In general, an explained variance of $\geq 60\,\%$ was considered sufficient.

In a second stage of the research, partial least squares (PLS) regression was used for developing a predictive model for a dependent variable. Because of its ability to properly handle the many, possibly autocorrelated predictor variables, and the relatively few samples, this multivariate regression method was preferred from the wide range of machine learning techniques (Wehrens and Mevik, 2007). The optimal number of components (ncomp) was selected using m-fold cross-validation (m-fold CV). A distinction was made between random, genotype- ($CV_{\mathbb{G}}$) and environment- ($CV_{\mathbb{E}}$) cross-validation (CV) for discriminating test and training data. The last two types of CV, samples from respectively a single genotype or environment were iteratively left out of the training set and were used as test set to estimate the model performance. Random CV was performed by each time randomly selecting 10 % of the samples as test set whereas the remaining 90 % was used as training set. The adjusted root mean squared error of prediction (RMSEP) was plotted in function of the ncomp for all CV-techniques. The optimal number—defined as the lowest ncomp at which the adjusted RMSEP was lowest—was selected for further evaluation. Overall model performance was expressed by means of the (relative) mean absolute error (MAE) as calculated following Equation 3.4.

$$\begin{aligned} MAE &= |[actual\ values] - [predicted\ values]| \\ rMAE &= \frac{MAE}{[actual\ values]} \times 100 \end{aligned} \tag{3.4}$$

3.3 Results and discussion

The compilation of a comprehensive data set of yielding parameters and kernel morphological, compositional and functional attributes, allowed to accurately study the relationship with bread quality. Firstly, an overview in the determinative quality characteristics for bread is presented. Subsequently, the relation

with environment ($\mathbb{E}$) and genotype ($\mathbb{G}$) is investigated to estimate the magnitude of both factors on bread quality.

A second part of the results and discussion section focuses on the development of a model for quality prediction. This includes selecting the variables which contribute most to the model and translating them to possible underlaying mechanisms. Furthermore, the repercussions of the used analytical techniques on quality screening are highlighted. The chapter is concluded with an overview of the factors which lay at the basis of the observed variation.

3.3.1 Sample characteristics determining for end-product quality

3.3.1.1 Variation in end-product quality

In industry, quality of white pan bread is frequently narrowed down to a high loaf volume and a uniform crumb structure. The former was found to be strongly varying between genotypes and environments (Figure 3.3) with bread produced from wheat grown in Ghent and harvested in 2016 being on average markedly larger (4756 $\pm$ 466 ml kg^{-1} flour) than breads from cultivars grown in 2017 (4303 $\pm$ 641 ml kg^{-1} flour) or 2018 (4326 $\pm$ 410 ml kg^{-1} flour). Moreover, within harvest year 2018, differences in the loaf volume (per kilogram flour) (VOLfc) for samples from Ghent (G), Koksijde (K) and Tongeren (T) were observed although these were smaller and may be attributed to the included cultivars. For 'Bergamo', 'Graham', and 'RGT Sacramento', significantly higher VOLfc was obtained compared to Ghent whereas similar or equal volumes were found for 'Gedser', 'Mentor' and 'RGT Reform'. In addition to the significant effect of environment ($\mathbb{E}$), the genotype ($\mathbb{G}$) effect was even stronger with 49 % of the variation being attributable to this factor (broad-sense heritability (H^2)) (Table 3.4). Cultivar 'KWS Ozon' had a consistently low VOLfc whereas highest volumes were observed for REF and MEN. A minor but highly significant interaction effect can be observed, for example for 'Cellule'. VOLfcs for this cultivar were among the lowest for 16.G (4282 $\pm$ 61 ml kg^{-1} flour) and 17.G (3929 $\pm$ 14 ml kg^{-1} flour) whereas it was ranked fifth best for 18.G with a volume of 4369 $\pm$ 50 ml kg^{-1} flour.

By means of principal component analysis (PCA), it could be illustrated that VOLfc is a main discriminative factor when studying bread quality (Figure 3.4). However, variability explained by the first two principal components (PC) was only 52.6 % whereas the addition of a third component increased the explanatory power by 10.7 %. Besides loaf volume, crumb texture and color (*i.e.* lightness) show to have the highest contributions (indicated by the green color of the arrow in the loading plot). Crumb hardness (HARD) and the related crumb cohesiveness (COH) and crumb resilience (RES) (as well as the change over a 4-day period) are varying between different environments (2016 *vs.* 2018) and, to a larger extent, within 2017. Crumb lightness (L_{crm}) is highly correlated with the second PC and shows no correlation with volume or gas cell properties of the crumb. In contrast, a strong relation between the relative gas cell area (rGCarea) in the crumb and the COH appears to be present. However, a further distinction on the 3rd PC is only present for rGCarea together with the relative increase (Δ) in the hardness (and thus, chewiness).

Current findings show that most breads with a high VOLfc also experience a high oven spring (OvnS) which is also confirmed by the significant positive correlation ($p \leq 0.001$, $r = 0.688$) between both

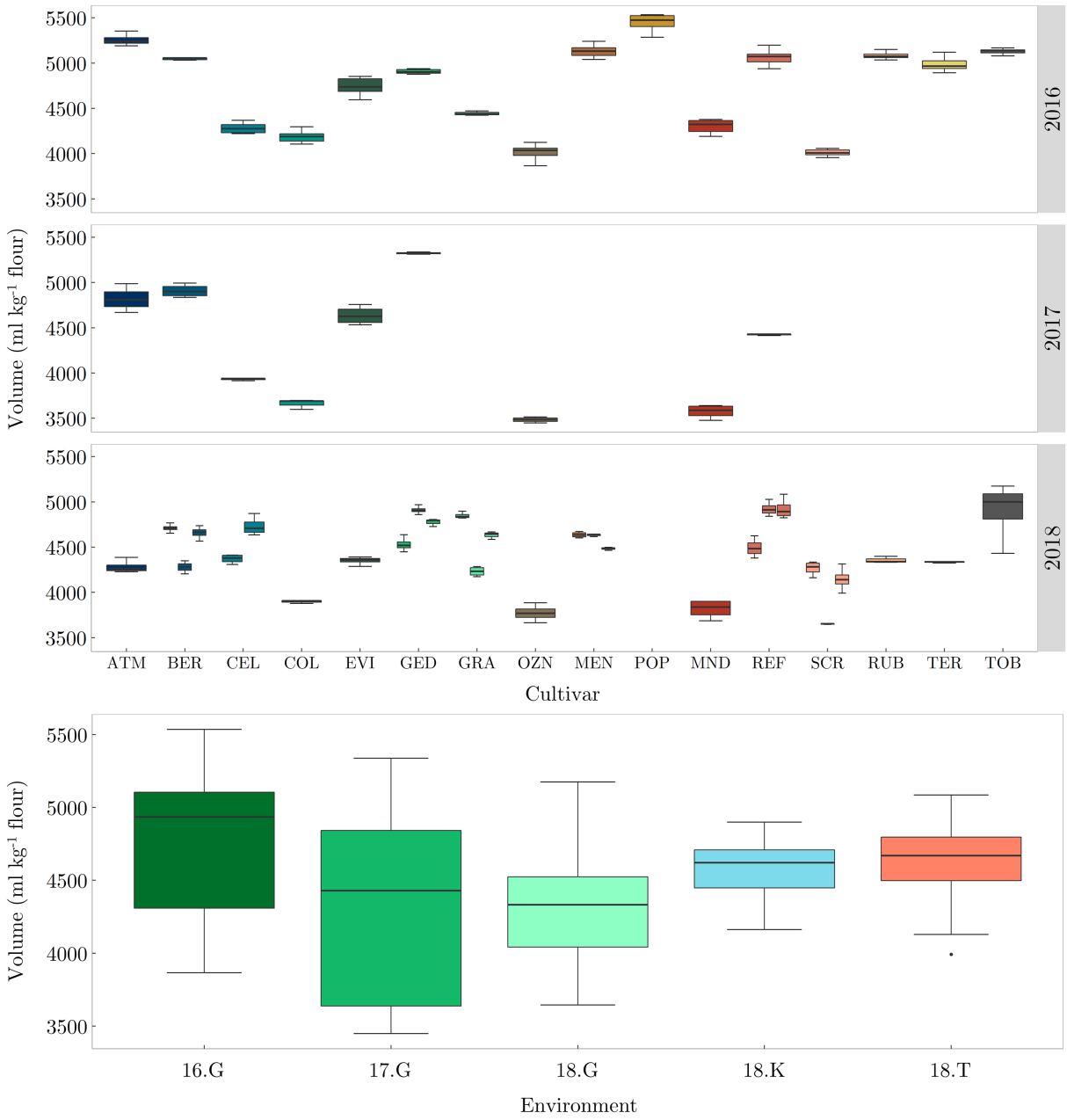

Figure 3.3: Top: loaf volume per kilogram flour (VOLfc) per year, genotype and location (for 2018, Koksijde, Ghent, Tongeren) determined on the pan breads. **Bottom**: VOLfc per environment.
Coding for the genotypes and environments can be found on page xii.

variables. However, relative OvnSs outside the range of 5–15 % show to be less correlated with the VOLfc. The low correlations between the height-to-width-ratio (HtWr) and any of the other bread quality attributes may be an indirect effect of the different bread type (plate bread) used for obtaining this measure. Despite wheat doughs which cannot retain their shape during baking will have a low HtWr, they can experience a high OvnS in case the dough is supported by the shape of the baking tin.

Although texture was measured on CP-breads, a strong negative correlation between VOLfc and HARD was observed ($p \leq 0.001$, $r = -0.796$). A limited number of breads did not fill the entire closed pan shape because of a limited proofing and/or reduced OvnS. Hence, it can be postulated that the volume effect was

not excluded. Nevertheless, despite the linear negative correlation, a clear distinction between two groups of samples (different hardnesses) at equal volumes was observed.

Table 3.4: Variation in bread quality attributes per environment and indication of the main influencing factors.

	Mean per environment[1]					Influencing factors[2]			
Quality attribute	*16.G*	*17.G*	*18.G*	*18.K*	*18.T*	$\mathbb{G}$	$\mathbb{E}$	$\mathbb{G}\times\mathbb{E}$	H^2 (%)
VOLfc (ml kg^{-1} flour)	4756 ± 466	4303 ± 641	4326 ± 411	4581 ± 197	4620 ± 258	***	***	***	49
OvnS (%)	-	6.70 ± 4.37	6.15 ± 4.33	9.40 ± 5.64	5.90 ± 3.59	***	***	***	42
HtWr	0.642 ± 0.044	0.631 ± 0.042	0.662 ± 0.054	0.641 ± 0.067	0.628 ± 0.065	**	n.s.	***	30
rGCarea (%)	17.1 ± 1.6	15.6 ± 1.4	16.0 ± 2.1	17.9 ± 2.1	17.9 ± 1.8	***	**	***	44
$\mathbf{L}_{crm}$	-	73.6 ± 2.9	73.5 ± 2.8	69.3 ± 2.5	68.8 ± 2.6	***	n.s	***	47
$\mathbf{a}^*_{crm}$	-	17.5 ± 1.6	17.1 ± 1.9	16.0 ± 1.2	17.1 ± 1.7	***	n.s	***	58
HARD (N)	0.074 ± 0.021	0.137 ± 0.062	0.149 ± 0.042	0.101 ± 0.022	0.103 ± 0.020	***	***	***	22
ΔHARD (%)	93.8 ± 16.5	79.4 ± 64.7	66.6 ± 16.8	63.3 ± 9.9	74.5 ± 11.2	-	-	-	-
RES	0.39 ± 0.04	0.35 ± 0.05	0.31 ± 0.04	0.33 ± 0.03	0.36 ± 0.03	***	n.s.	***	34
ΔRES (%)	-41.8 ± 3.5	-35.4 ± 5.9	-35.2 ± 4.1	-37.4 ± 2.8	-39.7 ± 2.9	-	-	-	-

[1] Empty fields indicate that no data was available for the this environment.
[2] In case only a single value per combination (genotype, environment) was available, no two-way ANOVA could be performed (indicated by '-'). As for all quality attributes a significant interaction effect is observed, significant differences between environments are not indicated.

As indicated in Table 3.4, a relatively high H^2 value was found for VOLfc and OvnS, crumb color (redness (a^*_{crm}) and lightness (L_{crm})) and the rGCarea. Color attributes were not significantly affected by environment although a highly significant interaction effect was observed. A similar finding was obtained for the HtWr although a lower H^2 also indicates that this parameter is environment dependent. As dough rheology is highly dependent on mixing intensity and other conditions during bread making, extra variation may have been introduced in this way. It can be seen from its direction in the loading plot that HARD and gas cell properties (rGCarea) are the main attributes to discriminate between environments which is in accordance with their lower H^2 of 22 % and 44 %.

Despite its relation with the VOLfc, crumb HARD showed to provide additional information on bread quality. Moreover, the relative increase in HARD (*i.e.* staling rate) over a 4-day period was found to be strongly influenced by the environment with almost a 100 % increase for 16.G whereas 18.G and 18.K showed only a 65 % increase. It can however be concluded that the VOLfc is a relevant indicator of bread quality as it majorly contributed to explaining the variability in the studied sample set. As this also remains one of the main challenges in industry, the remainder of the study will focus on developing a predictive model for VOLfc. Due to its high H^2 (49 %), models will be cross-validated for both genotype and environment.

> As the main research objective was to use the model output for obtaining an improved understanding of the driving factors in bread volume development, primary variables (compositional and functional attributes) should be chosen as input variables. Inclusion of secondary variables (yielding and kernel properties) which are strongly correlated to environment should be avoided as these will suppress the contribution of the primary variables.

3.3.1.2 Determinative factors for quality prediction

Partial least squares (PLS) regression was used as a modeling technique to predict the VOLfc. From this, it was attempted to further unravel which elements contribute to loaf volume development. Due to the

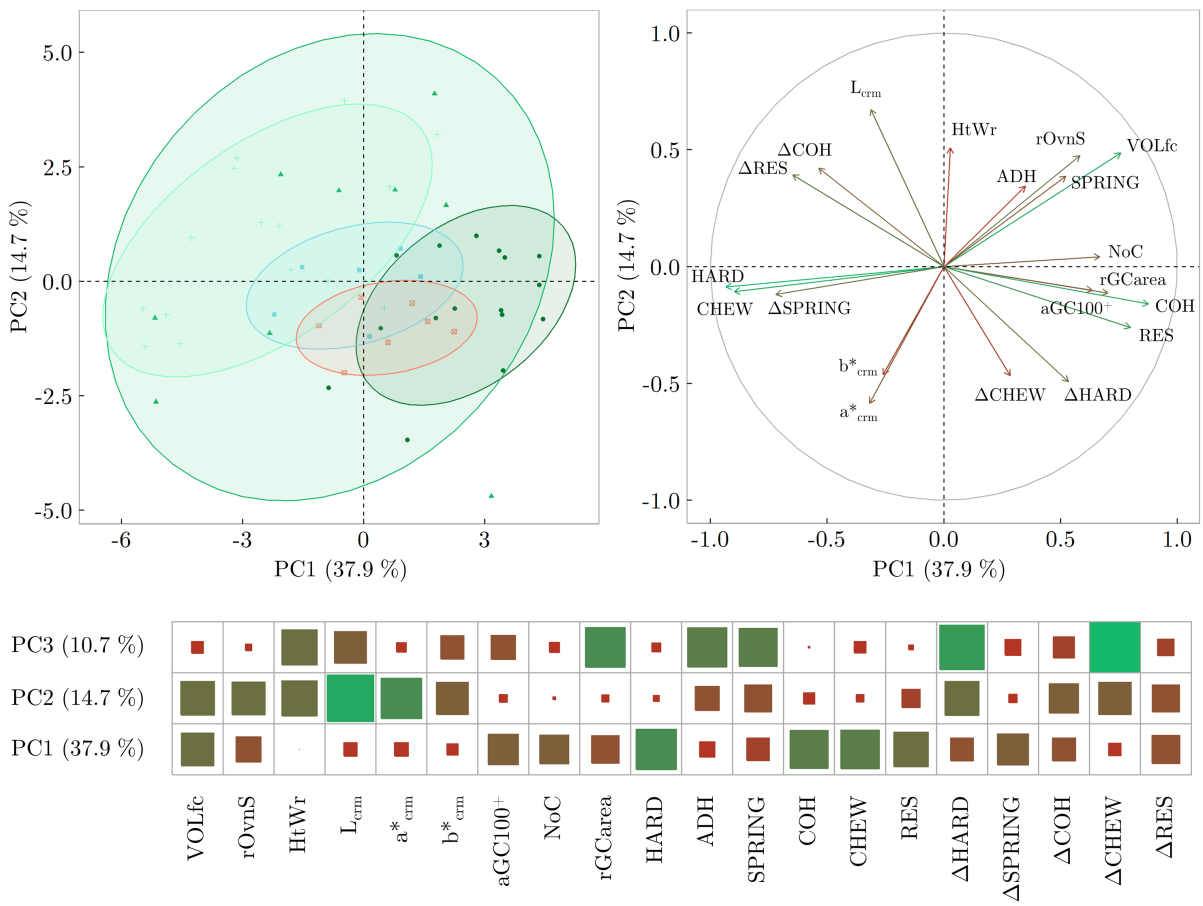

Figure 3.4: Top: score (left) and loading (right) plot from a principal component analysis using bread quality attributes. Samples are classified according to the environment. Two first principal components (PCs) together explain 52.6 % of the variability in the dataset. **Bottom**: correlation plot showing the contributions (red to green, low to high) of the variables to the first three PCs.
Coding for the genotypes and environments can be found on page xii.
Crumb lightness (L_{crm}); crumb chewiness (CHEW); crumb yellowness (b^*_{crm}); crumb redness (a^*_{crm}); absolute number of gas cells lager than 100 pix^2 (aGC100$^+$); number of gas cells (NoC); crumb adhesiveness (ADH).

high number of available parameters (177 in total), multiple sets of variables were predefined based on their common ground to introduce them in a stepwise manner. Simultaneously, PCA is performed on the same dataset to enhance data exploration, to understand the key variables in the data and to spot possible outliers.

A first model (**model 1**) was build using all compositional and functional attributes determined on wholemeal or flour. Fifty two samples covering 5 environments (16.G, 17.G, 18.G, 18.K and 18.T) were used as a training set. Cross-validation (CV) (regular, genotype and environment CV) was performed to obtain an estimate of the model performance. Figure 3.6**A** shows the score plot of the PCA which explained only 35.1 % of the variability in the dataset in the first two components. A clear distinction between environment 16.G and all other environments is noticeable. Furthermore, samples cultivated in Ghent are clustered together compared to environments 18.K and 18.T. Separation of 16.G was mainly obtained by a higher peak viscosity (PV) and breakdown (BD) whereas samples from 2017 and 2018 had increased peak temperature (PTemp) and increased values for final viscosity (FV) and holding strength (HS) resulting in an overall increased setback. Interestingly, samples in quadrant IV (mainly samples grown in Ghent during 2017 and 2018) also had a higher Gluten Index (GI) and, in contrast to

samples in quadrant II, a lower concentration weak gluten. In addition to the aforementioned variables, water absorption at 14 % moisture (WA_{14}) and the content of amylopectin (AMP) B_3^+-chains (degree of polymerization (DP) ≥ 36) were negatively correlated with pasting temperature (Tg).

Genotype-cross-validation ($CV_{\mathbb{G}}$) showed the ability of the obtained model to accurately predict the loaf volume of an unknown genotype using a 12-component model. The overlap of the ellipses in the score plot of the PCA already implied the inability of the data to distinguish between environments as was also seen from the increasing root mean squared error of prediction (RMSEP) when environment-cross-validation ($CV_{\mathbb{E}}$) is applied. As $CV_{\mathbb{G}}$ provides the most reliable performance indicator, this model is used. Mean absolute error (MAE) of model 1 was 281 ml kg^{-1} (6.3 %). However, the error rate drastically decreased for actual VOLfcs of 4000–4750 ml kg^{-1} flour (Figure 3.5**a**).

By applying variable importance in projection (VIP), noisy variables can be distinguished from the variables which significantly contribute to the predictive power of the model. Although the loadings of the former will be drastically reduced by PLS regression, introduction of noisy variables forces the loadings in their direction to be attenuated thereby causing inconsistencies (Chun and Keleş, 2010). To overcome this issue, a second model using only the variables with a relative VIP-score higher than or equal to the third quartile was composed. The 26 selected variables included all parameters of the Glutomatic analysis for both wholemeal and flour (except strong gluten), dough rheological attributes Farinograph dough development time (DDt), water absorption at 500 BU (WA_{500}), stability (STAB) and the Alveograph elasticity index (AL_{Ie}). Also pasting parameters HS and FV under enzyme-inhibited ($_{EI}$) conditions for flour and pasting temperature 1 (Tg1) for wholemeal (non enzyme-inhibited, $_{NEI}$) and F (both $_{EI}$ and $_{NEI}$) greatly contributed. Finally, protein composition of both wholemeal and F—more specifically the relative amount of glutelin—and granule size properties (D_{10} and the proportion of B-type granules) of wholemeal starch extracts aided in obtaining a robust and accurate prediction.

> Variable selection prior to partial least-squares regression on the basis of PCA is unfavorable as it is difficult to comprehend the high-dimensional relations between variables. PCA should only be used to provide insight in the variability in the data. Variable selection can possibly improve prediction performance (*i.e.* lower MAE) due the removal of noisy data. Optimally, this is nested in the cross-validation loop. The main difficulty however lies in setting the thresholds during this process as commonly, a gradual decrease in relative contribution is noticed.

A model on the basis of the selected variables (**model 1b**) performed significantly better (number of components (ncomp) = 6, $CV_{\mathbb{G}}$), as the MAE further decreased to 274 ml kg^{-1} flour (6.1 %). This improvement should however be nuanced as the variable selection was not nested in the CV-scheme. Optimally, this is done to prevent data leakage and to optimize the selection procedure. For example, a backwards selection strategy, each time removing 5 % of the remaining variables, can be used to overcome this issue (Rosenberg et al., 2010). Single selections, as performed in the current research, may possibly result in an overestimation of the performance increase obtained due to variable selection. Apart from the significance of the improvement, it can be questioned whether or not variables which are measured on both wholemeal and flour provided supplementary or complementary information. In an industrial setting, this would also require multiple operations and would make predictions even more depending on

the milling conditions. As shown by Bonfil and Posner (2012), milling may have had a major impact on GI through differences in kernel size and hardness. Therefore, it is investigated if wholemeal parameters can be substituted by their flour counterpart or, in case both occur in the list of variables, if the wholemeal variable can be removed (**model 2**). Results of the PCA and PLS regression are presented in graphs **B/b** in Figure 3.5. The obtained 20-parameter dataset (53 samples) provided poor separation between environments compared to the 26-parameter dataset. In addition, model performance decreased to a MAE of 320 ml kg^{-1} flour (7.1 %) with only a narrow range between 4000 and 4250 ml kg^{-1} flour for which good predictions were obtained. This implies that wholemeal-parameters provide additional information relevant for predicting VOLfc of flour based bread although, in essence, the same attribute is measured. A similar finding was postulated by Gélinas and McKinnon (2011), who observed a bias between wholemeal and flour measurements. These authors attributed their findings to the combined measurement of differences in gluten content and the water absorption of the remaining components. Sieving of the wholemeal further improved correlations with flour parameters as well as improvement of correlations with secondary parameters (DDt and WA_{500}) (Kulkarni et al., 1987).

Sorting of the remaining 26 variables of **model 1b** according to their relative importance showed the persistently high contribution of HS and FV for flour ($_{EI}$) and Glutomatic water binding to the wet gluten (WBwg), Glutomatic dry gluten (DG) and Glutomatic wet gluten content (WGC) measured on the wholemeal. The latter parameters may also provide information on the amount of water bound to (in)soluble fiber particles or non-starch polysaccharides present in the wholemeal, thereby being useful for improving predictions (Bonnand-Ducasse et al., 2010). The relation observed for the WBwg of wholemeal and flour was not linear outside the range of 13–16 % for wholemeal with a stagnation in the values for flour outside this range.

> Presumably, supplementary information is provided by Glutomatic measurements performed on wholemeal compared to the analysis results from flour as model performance decreases upon removal of these attributes. The water binding to remaining (in)soluble fibers or non-starch polysaccharides in the washed out gluten may lie at the basis of this effect.

In order to better understand the origin of the high contribution of Glutomatic parameters (measured on wholemeal), a new model (**model 3**) was constructed after removing variables of this technique from the dataset. The remaining 91 parameters could explain an almost equal amount of variation in the first two PC (36.8 %). In model 1, GI and the concentration of weak gluten allowed a distinction on the diagonal between quadrants IV and II. Hence, a less distinct separation between samples from 17.G and 18.G was obtained by using the variables in model 3. This is partially covered by the higher contribution of the softening (SOFT) and WA_{14}. However, mainly pasting parameters (BD, PV, PTemp and Tg) provide separation in the first two dimensions. A limited separation on the third PC (explaining an additional 8.5 % of the variability) was obtained by B-type granules (in starch extracts from wholemeal) and was negatively correlated with setback from trough (SBt) and FV.

Lowest RMSEP was obtained for $CV_{\mathbb{G}}$ at 11 components, resulting in a model with a MAE of 296 ml kg^{-1} flour (6.6 %). The comparison of actual versus predicted values in 3.5**C** shows the similar predictive power as model 1(**a**), although values above 5000 ml kg^{-1} flour (both positive and negative) are positioned

further away from the ideal prediction line. Interestingly, these 'outliers' comprise three samples from 'Gedser' (16.G, 17.G and 18.T). Main variables contributing to the model are dough rheological properties DDt, Alveograph configuration ratio (AL-PL) and the Farinograph quality number (possibly due to its close relation with DDt). Additionally, the relative concentrations of long, short and medium length amylopectin chains (based on high-performance size-exclusion chromatography (HP-SEC) profiles) were introduced as important parameters for quality prediction. Similar to the findings for model 1, HS, FV and Tg remained important. Only 4 parameters (Tg, protein concentration (PROC), Hagberg falling number (HFN) and the proportion of B-type granules) were coming from measurements performed on wholemeal.

The substitution of the Glutomatic parameters by Farinograph and Alveograph attributes may indicate various underlaying mechanisms valuable for loaf volume prediction. In all three techniques, mixing occurs thereby promoting gluten network formation in the presence of water. The behavior of the gluten network in terms of extensibility and elasticity may be enclosed in the selected variables. DDt is associated with stronger gluten and higher protein contents (Barak et al., 2013) whereas the configuration ratio (AL-PL) allows to discriminate between weak, soft, strong and stiff doughs as an optimum can be defined for this parameter (Fustier et al., 2009). Secondly, interaction between protein and starch (and other components present in te matrix), and structural changes taking place during mixing are measured (Schiedt et al., 2013). Although it is expected that all components other than gluten-forming proteins are washed out (Cesevičienė et al., 2012), small starch granules (C- and B-type granules) are more easily incorporated in the gluten network upon mixing (Park et al., 2009) and may thus contribute to the values obtained for water binding. Moreover, Noort et al. (2010) have clearly shown the negative influences of wheat fiber (constituents) on gluten network development. These will however also be incorporated by functional analysis.

Inclusion of HS and FV, as well as the AMP chain-length distribution (CLD), may also indicate the importance of viscosity in loaf volume development. On its turn, this may result from the hydration of starch molecules (in their granular form) and thus, water availability, or the formation of a starch(-protein) network (Kowittaya and Lumdubwong, 2014). A vast number of reports suggest that physicochemical properties of starch are determined by the AMP fine structure (Zhu et al., 2010; Kowittaya and Lumdubwong, 2014). However, literature on the direct relation between pasting parameters and bread quality is relatively scarce. This allows room for discussion on the modes of action contributing to loaf volume development exposed through pasting properties of flour in excess of water.

With the current modeling approach of PLS regression being a black-box simulation—contrary to a mechanistic model in which a combination of physical laws are used for making accurate predictions—it is questionable whether or not the model outcome allows any physical reasoning about the phenomena occurring during volume development (Kristiawan et al., 2016). In this chapter, it was attempted to partially overcome this shortcoming by the introduction of parameters in a stepwise fashion and by selectively removing variables on the basis of newly obtained insights.

3.3.1.3 Implementation in HPLC-technique

Building on the findings from Chapter 2, an attempt was made to improve predictions by incorporating data from the prolamin (PRO) and glutelin (GLU) fraction obtained through high-performance liquid

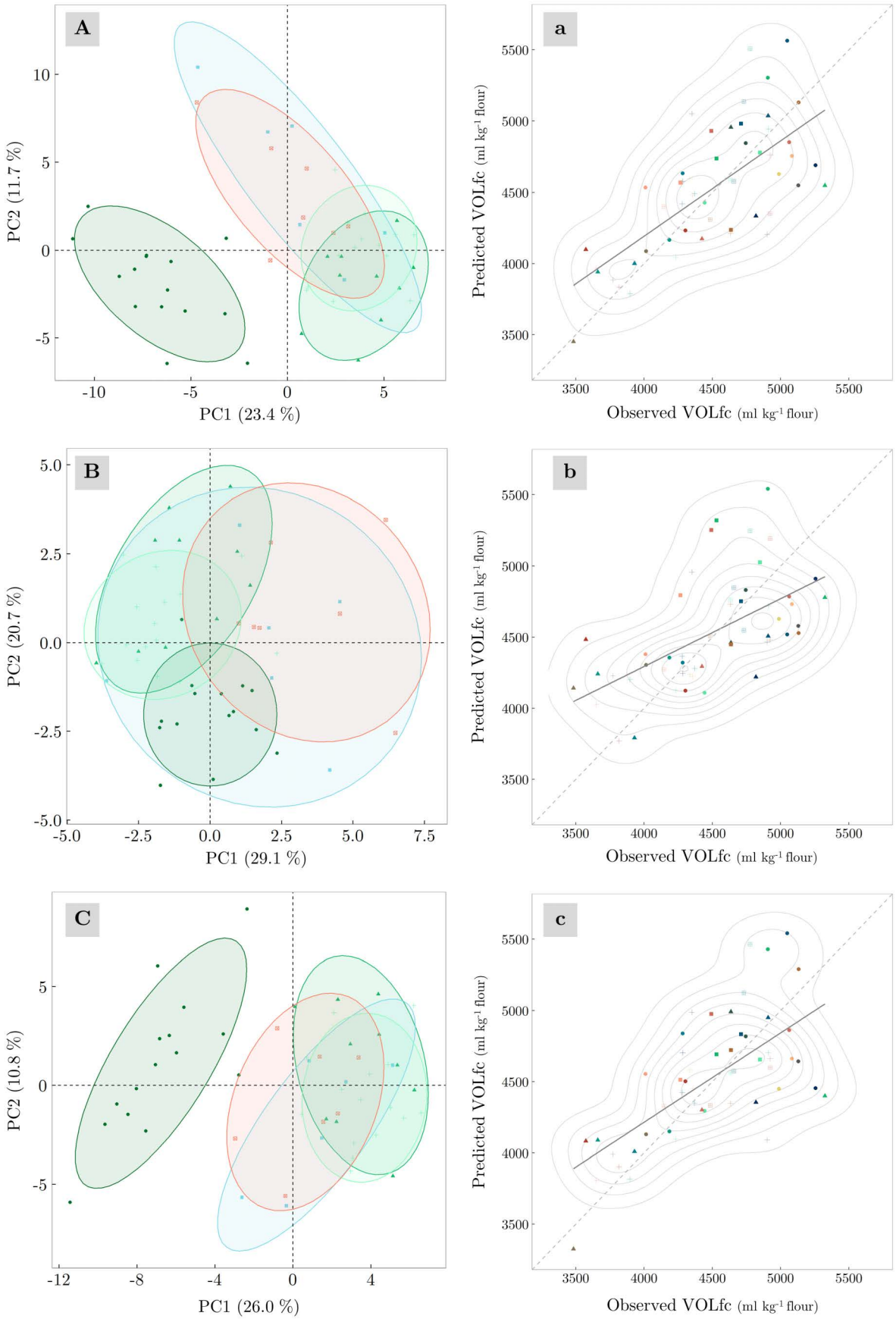

Figure 3.5: Left column: Score plots (two first principal components (PCs)) for the principal component analysis and **right column**: comparison between observed and predicted values of **A** model 1 (full), **B** model 2 (selected variables, flour) and **C** model 3 (full model, no Glutomatic data).
Coding for the environments and genotypes can be found on page xii.

chromatography (HPLC). In this way, it was investigated if the HPLC-technique could substitute other protein-related variables (while maintaining the predictive power) and if the combination with starch attributes would enhance its ability to accurately predict VOLfc.

> Area data from all protein fractions obtained through the RP-HPLC-approach has shown to be valuable for predicting both functional parameters of wheat flour dough (water absorption, dough rheology) and end-product quality attributes (loaf volume). Combined with the high resolution of the technique, it can be considered valuable for substituting multiple low-resolution techniques otherwise providing insight in the functional behavior of the flour.

A simplified overview of the variability present in the original data (not including chromatographic data) is presented in Figure 3.6. Although only 34.3 % is explained by the first two PCs, an almost complete distinction between environments was obtained. Samples grown during cultivation season 2015–2016 in Ghent (16.G) were clearly separated from those grown at virtually the same location in 2016–2017 and 2017–2018 (17.G and 18.G, respectively). Moreover, a closer relation between all samples grown during the last season could be observed by the overlap between the three environments (18.G, 18.K and 18.T). Also the introduction of a third PC, explaining an additional 8.0 % of the variability, did not separate samples from Koksijde and Tongeren.

From the loading plots, it can be observed that separation in the first two dimensions could be mainly attributed to the pasting properties (Tg, FV, setback), the test weight (TW) (yielding property) and the HFN. Despite viscosity lies at the basis of the latter measuring technique, no relation with PV was obtained implying that the variable provides information on the variation in the α-amylase activity. The overall and specific overall extraction rate (ER) (total, breaker rolls and reduction rolls) were most strongly correlated with the second PC, together with gluten properties (weak gluten content and GI) and flour particle size properties. This relation may be a result of differences in kernel hardness with a higher ER being accompanied by coarser particles. As the endosperm from hard kernels is separated easier from the outer layers, these may already be smaller than 180 µm after passing the first breaker roll (B_1). Subsequent milling steps (breaker rolls 2–3 and reduction rolls 1–3) are followed by a 150 µm sieve. Moreover, less endosperm will stick to the bran and shorts leading to a lower concentration of finisher flour when endosperm is harder and more easily to fracture (due to the harder endosperm).

Interestingly, the obtained separation using PCA on all original data corresponds well with the classification of the data when using HPLC-data for predicting the environment (Figure 2.14, page 84). This finding indicates the ability of the HPLC-data to distinguish the examined samples to a same extent as a combination of all variables. However, it can be expected that noisy variables are abundantly present in the PCA resulting in a diminished separation in the first two component. In addition, the fact that both datasets allow a similar separation does not guarantee an enhancement of predictions upon combining the HPLC-data.

A combined model was developed by merging the 23 variables from model 3 (Glutomatic properties excluded, variable selection applied) with the HPLC-data from the PRO (61 variables) and GLU (74 variables). This resulted in a total of 158 variables. The model outcome (**model 4**: ncomp = 3, $CV_{\mathbb{G}}$, MAE

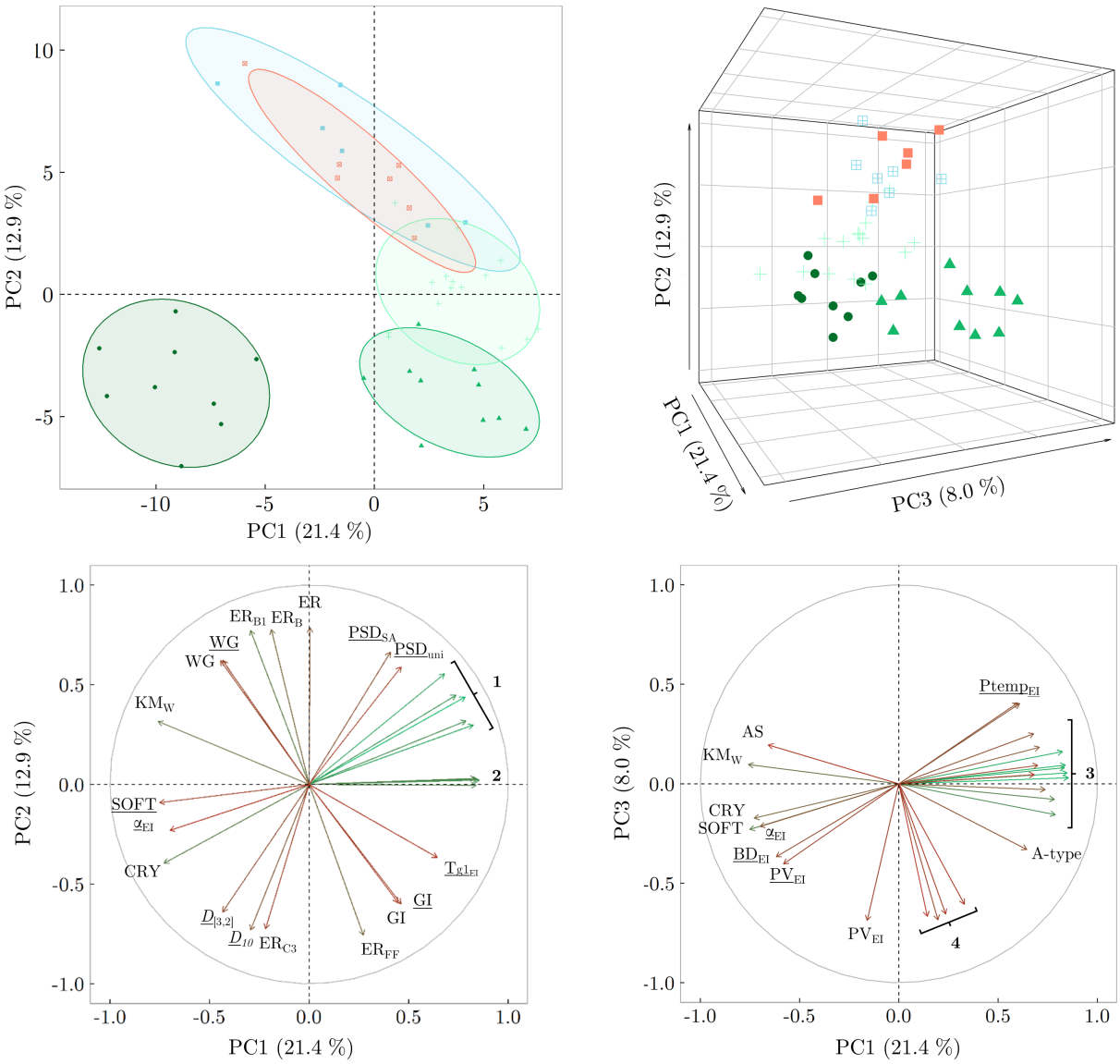

Figure 3.6: Top: score plots for the two or three first principal components (PCs) of the principal component analysis using yielding parameters, kernel morphology, flour particle size and extraction rates and compositional and functional attributes of protein and starch. **Bottom**: Accompanying loading plots (component 1+2 and 1+3) showing the first 20 % of the variables contributing most to the components.
Group 1: Tg_{EI} (WM), TW, HFN (F), SBp (F), HFN (WM). **Group 2**: FV_{NEI} (F), SBt_{NEI} (F) and β_{NEI} (F). **Group 3**: pasting parameters related to peak position (x and y), AMP CLD (medium), HFN and TW. **Group 4**: SBt, FV and HS (under enzyme inhibited conditions, WM), ER_{C1}.
Coding for the environments can be found on page xii.
Weak gluten (WG); relative crystallinity (CRY); surface mean ($D_{[3,2]}$); finisher flour (FF); particle size uniformity (PSD_{uni}); particle size surface area (PSD_{SA}).

$= 306$ ml kg^{-1} flour (6.8 %)) is not discussed as the increased MAE implies the inclusion of a high number of meaningless variables. Therefore, it was chosen to directly select the most contributing variables using VIP (**model 4**b). The remaining 40 variables did not allow to distinguish between environments in the first 2 PCs (explaining 24.1 % and 13.0 % of the variance respectively) of the PCA. Besides a selection of variables (*i.e.* peak areas) from both PRO and GLU, Farinograph parameters DDt and the quality number, the HS ($_{EI}$) and Tg (under $_{EI}$- and $_{NEI}$-conditions), the PROC and the AL-PL were retained. All selected

parameters were also determined on the flour in contrast to earlier results in which wholemeal parameters were preferred to flour properties.

When using the proposed ncomp on the basis of $CV_{\mathbb{G}}$ (ncomp = 3), model accuracy increased to a MAE of 262 ml kg^{-1} flour (5.8 %). As shown in Figure 3.7, satisfactory predictions were obtained for samples with a VOLfc $\geq$ 4000 ml kg^{-1} flour with two samples from 2017 ('KWS Ozon' and 'Atomic') and a single sample from 18.G ('Atomic') being gravely over- and underestimated, respectively. In contrast to all earlier approaches using solely the original data, a proper model with $CV_{\mathbb{E}}$ could be developed (ncomp = 1), although this was accompanied by a higher MAE of 295 ml kg^{-1} flour (6.6 %). Mainly samples with an actual VOLfc above 4500 ml kg^{-1} flour were markedly underestimated by the model.

Proposition

Conventional parameters currently used for estimating wheat quality were found not to be good predictors for bread quality. Dough rheological properties also provide a too narrow insight in baking quality and have to be combined in multivariate data analysis to obtain enhanced predictions. Mainly parameters related to dough development and the balance between extensibility and elasticity contributed most to the predictions. Inclusion of dough rheological attributes to a set of compositional starch and protein-related variables drastically improves the power of the model in contrast to using solely compositional attributes.
Water binding appears to be highly relevant as Glutomatic waterbinding to the wet gluten was consistently ranked high. Remarkably, the parameters of wholemeal and flour provided supplementary information implying that not only the water binding of the gluten protein was relevant. Inclusion of starch properties also improved model accurateness, mainly when including information on the pasting behavior of the flour (holding strength, final viscosity and pasting temperature) and fine structural properties of amylopectin. Also granular properties (proportion of B-type starch granules) were found to contribute, although this was to a lesser extent.
Inclusion of high-resolution chromatographic data further improved the accurateness of the model, thereby suppressing the importance of properties obtained from other conventional protein-related techniques. Remarkably, starch properties remained equally important implying their relevance in predicting the bread loaf volume.

3.3.2 Variation in determining properties

The empirical models discussed in the previous sections allowed for the selection of the main variables involved in bread volume development. An overview of the observed variation in function of the environments for these parameters and other commonly determined properties is presented in Table 3.5. By identifying the factors which mainly contribute to fluctuations (*i.e.* $\mathbb{G}$ or $\mathbb{E}$), an improved understanding of how to steer bread quality through cultivation can be achieved.

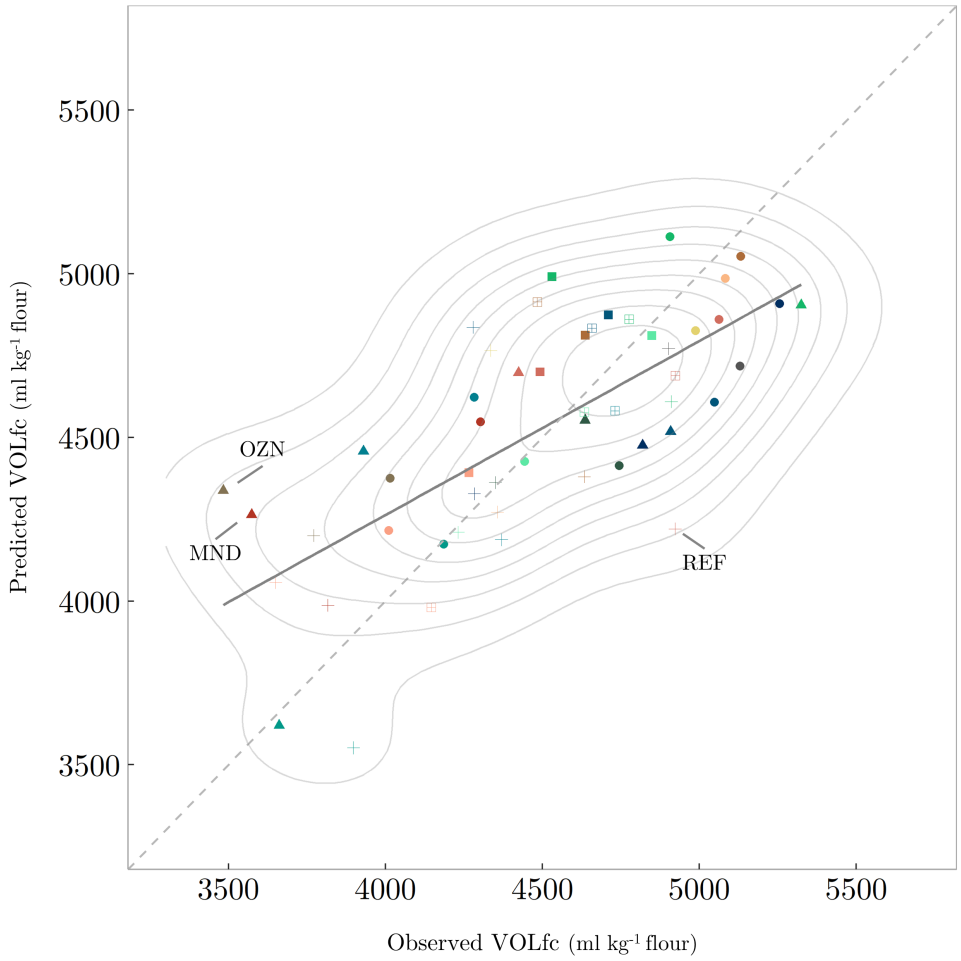

Figure 3.7: Comparison between actual and predicted values of a partial least squares regression model comprising 40 variables (remaining selection based on 75th quartile of highest ranked variables) with 3 components (optimal number obtained through genotype cross-validation). Dashed line illustrates ideal prediction, solid gray line shows the linear correlation between samples, thin light gray polygons indicate the density of the number of observations. Coding for the environments can be found on page xii.

3.3.2.1 Protein content

PROC of the and the flour is one of the most established compositional attributes during wheat quality screening due to its (limited) relation with VOLfc. Hence, it is used for price setting in the wheat market. From the current results, solely an $\mathbb{E}$-effect could be noted with a consequently higher PROC for samples cultivated in Tongeren ($F(8,153) = 62.18$, $p \leq 0.001$). PROC of wheat grown in Koksijde was markedly lower in 2016 and 2017 whereas a similarly high content was obtained for samples harvested in 2018. A possible explanation may be found in the prevailing cultivation conditions, more specifically in the temperature during grain filling (Nuttall et al., 2017), as temperatures were consistently higher for Tongeren during June and July. However, also in Ghent, temperatures were equally high during this period. However, application of the 3rd N-fraction was performed generally later which may have impacted the N-uptake.

Across environments, no significant differences between genotypes were found ($F(15,146) = 0.94$, $p = 0.526$) with an absolute difference of 1.32 % between 'RGT Reform' and 'Tobak' which had the respectively highest and lowest PROC (12.34 $\pm$ 1.19 % and 11.02 $\pm$ 0.37 %). In an industrial setting, the

observed difference would however result in a lower classification of the wheat (depending on the used grading system) and thus, a lower price.

For the PROC of the flour, also no significant $\mathbb{G}$-effect could be noted ($F(15,42) = 0.53$, $p = 0.912$) whereas the variation between environments was found to be similar to the results for wholemeal flour ($F(4,53) = 33.87$, $p \leq 0.001$). Within 2018, wheat cultivated in Ghent had a significantly lower PROC (9.61 $\pm$ 0.51 %) compared to samples grown in Koksijde (11.05 $\pm$ 0.72 %) or Tongeren (11.52 $\pm$ 0.64 %). Interestingly, the absolute protein loss during flour extraction was around zero percent (-0.52–0.25 %) for samples from 16.G which was significantly lower than averages for other environments (1.24–1.65 %). This may be related to higher ERs resulting from a maintenance of the mill. As the spatial distribution of proteins in the wheat kernel is characterized by an increase towards the outer layers of the kernel, higher ERs will also yield higher PROC.

3.3.2.2 Starch composition

Although amylose (AM) concentration was not included in the model, it is worth mentioning as literature on the influence of $\mathbb{E}$ on the concentration in wheat (flour) is scarce. As the sample set only comprised of conventional wheat cultivars (in terms of the starch genes), a relative low H^2 of 23 % was found. However, significant differences between genotypes were observed ($F(14,112) = 2.84$, $p = 0.001$) with 'Atomic' and 'Evina' having a significantly higher AM concentration in starch (22.6 $\pm$ 3.3 % and 22.0 $\pm$ 3.0 %) compared to all other cultivars (17.1–20.8 %). 'RGT Sacramento' had a considerably lower AM concentration of 15.7 $\pm$ 3.0 %. Although a significant $\mathbb{E}$-effect was absent ($F(2,112) = 2.31$, $p = 0.104$), a highly significant $\mathbb{G} \times \mathbb{E}$-interaction was observed ($F(39,112) = 6.43$, $p \leq 0.001$) explaining 51 % of the variance.

In contrast to the AM concentration, the relative concentration of short AMP chains (fourth peak in HP-SEC chromatograms) was found to decidedly contribute to the prediction of VOLfc. All effects were also significant with $\mathbb{G} \times \mathbb{E}$ explaining 39 % of the variance. $\mathbb{G}$ and $\mathbb{E}$ apart contributed to a similar extent indicating an equal contribution of both factors. Hence, it can be postulated that this attribute is very dependent on both prevailing cultivation conditions ($F(2,102) = 8.54$, $p \leq 0.001$) and the genotype ($F(14,102) = 8.80$, $p \leq 0.001$). The average concentration of short chains was $\approx$ 39 % except for 18.K which contained significantly less sort chains (32 %). It is noteworthy that the data obtained through high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) did not confirm this finding. No significant differences in the proportion of A-type chains (DP 5–12) between environments was noted.

> Amylose content showed no direct relation with bread quality (*i.e.* loaf volume) whereas the relative concentration of short amylopectin chains (on the basis of size-exclusion profiles) was selected as contributing parameter in various partial least-squared regression models.

3.3.2.3 Damaged starch content

The damaged starch content in wheat flour is considered determinative in loaf volume development as it provides directly accessible glucose for the yeast to convert to CO_2. Besides kernel hardness and morphology (plumpness, circularity), milling properties may influence the content. As the latter was kept constant during this research, the variation observed for this parameter can be solely attributed to the main effects. A virtually equal proportion was explained by $\mathbb{G}$ (42 %) and $\mathbb{E}$ (39 %). Besides the main effects, a highly significant interaction term was found ($F(37,95) = 43.75$, $p \leq 0.001$). When considering solely the main effects, 'KWS Ozon' had a significantly higher damaged starch content of 7.5 ± 0.2 %. Significant differences were also observed between other cultivars ($F(15,95) = 95.37$, $p \leq 0.001$) with values ranging from 5.7 ± 0.2 % to 6.7 ± 0.5 %. The observed range corresponds with the values recommended for breadmaking. Environment also significantly impacted the concentration with values for Ghent (6.2–6.5 %) being significantly higher ($F(2,95) = 87.90$, $p \leq 0.001$) compared to samples from 18.T (6.1 ± 0.3 %) or 18.K (5.9 ± 0.4 %).

3.3.2.4 Hagberg falling number

HFN of both wholemeal and flour were mainly influenced by $\mathbb{E}$ with respectively 67 and 54 % of the variability being explained by this factor. A significantly lower HFN ($\leq$ 269 s) was found for samples grown in 2016 ($F(4,472) = 174.90$, $p \leq 0.001$) with an even lower value for Tongeren (148 ± 53 s). Considerable differences between cultivars over multiple locations were also observed ($F(15,472) = 67.48$, $p \leq 0.001$) with values ranging from 378 ± 51 to 214 ± 58 for 'Mentor' and 'Popeye' respectively. Furthermore, the expected decrease in HFN related to flour extraction (and removal of the enzyme-rich outer layers as well as fibers) was also significantly differing per environment with a 5.0 % decrease for 16.G compared to a significantly higher decrease for 17.G (21.6 %) and 18.G (10.7 %). Despite higher ERs for 2016 samples, less α-amylases appeared to be incorporated in the flour. Values range from 392 ± 39 for 'RGT Reform' to 299 ± 59 for 'Collector'. 'Popeye' was however characterized by a significantly lower ($F(15,95) = 102.61$, $p \leq 0.001$) HFN of 187 ± 7. $\mathbb{E}$-effect and the interaction term also remained significant with an similar H^2 of 26 %.

As the HFN is a viscosity based technique, its applicability to measure the α-amylase activity in a $\mathbb{G} \times \mathbb{E}$-study can be argued as the difference is related to compositional changes which also impact the viscosity of starch pastes. Despite only weak correlations with compositional attributes (relative crystallinity, AMP CLD, and starch granule size distribution) were found, the HFN of flour was negatively correlated ($r = -0.536$, $p \leq 0.001$) with the concentration of C-type ($\leq$ 5 µm) starch granules. This correlation was however not observed for wholemeal. Additionally, setback from peak (SBp) was found to be majorly affected by the α-amylase activity for measurements conducted in distilled water. A linear relation ($r = 0.840$, $p \leq 0.001$) was noted between HFNs of 200–500 s and SBps of -1000–1500 mPa s.

3.3.2.5 Zeleny sedimentation value

As the Zeleny sedimentation value (ZEL) is assumed to provide insight into the water binding capacity of the gluten and the total amount of gluten-forming proteins present in the sample, it is frequently

used in industry for the rapid quality screening of wheat flour. With values ranging from 25 $\pm$ 3 for 'Gedser' to 46 $\pm$ 5 for 'Evina', a main $\mathbb{G}$-effect is present ($F(15,42) = 709.41$, $p \leq 0.001$, $H^2 = 62\,\%$). In addition, two-way ANOVA indicated the presence of a significant $\mathbb{E}$ ($F(2,42) = 166.48$, $p \leq 0.001$) and $\mathbb{G} \times \mathbb{E}$-effect ($F(38,42) = 261.81$, $p \leq 0.001$) although significant differences between the tested environments were absent due to the high standard deviation resulting from the main genotypic variation. In general, samples from 2018 have a higher value with exception of 18.K for which comparable values as for 17.G were obtained.

As the measure allows one to distinguish between genotypes, it is mistakingly postulated to be correlated with baking quality as this is also genotype dependent and affected by gluten properties. However, samples showing the highest VOLfc (*e.g.* 'Gedser') had the lowest value for ZEL whereas for 'KWS Ozon', the contrary was observed. Interestingly, a strong correlation with the Alveograph W-value was found ($r = 0.805$, $p \leq 0.001$) implying a relationship with dough rheological properties.

> Conventional rapid screening techniques such as the Hagberg falling number and the Zeleny sedimentation value both allow to discriminate between genotypes and are characterized by a high heritability. However, no direct correlation with baking quality could be established supporting their invalid use for rapid grain quality screening. Due to the indirect measurement (viscosity and water binding capacity, respectively) of the traits of interest (α-amylase activity and gluten quality), their future applicability should be brought under discussion.

3.3.2.6 Gluten quality (Glutomatic)

Another commonly used technique for quality screening is the Glutomatic analysis (Perten Instruments, Hägersten, Sweden) in which gluten-forming proteins are separated from starch and water extractable proteins after which they are separated and dried. In this way, information on the quantity and water binding capacity of the gluten is obtained. During model development, it was noticed, that measurements on wholemeal and flour (while theoretically measuring the same properties of the gluten) provided supplementary information. Moreover, analysis of variance indicated significant effects for all factors for wholemeal measurements whereas no significant effect for $\mathbb{G}$, $\mathbb{E}$ or their mutual interaction was found for flour measurements.

WGC and the therefrom calculated WBwg of wholemeal was varying between both environments ($F(4,401) = 21.19$, $p \leq 0.001$) and genotypes ($F(15,401) = 15.21$, $p \leq 0.001$) with 'Gedser' containing a significantly higher amount of gluten and/or bound water. In contrast, 'RGT Mondio' and 'KWS Ozon' contained significantly less gluten or could bind less water. In addition, the DG-content was found to be a highly contributing factor during modeling with a similar ranking order as obtained for variables WGC and WBwg. This may imply that not the water binding capacity but rather the gluten *content* is differing between genotypes, assuming the total evaporation of water during the four minute drying period. Environmental influence was consistent for all Glutomatic parameters measured on wholemeal with 17.G having a significantly lower gluten 'quality' compared to all other environments. In addition, wheat cultivars grown in Koksijde and Tongeren during 2018 had a slightly higher concentration of gluten although this might also be related to the genotypes enclosed in the set.

When comparing WGC and WBwg of flour and wholemeal, some shifts in the order of the genotypes could be noticed. 'Graham', which had the 2nd lowest WGC for wholemeal, was ranked 6th for flour. A similar improvement was observed for 'RGT Sacramento' and 'Popeye', whereas 'Tobak' had a significantly lower WGC in flour compared to its wholemeal counterpart. This also resulted in a lack of significant correlations between flour and wholemeal parameters. In contrast, gluten retained on the sieve (*i.e.* strong gluten) were correlated with the Alveograph baking strength (AL-W) as well as the GLU content as obtained through the HPLC-analysis.

> Lacking correlations between measurements on wholemeal and flour using the Glutomatic apparatus imply the inclusion of secondary attributes or an increased measurement error. Despite its main contribution in predicting the loaf volume (not as a single parameter however), execution of the technique is prone to error and cannot be applied to all flour types.

3.3.2.7 Flour pasting properties

Besides protein and starch content and composition, the variation in the functional attributes related to cultivation conditions may provide additional insight into underlying mechanisms for bread quality. Both HS and FV of flour, measured under enzyme-inhibited conditions, received high VIP-scores in various models. As only a single measurement per sample was performed, one-way ANOVA was used to study the effects of the main factors. A marginally significant $\mathbb{G}$-effect ($F(14,40) = 1.96$, $p = 0.049$) was found for the HS with post-hoc analysis showing a significantly higher value for 'RGT Reform' (2097 $\pm$ 85 mPa s) whereas 'Graham' had the lowest value (1690 $\pm$ 275 mPa s). A more prominent variation was observed between environments ($F(4,50) = 3.58$, $p = 0.012$) with 16.G having a significantly higher HS compared to all other environments (2047 $\pm$ 113 mPa s *versus* 1846–1949 mPa s).

Opposed to the numerous correlations between pasting measurements, strong significant correlations with compositional attributes were scarce. SOFT and HFN were related with PTemp ($_{NEI}$) (r = -0.700, r = 0.816, p $\leq$ 0.001) whereas pasting rate (α) was significantly correlated (p $\leq$ 0.000) with the relative crystallinity of the flour (r = 0.780), PROC (r = 0.635) and the ratio of HMW-GS to LMW-GS (HMW:LMWr) (r = 0.719).

Although pasting behavior is mainly attributed to starch properties, an influence of protein content and composition has been postulated by various researchers. However, current results contradict this as an increased PROC or a higher HMW:LMWr positively influenced α thereby implying an increased granule swelling. Differences in the granule architecture, the AMP CLD or the presence of amylose-lipid complexes may impact this relation. In addition, a negative correlation between granule relative crystallinity and SBp (r = -0.760, p $\leq$ 0.05) was observed which may be attributed to an increased AMP concentration. However, no direct correlation with the latter variables was observed as some samples showed an increased relative crystallinity ($\geq$ 15 %) at equal concentrations of AMP.

> Pasting properties were mainly influenced by the cultivation environment and showed to be influenced by both starch attributes (crystallinity) and protein properties (protein content). Although this

Table 3.5: Variation in compositional and functional attributes in function of the environment and genotype (H^2).

	Mean per environment[1]									Influencing factors			
Quality attribute	*16.G*	*16.K*	*16.T*	*17.G*	*17.K*	*17.T*	*18.G*	*18.K*	*18.T*	$\mathbb{G}$	$\mathbb{E}$	$\mathbb{G}\times\mathbb{E}^2$	H^2 (%)
PROC$_{WM}$ (%)	11.19 ± 0.51	10.95 ± 0.45	13.29 ± 0.58	11.84 ± 0.28	11.03 ± 0.30	12.78 ± 0.44	11.19 ± 0.44	12.16 ± 0.63	12.44 ± 0.38	n.s.	***	n.s.	6
PROC$_{F}$ (%)	11.65 ± 0.42	-	-	10.28 ± 0.61	-	-	9.61 ± 0.51	11.05 ± 0.72	11.52 ± 0.64	n.s.	***	-	-
DS$_{F}$ (%)	6.2 ± 0.5	-	-	6.5 ± 0.6	-	-	6.3 ± 0.5	5.9 ± 0.4	6.1 ± 0.3	***	***	***	42
AM (%)	19.4 ± 3.8	-	-	21.6 ± 4.5	-	-	18.7 ± 3.9	15.9 ± 4.4	17.4 ± 3.1	**	n.s.	***	23
AMP SC$_{SEC}$ (%)	38.9 ± 3.8	-	-	39.2 ± 3.3	-	-	39.6 ± 4.3	32.9 ± 6.1	38.4 ± 5.5	***	***	***	29
HFN$_{WM}$ (s)	269 ± 52	269 ± 74	148 ± 53	436 ± 49	356 ± 50	384 ± 65	430 ± 38	436 ± 20	-	***	***	***	26
HFN$_{F}$ (s)	255 ± 43	-	-	348 ± 33	-	-	380 ± 25	384 ± 22	367 ± 34	***	***	***	30
ZEL$_{F}$ (ml)	33 ± 8	-	-	35 ± 7	-	-	39 ± 7	34 ± 7	36 ± 8	***	***	***	62
WBwg$_{WM}$ (%)	14.4 ± 1.8	14.4 ± 1.3	14.9 ± 2.3	10.1 ± 3.7	14.2 ± 1.0	0.0 ± 0.0	14.2 ± 1.8	15.9 ± 1.7	16.0 ± 1.4	***	***	***	33
WBwg$_{F}$ (%)	15.2 ± 1.6	0.0 ± 0.0	0.0 ± 0.0	14.9 ± 1.8	0.0 ± 0.0	0.0 ± 0.0	13.8 ± 1.6	15.8 ± 3.2	17.8 ± 1.8	n.s.	n.s.	n.s.	18
WGC$_{WM}$ (%)	21.3 ± 2.6	21.3 ± 1.7	22.0 ± 3.3	15.7 ± 5.4	2.9 ± 1.3	0.0 ± 0.0	21.0 ± 2.3	23.9 ± 2.4	24.0 ± 1.9	***	***	***	34
WGC$_{F}$ (%)	22.8 ± 2.3	0.0 ± 0.0	0.0 ± 0.0	22.4 ± 2.4	0.0 ± 0.0	0.0 ± 0.0	21.0 ± 2.7	23.9 ± 4.7	26.8 ± 3.1	n.s.	n.s.	n.s.	18
DG$_{WM}$ (%)	6.9 ± 0.9	6.8 ± 0.5	7.0 ± 1.0	5.4 ± 1.7	6.7 ± 0.4	0.0 ± 0.0	6.7 ± 0.7	7.8 ± 0.8	7.9 ± 0.6	***	***	***	37
HS$_{EI.F}$ (*mPa.s*)	2047 ± 113	-	-	1842 ± 264	-	-	1875 ± 125	1949 ± 122	1846 ± 140	*	*	-	-
Tg1$_{EI.F}$ (°C)	58.6 ± 0.6	-	-	60.6 ± 0.6	-	-	59.8 ± 0.5	59.1 ± 0.6	58.4 ± 0.4	n.s.	***	-	-
Tg1$_{NEI.F}$ (°C)	58.0 ± 1.6	-	-	60.3 ± 0.6	-	-	59.4 ± 0.6	58.7 ± 0.5	58.3 ± 0.3	n.s.	***	-	-
DDt (min)	1.61 ± 0.23	-	-	2.02 ± 0.55	-	-	1.77 ± 0.29	2.38 ± 0.58	2.26 ± 0.61	n.s.	***	-	-
WA$_{500}$ (%)	59.0 ± 2.5	-	-	57.0 ± 2.7	-	-	56.9 ± 2.6	59.5 ± 2.5	58.5 ± 1.9	***	n.s.	-	-
AL-PL	2.95 ± 1.73	-	-	1.83 ± 1.06	-	-	1.38 ± 0.87	1.48 ± 0.53	1.06 ± 0.29	***	***	***	59

[1] Empty fields indicate that no data was available for the this environment.
[2] In case only a single value per combination (genotype, environment) was available, no two-way ANOVA could be performed resulting in no significance for the interaction effect and the H^2.

measurement is fundamental (not model-system based), it persistently contributed to the prediction of loaf volume. In this way, the importance of starch swelling and the impact of its components on starch-gluten interaction (network development) and viscosity is conceived.

3.3.2.8 Dough rheology

Dough rheological attributes have been proposed as good measures for estimating baking quality of wheat, as the volume increase during baking is highly dependent on the elasticity and extensibility of the dough. However, performing such measurements requires that the wheat kernels be cleaned and milled and the flour to be left for multiple weeks (4–8 weeks) to obtain representative measurements. Nevertheless, they provide information on the interactions between starch and protein taking place during dough development which cannot be accurately predicted using the current limited set (in terms of samples and variables) of compositional attributes. Hence, inclusion of DDt, WA_{500} and AL-PL provided crucial information to accurately predict the VOLfc.

No significant differences between $\mathbb{G}$ were observed for DDt ($F(15,38) = 0.85$, $p = 0.624$) with a narrow range from 1.4 ± 0.4 to 2.3 ± 0.6 min. In contrast, significantly higher values were found for samples from Koksijde and Tongeren (2018) compared to Ghent (2016, 2017 and 2018) which required a markedly shorter time to develop a gluten network ($F(4,49) = 5.95$, $p \leq 0.001$). Although a major effect of gluten composition is expected (Barak et al., 2013), no relation with any of the protein-related parameters could be established (r-values < 0.500).

Opposite to results for DDt, WA_{500} was significantly influenced by $\mathbb{G}$ ($F(15,38) = 4.18$, $p \leq 0.001$) whereas no significant differences between $\mathbb{E}$ could be noted ($F(4,49) = 2.48$, $p = 0.056$). Water absorption of flour is, analogous to the ZEL, an important quality parameter of wheat flour. Higher absorption rates are desired as it is expected to translate in stronger gluten. Moreover, a high stability of this trait over harvest years is preferred as fluctuations require the (industrial) production process to be adapted. In this light, the absence of a significant environmental effect is positive. However, an absolute decrease in the average WA_{500} of 2.0 % (16.G *vs.* 17.G and 18.G) would be considered detrimental in industry. 'KWS Ozon', for which earlier the lowest values for WBwg and WGC were reported, showed a significantly higher WA_{500} (61.9 %) whereas flour from 'Graham' and 'Rubisko' absorbed significantly less water (55.3 and 54.8 % respectively).

AL-PL was mainly influenced by $\mathbb{G}$ ($F(15,159) = 31.20$, $p \leq 0.001$) indicated by a relatively high H^2 of 59 %. Significant differences between all genotypes were noted with 'KWS Ozon' displaying again the highest ratio (4.48 ± 1.50), indicating a stiff and less extensible dough. This relation is however not directly relatable to VOLfc. For example: 'Gedser' and 'RGT Reform' had a consistently high VOLfcs over all environments while showing a low AL-PL. 'Atomic', on the other hand was ranked 3rd highest (2.45 ± 0.60) with equally high loaf volumes. From this result, it can be noted that a minimal ratio however had to be obtained before dough rheology was affected as AL-PL-values remained constant (≈ 1.00) below a HMW:LMWr of 0.50. At higher ratios, a significant linear correlation with the HMW:LMWr was noted ($r = 0.704$, $p \leq 0.001$). Both findings indicate the importance of protein composition as well as the interaction with other components in the flour for dough rheology and eventually, the loaf volume development.

Dough rheological attributes remain of crucial importance for predicting baking quality as it provides insight in the interaction between components present in the wheat flour. The latter cannot be captured or estimated using small datasets of compositional data. In contrast, the methods are labor and time intensive and require flour as starting material making them unfavorable for rapid quality screening. Moreover, they are no good predictors on their own and have to be supplemented with other (compositional) attributes.

3.4 Conclusion

The current study intended to develop a model for wheat bread quality prediction using an optimized number of variables, thereby focusing on gaining knowledge on their contribution and the underlying mechanisms they represent. Moreover, their variability in function of environment and genotype was investigated. In this way, the feasibility and effectiveness of different strategies to steer wheat quality could be estimated.

The use of conventional quality parameters—including the protein content, Zeleny sedimentation value, Glutomatic parameters and dough rheological attributes—were not correlated with the loaf volume (corrected for flour weight), the main quality attribute of bread, when considered separately. Strong one-to-one correlations were lacking, presumably due to the multi-dimensional interaction effects which largely interfere with these relations. However, no sufficient prediction of the loaf volume of 52 wheat cultivars could be obtained, even when considering the parameters in a combined fashion. Using partial least-squares regression, a predictive model with a mean absolute error of 9.8 % was obtained. This finding implies that the application of advanced statistical techniques in combination with poor, low-resolution data does not allow an accurate and robust prediction of complex attributes as loaf volume.

By stepwise evaluation of the variables introduced in the models, insight in the relevant parameters was obtained. Predictive power was lowered upon removing wholemeal parameters or substituting them with their flour counterparts. Therefrom, it was postulated that wholemeal measurements provided supplementary information, especially for Glutomatic properties. This might be related to the water absorption of fibers, non-starch polysaccharides or starch. The model using solely compositional attributes related to both starch and protein, had a significantly diminished accurateness compared to a model which also included dough rheological properties. Farinograph dough development time and water absorption were selected together with the Alveograph configuration ratio (dough rheological attribute) as most contributing variables. It can be postulated that, when using a limited dataset (in terms of samples) with a relatively narrow variance in its chemical composition, functionality cannot be apprehended. Hence, functional properties were selected together with compositional properties to obtain accurate predictions.

Alternatively, implementation of high-resolution chromatographic data as discussed in Chapter 2 significantly improved predictions when being combined with a selection of compositional and functional variables from earlier models (mean absolute error of 5.8 %). Even when combining this data with solely compositional attributes (no functional properties), prediction improved compared to the model without data on the *protein* functionality. These findings supports that the HPLC-based approach supplies supplementary information, partially covering functional attributes, which can be gathered at a greater

ease compared to the conventionally used techniques. However, this demands for the employment of advanced modeling techniques in industry for quality screening. In contrast to the currently used single or multiple linear regression models, these may provide enhanced and more robust (through cross-validation) predictions. Moreover, their ability to deal with a large number of variables while reducing the possibly detrimental effects of noisy data supports their use. From a research point of view, this also allows for a more thorough selection of variables to capture the complex driving mechanisms behind the variable of interest (end-product quality attribute).

For both bread quality and determinative variables, the contribution of the main factors, environment ($\mathbb{E}$) and genotype ($\mathbb{G}$) was studied. Most compositional attributes had a broad-sense heritability around 30 % implying the ability to manipulate bread quality by both genotype selection and application of specific cultivation conditions. In contrast, environment is the main determinant in the functionality (pasting behavior and dough rheology) of wheat flour. Due the wide diversity in cultivation conditions between environments in terms of controllable (*e.g.* fertilization) and uncontrollable (*e.g.* soil, meteorology) variables, it was unfortunately not feasible to outline approaches needed to steer these quality attributes.

A high genotype-dependency was found for loaf volume (49 %) whereas also the environment and the mutual interaction ($\mathbb{G} \times \mathbb{E}$) influenced this main quality attribute. Additionally, gas cell distribution in the crumb and color attributes were found to be mainly genotype-dependent. Interestingly, the height-to-width-ration, which is indicative for the dough visco-elastic behavior during proofing and baking, was found not to be significantly affected by environmental conditions, but it was subject to a high interaction-effect.

With the current knowledge, it is recommendable to setup single-location trials in which cultivation conditions are specifically varied to study their impact on the selected quality attributes or (newly introduced) related characteristics. However, as values for broad-sense heritability are highly dependent on the included diversity in genotypes and environments, a similar study as the present one—in which relevant attributes are determined at all levels (from crop to end-product)—has to be continued or even expanded in order to encompass a larger variation for both $\mathbb{G}$ and $\mathbb{E}$. Hence, in addition to the diversity included in the obtained sample set, major points of attention should be the comprehensiveness of the screened quality attributes and which repercussions this has for studying structure–functionality–end-product quality–relationships. In order to gain insight in wheat quality as early in the wheat-to-bread-chain as possible, a clear focus must lay on measures related to growth conditions and plant development as well as on kernel characteristics and wholemeal properties.

Highlights

- Loaf volume (per kilogram flour) is the main quality attribute for bread besides the initial crumb hardness and chewiness and the relative increase over a four-day period.
- Non of the conventional flour characteristics is solely a good predictor for loaf volume, even when considering them using advanced modeling techniques.
- Measures for wholemeal and flour (pasting behaviour, glutomatic, *etc.*) may provide supplementary information aiding in an improved predictive power.
- High-resolution data on protein composition can partially substitute information enclosed by conventional techniques although this currently has to be combined with functional properties, mainly dough rheological attributes.
- Main compositional and functional differences are observed between harvest years whereas location and genotype ($H^2 \approx 30\,\%$) are found to be less determinative.
- Starch-related attributes with regard to the molecular structure and pasting behavior, significantly contribute to loaf volume development in combination with protein composition and water binding properties.

CHAPTER 4

Steering wheat quality through Nitrogen and Sulfur fertilization

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4.1 Introduction

As discussed in Section 1.5.3 (page 40), fertilization is one of the most commonly applied crop husbandry practices to steer wheat quality. Mainly nitrogen (N) and sulfur (S) fertilization are broadly applied to increase wheat yield, grain protein content and the *composition* of proteins. Since 1992, the use of N-fertilizers in both its mineral and organic form, is however legally restricted in the European Union by the implementation of the Nitrates Directive (91/676/EEC (Council, 1991)). This directive is developed to limit environmental threats such as eutrophication and an unfavorable soil N-balance resulting from intensive livestock farming and N-fertilizer applications to crops. Besides dosage limitations, the Nitrates Directive makes it compulsory to implement good farming practices in all member states comprising (a) the identification of water polluted or at risk of pollution, (b) the designation of Nitrate Vulnerable Zones, (c) the establishment of action programs to be implemented by farmers, and (d) the setup of a national monitoring and reporting system (Environment Directorate General of the European Commission, 2019).

> Nitrogen is a crucial element in plant growth and for the synthesis of storage proteins. It impacts all phases throughout crop development with a general tendency towards a higher productivity and higher grain protein contents. Availability in the soil has to be balanced with the amount of N which is administered as both a shortage or an excessive use can have negative effects. To achieve a certain protein concentration and composition, while ensuring a sustainable production, optimized N-fertilization practices are essential.

In general, the implementation of legislative measures in the member states is fairly comparable regarding restrictions for where and when to apply fertilizer and manure, but very different regarding application limits for N-fertilization. Despite the Nitrates Directive has already markedly contributed to a decrease of the soil N-balance since 1995, particularly in Belgium, Denmark, Ireland, the Netherlands and the United Kingdom, the southeast of the Netherlands, the Flemish Region (Belgium) and Brittany (France) remain to be regions of major concern. Main reasons are the persistently high N-surpluses and leaching fractions to ground water as well as a tenacious exceedance of the water quality standards (Van Grinsven et al., 2012). As a result, the most recent revision of the action plan (on the basis of the revised Nitrates Directive) in Belgium, the so-called 'MestActiePlan', version 6 (MAP 6), proposes more stringent measures. This comprises a nuanced distinction in the area types going from 0 (water quality is better than the quality standards) to type 3 (water quality does not meet requirements). For example, area types 1–3 (59 % of the agricultural area in Belgium, based on groundwater and surface water) are obligated to implement catch crops (if possible) to further reduce N-leaching from the soil. For areas designated as types 2 and 3 (respectively medium and large distance from reaching the water quality standards), a further reduction of the maximum dosage of active N (10 to 20 % less by 2022, respectively) will be enforced. The general tendency is that, in areas where a more extensive improvement has to be made, the focus will go to apply fertilization (a) using the correct dosage and type of fertilizer, (b) applying it at the correct time, and (c) by means of the optimal technique. (Denys, 2019).

In Belgium, a maximum of 160 (sand) or 175 kg ha^{-1} yr^{-1} (other soil types) of active N is allowed in area types 0 to 2 whereas for area type 3, a 5 % reduction (8 or 9 kg ha^{-1} yr^{-1}) is already in action. In 2022, the maximum dosage rate in area types 2 and 3 have to be further reduced to 144 and 128 kg ha^{-1} yr^{-1} for sandy soil or 157 and 140 kg ha^{-1} yr^{-1} for other soil types. As winter wheat can be classified as a derogation crop (by sowing a catch crop after harvest), a maximum of 180 and 195 kg ha^{-1} yr^{-1} applies for sandy or other soil types respectively.

A valid concern for farmers in the European Union is the limiting effect on wheat quality of these ever more stringent measures. Despite crop yield and plant growth are taken into account when revising the Nitrates Directive, other quality attributes such as grain protein content and composition and, ultimately, the suitability for bread production, are not evaluated. By persistently lowering the maximum amount of active N which can be used annually, the optimal application (timing, dosage per fraction, technique, and type of fertilizer) becomes even more crucial. Although it can be debated if such stringent measures are required (Zavattaro et al., 2016), it is generally accepted that lowering N-fertilization dosage rates is required to maintain or improve sustainability and water quality on a long term. As protein composition is the major trait when cultivating high quality wheat, the use of S-fertilizer can be proposed to compensate for the reduced N-application. Such additional applications are however cost ineffective and have to be applied carefully in order to obtain a positive effect. Moreover, as S-to-N ratio is also found to be of importance, a reduced N-addition will also require a revision of the S-fertilization advices. Another reason for the increasing interest in S-fertilization is the depletion of the soil in the past decades. This effect results from a decreased atmospheric deposition induced by the successful policy to reduce S emissions from industry (Erisman et al., 2007).

4.2 Research approach

The current study was performed on the basis of two field trials conducted during years 2016–2017 and 2017–2018. Initially, only N-fertilization was studied comprising the combined effect of dosage rate and fractionation. In the second year, also a null treatment (no N administered, N0) and blanc treatment (two N-fractions 85 and 35 kg N ha^{-1}, N2) were included to set a baseline for comparing the other treatments to. In both field trials, two levels of fractionation were tested (three and four fractions), each time comprising three dosage rates. The initially suggested total dosage rate, as determine by the National soil service of Belgium (Bodemkundige dienst van België) (BDB) using the N-Index method, was used as a starting point. In addition, minus 30 % and plus 30 % levels were tested. Besides management practices ($\mathbb{M}$) and environment (*i.e.* year, $\mathbb{E}$), the genotypic effect ($\mathbb{G}$) was screened. Therefore, five genotypes ('Cellule', 'Evina', 'KWS Ozon', 'RGT Mondio' and 'RGT Reform') were included in the first year whereas three were selected for the second year. This was necessary to comprehend the extensiveness of the performed analysis.

During the second year, S-fertilization was also included in the setup to shed light on the interaction between N- and S-treatments and to gain a basic insight in the potential quality improvement of S-fertilization treatments. As it is generally accepted that S-fertilization is applied for the cultivation of quality wheat (with a focus on high-demanding bakery products), it was chosen to combine this with four N-fractions which also is a common practice for quality wheat cultivation.

Table 4.1: Overview of the soil characteristics and the suggested (as determined by the National soil service of Belgium (Bodemkundige dienst van België)) and applied N- and S-fertilization treatments

		2016-2017		2017-2018	
Growth period		241 (29/11/2016–28/07/2017)		259 (29/10/2017–15/07/2018)	
Forefruit		Potato		Perennial rye grass	
Soil type		Sandy loam pH = 6.8		Light loam pH = 6.1	
N-index[1]		210		139	
Nitrogen	Fractions	3[2]	4[2]	3	4
	Suggested (kg N ha^{-1})	153 (58-33-62)	173 (58-33-62-20)	198 (85-43-70)	223 (85-43-70-25)
	−30 % (M)	127 (50-33-44)	141 (50-33-44-14)	179 (87-50-42)	198 (87-50-42-19)
	Advice (A)	145 (50-33-62)	165 (50-33-62-20)	197 (87-50-60)	224 (87-50-60-27)
	+30 % (P)	164 (50-33-81)	190 (50-33-81-26)	215 (87-50-78)	250 (87-50-78-35)
Sulfur	**Suggested** (kg S ha^{-1})	-	-	-	100
	−30 % (M)	-	-	-	70 (35-35)[3]
	Advice (A)	-	-	-	100 (50-50)[3]

[1] N-index as determined by the National soil service of Belgium (Bodemkundige dienst van België).
[2] Amount of N applied as treatment, excluding the 20 kg N ha^{-1} applied to improve plant health at seedling stage.
[3] Applied in two fractions together with the 3rd N-fraction or between the 3rd and 4th N-fraction from.

Table 4.1 provides an overview of prevailing cultivation conditions and the applied management practices in both trials. Meteorological conditions for both growth periods is shown in Figure 4.1 with an indication of the timings at which the main crop husbandry practices were applied. For both trials, the sowing density was 350 seeds per m^2 and 1.5×10 m plots were used. Soil composition and fertilization suggestions (N, P, K, S and other minerals) were obtained from the BDB. No significant differences between both environments were noted with exception from the calcium content for 2017 (280 mg kg^{-1}) compared to 2018 (90 mg kg^{-1}). It was attempted to deliver the suggested dosage for P, K and Mg to the best ability. No S was administered in the N-trial to prevent interaction effects. Due to the high amounts of precipitation in December 2016 and January 2017, wheat seedling and tillering occurred under suboptimal conditions. To enhance plant health, a small first fraction of N (20 kg ha^{-1}) was applied at the end of January to all plots.

All fertilization trials were performed against a relatively low background of initially present sulfur in the soil (2016–2017: 25.6 mg kg^{-1} and 2017-2018: 13.9 mg kg^{-1}). This was beneficial for the S-fertilization trial conducted in 2017–2018 as S-fertilization was recommended at a dosage rate of 100 kg S ha^{-1}. Besides the effect of S:N-ratio, attained by varying both the amounts of N (-30 % and suggested amount) and S (-30 % and suggested amount), timing of the application was investigated. All S-treatments were applied in two fractions, however, they were administered at the same time ('a', N+S) as the third N-fraction or between ('b', N|S) the third and fourth N-fraction (21 days after and 16 days prior to the 3rd and 4th N-application respectively). When applied together, S:N-ratio becomes potentially determining for wheat quality. Ratios were 0.313, 0.354, 0.446 and 0.505 for respectively -30 % S at A or M N-dosage and suggested S-dosage in combination with N-dosage rates A and M.

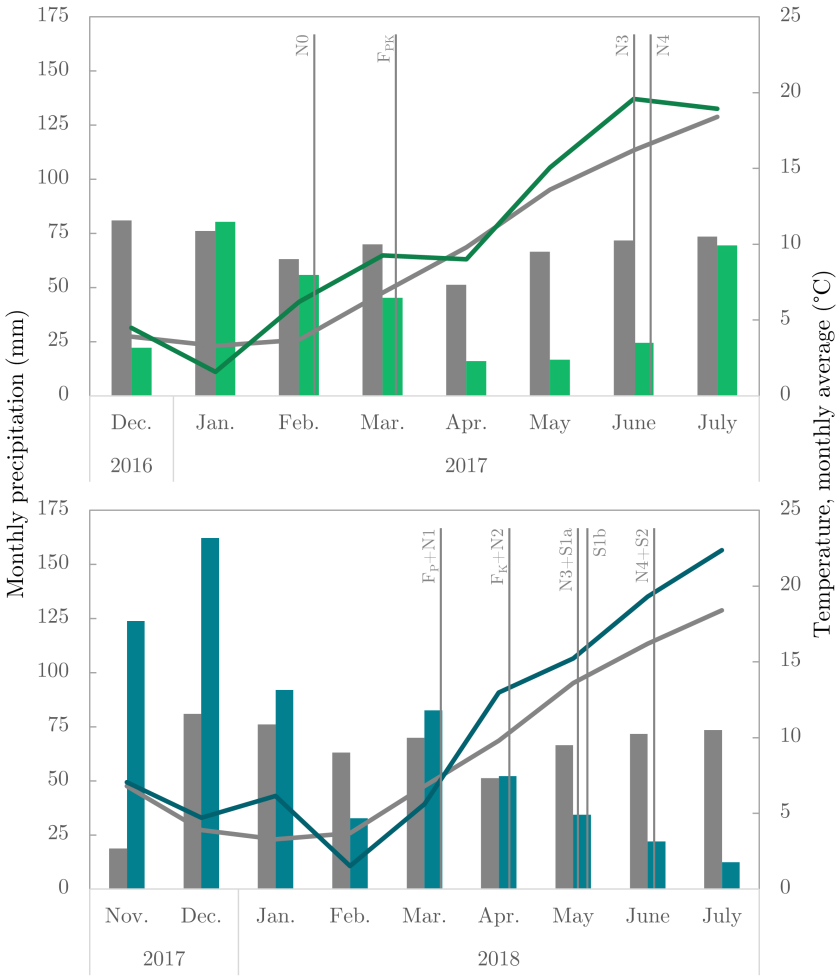

Figure 4.1: Overview of the prevailing climatological conditions—total monthly precipitation (bars) and average temperature (lines)—from a weather station in Bottelare (≤5 km from the field sites) (colored, 2017 ● and 2018 ●) compared to the longterm average (gray) (Koninklijk Meteorologisch Instituut van België (KMI), 2019).
N_i, nitrogen fraction; F_{PK}, general fertilization; S1a,b, sulfur application (a = combined, b = separate).

After harvest, samples from a single parallel, selected on the basis the field homogeneity, were cleaned and milled to flour according to the workflow described in Chapter 3 (Section 3.2.2). Analytical techniques used are similar to the previous chapter resulting in a comprehensive dataset of compositional and functional properties, supplemented with data from the breadmaking trials. The data was analyzed through conventional statistical approaches including analysis of variance and principal component analysis using R (version 3.4.3, (R Core Team, 2018)). The main research objective is to investigate how the two most common fertilization approaches can be used to steer wheat quality. In the light of the stringent nitrate directive, as discussed in the introduction, questions about the possibly accompanying limitations for quality wheat cultivation can be proposed. Within the overall framework of this research, it is attempted to provide an estimation of the relative contribution of management practices compared to genotypic ($\mathbb{G}$) and environmental ($\mathbb{E}$) effects. It was not within the scope of this specific study to elucidate the relationship between compositional and functional attributes and the bread quality.

Through this study, it was attempted to estimate the impact of fertilization on wheat *quality* with regard to breadmaking. Moreover, outcomes were evaluated in light of the European legislation on the application of mineral and organic N-fertilizers. As such, it was attempted to provide an answer on the question if the proposed limitations will impede the production of high quality wheat in Belgium. Moreover, the research could provide insight in which practices (timing, dosage rate, fractionation) are recommended to improve wheat quality under the prevailing environmental conditions.

4.3 Results and discussion

Due to the extensiveness of the trails in terms of analysis and treatments, results are split up per component. Both sections commence with a discussion of the compositional and functional attributes as well as on the quality of the bread. For all relevant attributes, it is investigated how these are affected by the main factors enclosed in the research affect. An overview is presented in Table 4.3. Overall correlations between these characteristics are displayed in correlation plots (Figures 4.4 and 4.12).

4.3.1 Nitrogen fertilization

4.3.1.1 Yield and kernel properties

The application of nitrogen fertilizer during the vegetative growth stages of wheat is assumed to have a main impact on plant development and plant growth, thus resulting in an increased grain yield. In the current research, a significant increase for the year-corrected Soil Plant Analysis Development (SPAD)-values (measured at Zadoks growth stage (Zadoks G.S.) 40) was observed when comparing the zero fertilization treatment (N0) with the other N-fertilization treatments (Figure 4.2). The blanc treatment (N2.A), however, had similar values as the treatments with three and four N-fractions. Furthermore, no effect of the total N-rate was noted with values ranging from 1.023 ± 0.060 to 1.034 ± 0.070 for -30 % (M) and +30 % (P) of the recommended dose respectively. Genotype influenced SPAD-values with 'Evina' having a significantly higher value then 'RGT Mondio', 'RGT Reform' and 'KWS Ozon' which, in turn, were significantly differing from 'Cellule'.

In contrast to the findings from various researchers (Le Bail et al., 2005; Islam et al., 2014), only a weak correlation between yield (YLD) and SPAD emerged from the data (Figure 4.4) after excluding the reference treatments (N0 and N2). This might be attributed to the timing at which measurements took place as grain weight is mainly sensitive to post-flowering conditions. A highly significant effect of all variables was found. Broad-sense heritability (H^2) however was low (19 %) due to the significantly lower yield for N0 (4.065 ± 0.204 kg ha^{-1}) compared to other N-treatments (on average 7.5 kg ha^{-1}). Remarkably, no significant differences in YLD were observed between the other treatments as shown in Table 4.2. When excluding the reference treatments, H^2 increased to 59 % with genotype being the only remaining significant variable. 'RGT Mondio', 'RGT Reform' and 'Cellule' had a higher average yield compared to 'Evina' and 'KWS Ozon'.

In addition to YLD and thousand kernel weight (TKW), test weight (TW) may be indicative for grain quality as this has been related to both the protein and the starch content (Schuler et al., 1995). For both yielding parameters, $\mathbb{E}$- and $\mathbb{G}$-effects were highly significant whereas management ($\mathbb{M}$) had no impact on TW. This is different for TKW for which a significant increase (9.2 % on average) was observed when no N was applied. The weak but significant correlation with the area size (AS) also implies that kernel filling was markedly impacted by N-fertilization. Cultivars showing the lowest yield ('Evina' and 'KWS Ozon') also had the highest TKW, supporting the relationship between both variables (Groos et al., 2003). TW also appeared to be less stable across years than TKW as is indicated by its respectively low and high H^2.

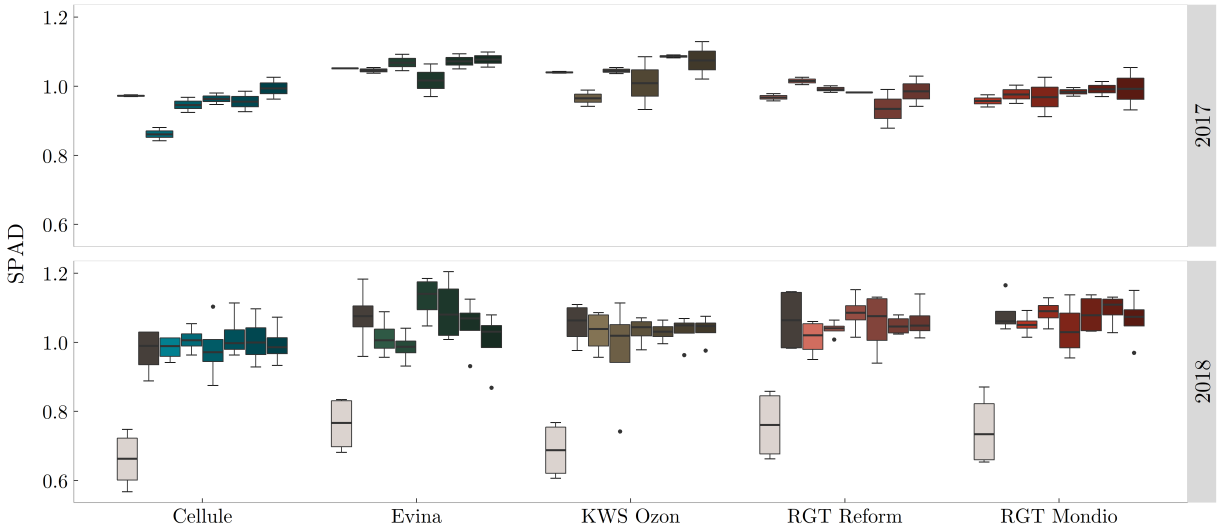

Figure 4.2: Year-corrected Soil Plant Analysis Development (SPAD) values measured in triplicate on the flag leaf at (Zadoks G.S. 40) on 10 plants per plot for all treatments (from left to right: *N0, N2.A,* N3.M|A|P, N4.M|A|P) and cultivars).

A lower average TW and higher TKW was observed for 2017. In contrast, high H^2 for both variables (ranging from 81 % to 92 %) were previously reported (Appels, 2008; Abinasa et al., 2011).

For all studied kernel morphological variables (AS, length-to-width-ratio (LtWr) and circularity (CIRC)), large variations between samples were observed. This results in both significant singular and complex interaction effects. In general, kernels form 2018 were significantly smaller, mainly for cultivars 'RGT Mondio' and 'Cellule'. A clear trend towards less circular kernels at more N-fractions and higher N-dosages was observed. This is in contrast with findings for AS and LtWr for which no clear trend between treatment was found.

> Grain yield was limitedly impacted by nitrogen fertilization with significantly lower values when no nitrogen was applied. Even at two fractions, obtained yields were comparable with three and four fractions (at all dosage rates). No linear relations with other yielding parameters or plant health were observed although an impact on grain filling is assumed by differences in the kernel morphology.

4.3.1.2 Flour composition

Upon flour extraction from the grain, damaged starch is produced by the high shear forces during milling. Kernel hardness and milling settings may influence this as was discussed by Van Der Borght et al. (2005). The former is also influenced by protein composition (presence and composition of puroindolines), implying that N-fertilization might also influence hardness (Oury et al., 2015). Indirectly, results did not support this hypothesis as a insignificant effect was found for management (*i.e.* fertilization treatment) on the damaged starch content of the flour. Genotype and, to a larger extent, year were determining for the damaged starch content with samples grown in 2018 having an on average $\pm$ 0.5 % higher content compared to 2017. Similar trends were observed for the Hagberg falling number (HFN) for which also a small (6.06 % of the variability) effect of the fertilization treatment was noted with N3.A having the lowest HFN (highest α-amylase activity) whereas for N4.M, a significantly lower activity was observed.

Table 4.2: Overview of the yielding parameters per treatment and environment for the N-fertilization trials.

		Plant growth and yield				Kernel morphology		
Envir.	**Treatment**	*SPAD*[1]	*YLD*	*TW*	*TKW*	*AS*	*LtWr*	*CIRC*
2016–2017	N3.M	0.92 ± 0.04	7.648 ± 0.280	82.40 ± 1.33	43.26 ± 3.16	15.40 ± 1.14	1.97 ± 0.06	0.729 ± 0.011
	N3.A	0.90 ± 0.06	7.935 ± 0.299	81.78 ± 1.37	42.47 ± 3.42	15.54 ± 0.84	1.97 ± 0.05	0.729 ± 0.007
	N3.P	0.92 ± 0.05	7.690 ± 0.451	81.89 ± 1.23	41.97 ± 2.20	16.22 ± 0.99	1.93 ± 0.07	0.738 ± 0.011
	N4.M	0.91 ± 0.02	7.446 ± 0.365	81.45 ± 1.26	41.21 ± 2.17	15.61 ± 0.48	1.98 ± 0.07	0.726 ± 0.013
	N4.A	0.93 ± 0.06	7.731 ± 0.373	81.56 ± 1.51	40.73 ± 2.63	14.69 ± 1.18	2.01 ± 0.05	0.722 ± 0.007
	N4.P	0.94 ± 0.04	7.354 ± 0.422	81.50 ± 1.42	42.13 ± 3.79	15.74 ± 0.81	1.96 ± 0.05	0.731 ± 0.006
	Mean	0.92 ± 0.05	7.634 ± 0.389	81.76 ± 1.28	41.96 ± 2.82	15.53 ± 0.97	1.97 ± 0.06	0.729 ± 0.010
2017–2018	N0	0.64 ± 0.05	4.065 ± 0.204	85.29 ± 1.89	45.24 ± 1.62	14.76 ± 0.71	1.80 ± 0.06	0.763 ± 0.003
	N2.A	0.92 ± 0.05	7.365 ± 0.648	84.71 ± 1.35	40.15 ± 3.55	13.79 ± 1.07	1.88 ± 0.06	0.749 ± 0.009
	N3.M	0.90 ± 0.03	7.703 ± 0.639	85.77 ± 1.80	41.10 ± 3.65	14.16 ± 0.87	1.90 ± 0.09	0.748 ± 0.016
	N3.A	0.91 ± 0.05	7.741 ± 0.665	85.76 ± 1.27	41.44 ± 2.94	14.09 ± 0.72	1.87 ± 0.04	0.750 ± 0.012
	N3.P	0.93 ± 0.07	8.008 ± 0.469	86.79 ± 1.93	41.36 ± 3.99	13.72 ± 0.89	1.90 ± 0.02	0.743 ± 0.007
	N4.M	0.94 ± 0.04	7.700 ± 0.755	85.72 ± 1.64	38.26 ± 3.18	13.50 ± 0.83	1.93 ± 0.04	0.738 ± 0.008
	N4.A	0.93 ± 0.04	7.687 ± 0.570	85.80 ± 1.43	43.87 ± 3.24	14.24 ± 0.76	1.85 ± 0.06	0.755 ± 0.009
	N4.P	0.91 ± 0.04	7.799 ± 0.597	86.25 ± 1.66	40.79 ± 2.67	13.98 ± 0.94	1.91 ± 0.11	0.745 ± 0.022
	Mean	0.91 ± 0.08	7.420 ± 1.002	83.18 ± 3.67	40.21 ± 3.69	13.77 ± 0.89	1.92 ± 0.07	0.742 ± 0.012

[1] Standardized per year using the maximum value over all samples.

However, no significant differences were found when studying the fractionation and dosage rate separately. This might be attributed to both the significant interaction-effects as well as to the inclusion of observations from 'KWS Ozon' and 'RGT Refrom' harvested in 2017 as a remarkably higher standard deviation could be noted for these samples.

A main quality characteristic for wheat flour is the protein concentration (PROC), as this is used for price setting and serves as a main quality attribute in wheat trading. The relationship between fertilization practices and grain PROC is often studied to find the optimal balance between investing resources (materials and time) in the crop and obtaining the highest value. Previous research stated that N-dosage rate increases PROC whereas splitting/fractionation may possibly impact protein *composition*. Besides a major determining effect of $\mathbb{E}$ (explaining 88 % of the variability) on the flour PROC, a less pronounced $\mathbb{M}$-effect was visible as is also illustrated in Figure 4.3. At three N-fractions, an increasing dosage rate resulted in an increase whereas no such effect was observed at four fractions. In addition, N3.P and N4.M resulted in a comparable PROC (2017: 12.8 *vs.* 12.2 % and 2018: 9.8 *vs.* 9.7 %) despite 17 kg N ha^{-1} more was given in the former treatment. Nevertheless, costs for applying the fourth fraction also have to be taken into consideration. Without any N-application or at two N-fractions, flour protein contents were 8.5 and 8.7 % which is approximately 1 % below the average for 2018 (9.6 %). In contrast, an absolute decrease of 2.3 % in the PROC was observed for 2018 compared to samples harvested in 2017. The main $\mathbb{E}$-impact also contributes to the low $\mathbb{G}$-effect.

The high temperatures at the time the 3rd and 4th N-fraction was administered may have reduced the amount of N that is absorbed thereby lowering the overall effect of the fertilization. Nevertheless, temperatures were higher in 2017 and both fractions were applied shortly after each other while the PROC was significantly higher compared to 2018. This partially contradicts the hypothesis that reduced uptake lies at the basis of the high environmental differences. Alternatively, it can be hypothesized that drought stress during 2017 has contributed to the higher protein contents as significantly less precipitation was noted from April to June 2017 compared to the same period in 2018 (Figure 4.1).

In addition to protein concentration, protein quality was studied by means of Glutomatic and empirically through studying dough properties (Farinograph and Alveograph measurements). Glutomatic wet gluten content (WGC) provides a basic insight in the protein composition, more specifically in the concentration of gluten-forming protein and the amount of water these bind. About one-third is determined by the genotype whereas the remaining two thirds come from environmental effects. In the current study, this comes to expression by the vast differences between the studied cultivars with 'Evina' and 'RGT Mondio' having the highest and lowest value (26.3 % and 17.7 %, respectively). Moreover, conditions during crop cultivation in 2016–2017 resulted in a 5 % higher WGC. Despite no strong statistical evidence was found the impact of fractionation or dosage of N, results showed a higher WGC at four fractions and +30 % N-fertilizer (N4.P). In contrast, flour from N3.P had on average an equally high WGC as flour from samples receiving four fractions at +30 %. When looking at the reference treatments, N0 and N2.A, significantly lower contents of wet gluten were observed when no additional N was supplied except for 'RGT Mondio' which also had the lowest PROC. Differences between treatments were also larger for 2018 compared to 2017 implying the presence of an important interaction with $\mathbb{E}$. The high positive correlations between parameters obtained from the Glutomatic (comprising the Glutomatic dry gluten (DG) and Glutomatic water binding to the wet gluten (WBwg)) resulted in similar findings as obtained

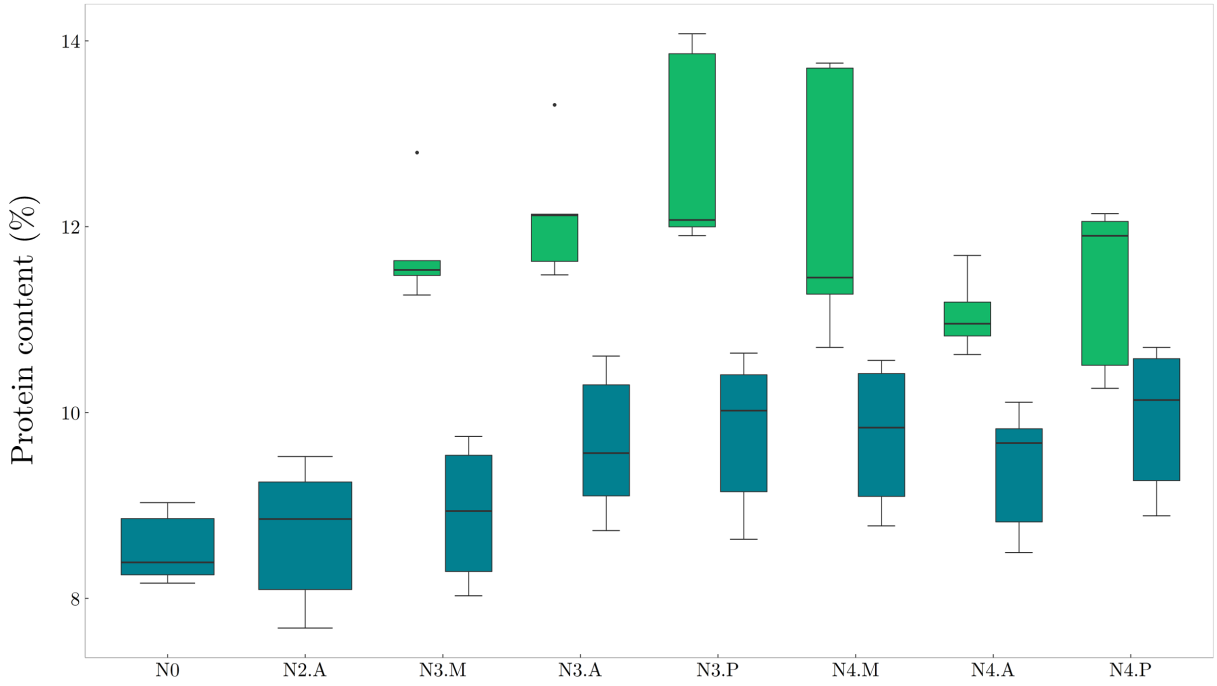

Figure 4.3: Overview of the variation in the protein content per treatment and environment (harvest years 2017 ● and 2018 ●).

through two-way ANOVA (Figure 4.4). Gluten Index (GI), on the contrary, behaved differently as for almost all samples, a value of 100 % was obtained. When evaluating the results from 2017 separately, a main genotype effect was observed while treatment did not clearly impact this quality attribute.

> Protein content is mainly environment dependent, presumably related to drought stress during plant growth and kernel filling, and fluctuates to a lesser extent between genotypes. The opposite was noticed for protein composition which is significantly influenced by the genotype although interactions with environment is clearly present. For both, the impact of the fertilization treatment was insignificant. High temperatures during the application of the third and fourth fraction may also have impacted uptake.

4.3.1.3 Dough properties

As only a single Farinograph measurement was performed for each sample, no full factorial analysis of variance could be performed for these parameters. Upon comparing mean values for the water absorption at 500 BU (WA_{500}) of 2017 and 2018, no significant differences ($p = 0.104$) were noted despite the 1 % higher WA_{500} for 2018 (56.3 ± 1.4 % *vs.* 55.4 ± 2.1 %). This increase is in contrast with the overall lower protein contents for 2018. This finding may support the involvement of protein composition to functionality. Genotype on the other hand greatly influenced WA_{500} with significant differences between all cultivars (in case reference treatments were excluded) following the next order: 'KWS Ozon'>'Cellule'>'Evina'>'RGT Mondio'>'RGT Reform' ranging from 57.0 ± 0.9 to 52.4 ± 0.7 %. No overall or factor-specific (fractions and dosage) differences could be observed between treatments. Zero fertilization had however a markedly lower WA_{500} of 53.3 ± 1.8 %. Similar trends were observed for

other Farinograph parameters (dough development time (DDt), stability (STAB), softening (SOFT)) with varying genotypic effects (H^2 of 59, 25 and 30 % respectively) and no significant impact of treatment, probably due to the high standard deviations. Nevertheless, trends for the latter factor could be observed for STAB and SOFT although these were not linear. Within treatments, large differences between the two harvest years were observed for N3.M and N4.A for DDt and SOFT. STAB was however clearly higher in samples harvested in 2017 (8.1 min *vs.* 2.5 min in 2018).

Analogously to findings from the Farinograph, Alveograph parameters were considerably influenced by $\mathbb{G}$ (H^2 of $\pm$ 30 %) as well as $\mathbb{E}$ with both 'KWS Ozon' and 'Cellule' and, in general, samples harvested in 2018 showing the highest Alveograph maximum required pressure (AL-P) or resistance to deformation. Alveograph curve length (AL-L) (as a measure for dough extensibility) was significantly higher in 2017 which implies the presence of an overall weaker gluten network in these samples. However, a varying order for AL-P and AL-L was observed for the different genotypes with 'Evina' having the highest AL-L-value (95.4 $\pm$ 36.7 mm) whereas it only had an average AL-P-value (80.3 $\pm$ 19.5 mmHg). Although insignificant, trends for N-dosage rate were observed for both parameters with a decrease at a lower dosage rate (mainly for -30 % (M) samples). N-fractionation also had a positive effect on AL-L with a higher extensibility when N was applied in four fractions. By taking the ratio (AL-PL), effects of genotype and environment are enlarged with a distinctively high value for 'KWS Ozon' (2.66) compared to other cultivars (1.65, 1.42, 1.08 and 0.72 for 'Cellule', 'RGT Mondio', 'Evina' and 'RGT Reform' respectively). Due to the high influence of both $\mathbb{G}$ and $\mathbb{E}$, a relatively limited effect of $\mathbb{M}$ was observed. Also, when including the reference treatments (N0 and N2.A), highest and lowest Alveograph configuration ratio (AL-PL)-values were obtained when respectively no or only two N-fractions were applied whereas three and four fractions resulted in intermediate AL-PL-values.

Differences on both a compositional level and in dough rheology indicate a shift in protein composition, mainly driven by environmental influences (*i.e.* year). Results imply the presence of a more extensible gluten network (higher AL-L and Farinograph STAB) at higher protein contents whereas an increased WA_{500} and AL-P were found when PROC decreased. This is in accordance with various studies showing the increase of monomeric prolamins—which mainly contribute to dough extensibility—at increasing protein contents (Konopka et al., 2007; Saint Pierre et al., 2008; Ashraf, 2014). Following Xue et al. (2016), prolamin-to-glutelin-ratio (PGr) was only increased through higher N-dosage regardless of the timing. This is however contrasted by the current findings.

> Protein content decreases by 1 % when no or limited nitrogen is applied whereas an interaction with the number of fractions was seen for three and four fractions. A main effect of the environment is noted with a 2.3 % difference between harvest years. Higher protein contents result in an accumulation of prolamins thereby impacting the dough rheological properties as weaker gluten networks are formed. No nitrogen application further lowers gluten strength.

4.3.1.4 Pasting behavior

Pasting behavior is a mainly starch related parameter and is therefore expected not to be influenced greatly by N-fertilization. Current results also showed that $\mathbb{M}$ (both combined or per factor) did not significantly

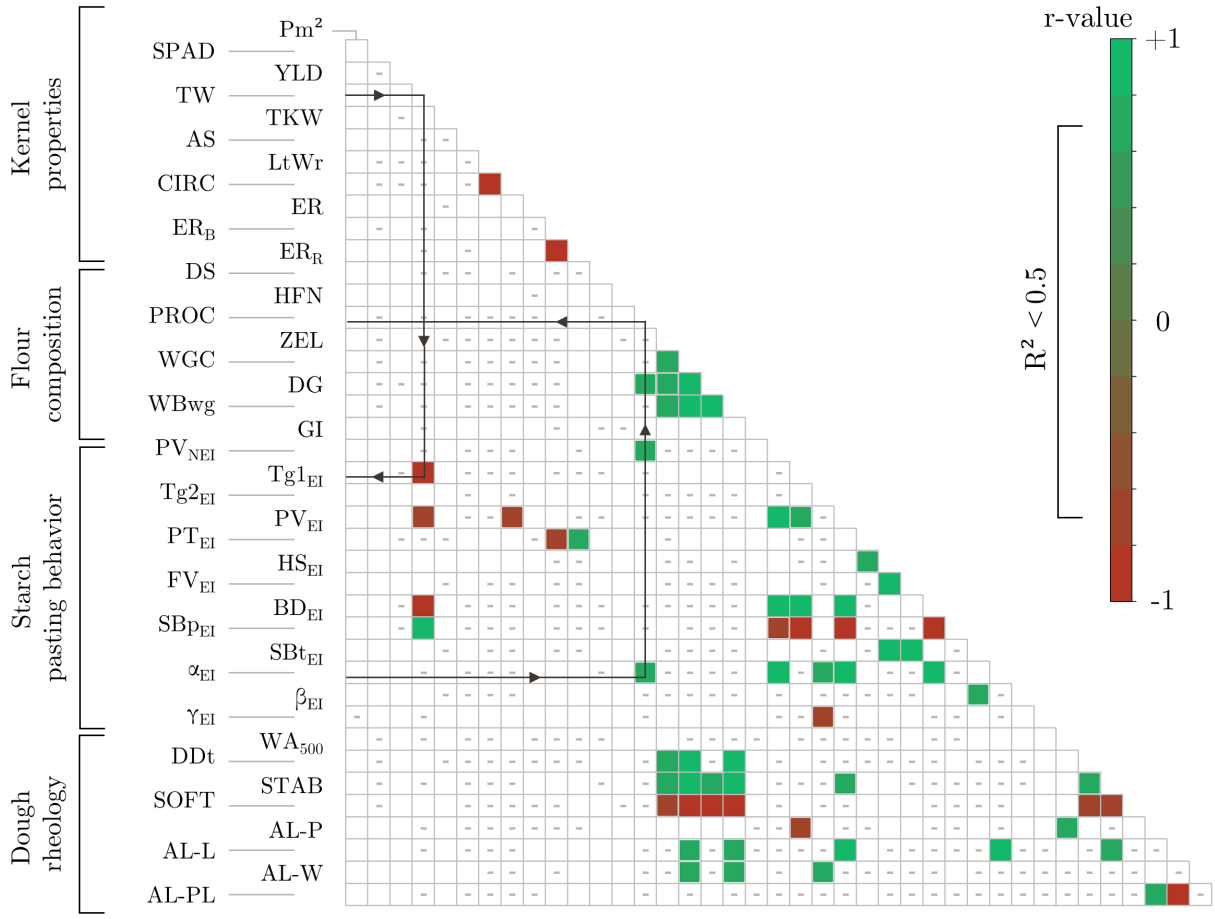

Figure 4.4: Lower correlation matrix displaying the mutual one-to-one correlations (Pearson correlation coefficient) between growth and yielding parameters, kernel characteristics (morphology and composition) and functional attributes on the basis of the mean values from the samples from the reference treatments (N0 and N2.A) and other N-related fertilization treatments.
Insignificant correlations ($p > 0.05$) were excluded (blank cells) whereas R^2-values smaller than 0.5 (absolute correlation coefficient (r) < 0.707) are denoted with a '-'. Colors indicate a negative (●) or positive (●) correlation. Two examples for significant, strong correlations are marked with lines.
Plants per squared meter (Pm^2).

affect any of the studied parameters. Despite no proof for the effect of fertilization on pasting behavior was found, a significant positive correlation ($r = 0.711$, $p \leq 0.001$) between peak viscosity (PV) (under non-enzyme inhibited conditions, $_{NEI}$) and PROC was observed. This is in contrast with expectations as a positive correlation implies that an inverse relationship between PROC and the total starch (TSt) content does not lay at the basis of this finding. Alternatively, this finding could be related to starch encapsulation in the protein matrix, although this phenomena is not yet fully elucidated in literature. When looking at the correlation plot (Figure 4.5), it can also be noted that the positive trend results from the difference between harvest years (higher PROC and PV_{NEI} for 2017 samples) with a insignificant correlation ($p = 0.885$) for 2017. For samples from 2018, the positive correlation between PROC and PV_{NEI} is preserved ($p \leq 0.001$, $r = 0.667$). A similar trend is observed for the pasting rate (α) under enzyme-inhibited conditions. The viscosity increase upon pasting may also be related to the water absorption capacity of the gluten protein.

Pasting temperature (at the first (Tg1) and second (Tg2) viscosity increase) showed to be significantly different when comparing the cultivars and was generally higher for samples harvested in 2017. Although correlations with the PROC were weak ($r = 0.553$ and 0.447, $p \leq 0.001$), a clear trend could be observed.

Starch encapsulation by protein may retard water absorption and swelling, thereby influencing the pasting temperature. Peak temperature (PTemp), was only influenced by $\mathbb{G}$ although a narrow range for this parameter was observed (92.3–93.3 °C for 'RGT Reform' and 'Evina' respectively). Peak viscosities under both non enzyme-inhibited ($_{NEI}$) and enzyme-inhibited ($_{EI}$) conditions were highly correlated, which thus implies an equal impact of the different factors. In 2018, PV was on average 27.0 and 20.3 % lower (for $_{NEI}$ and $_{EI}$ conditions respectively) compared to 2017 with a significant $\mathbb{G}$-effect. In contrast, holding strength (HS) and final viscosity (FV) were equal when comparing both harvest years, resulting in large differences for breakdown (BD) and setback (both setback from peak (SBp) and setback from trough (SBt)) values. 'RGT Reform' had the highest BD (2422 ± 105 mPa s) whereas the highest FV was noted for 'Evina' (4634 ± 188 mPa s).

Although there is no consensus on the effects of protein on the pasting viscosity and properties of cereals, it has been described by Ragaee and Abdel-Aal (2006) that pasting profiles (and more specifically, peak viscosities) can be used to differentiate between cereal flours in terms of protein quality characteristics. However, it was not elucidated which attributes lie at the basis of the interaction between protein denaturation and starch pasting. As the current results show, an increased PV is obtained at a higher PROC with assumed higher glutelin (GLU) concentrations (on the basis of previous dough rheological measurements when comparing both harvest years).

> Peak viscosity increases at higher protein contents for samples harvested in 2018 and decreases at increasing test weights for both harvest years. Significant environmental effects for the pasting temperature and peak viscosity were noted whereas other pasting properties were constant between harvest years.

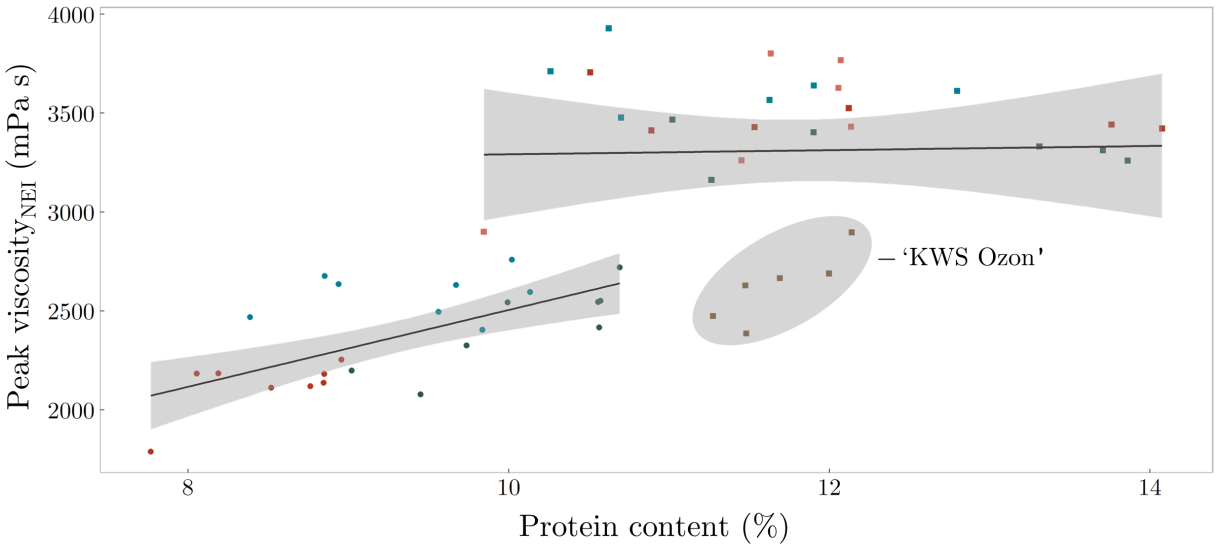

Figure 4.5: Peak viscosity (PV) (without enzyme inhibition) in function of protein concentration (PROC) with linear correlations (gray areas show 95 % CI) per harvest year (2017 ■ and 2018 ●).
Coding for the genotypes can be found on page xii.

4.3.1.5 Bread quality

Loaf volume is one of the main quality criteria of bread as it is the most coherent expression of quality differences in the end-product. A correction for the used amount of solid material (*i.e.* flour) by calculating the loaf volume (per kilogram flour) (VOLfc), results in a more nuanced insight in differences due to underlying compositional and functional attributes. When looking at Figure 4.6, a primary $\mathbb{G}$-effect can be observed as well as a significant difference between years. On average, breads produced from wheat harvested in 2017 had an 8.1 % higher VOLfc compared to 2018). In contrast to the high contributions of genotype (59 %) and environment (31 %) to explaining the variance in VOLfc, a marginal impact of management practices (2 %) was found. Although $\mathbb{M}$-effects are significant, effects are limited and surpassed by the $\mathbb{E}$- and $\mathbb{G}$-effects. A slight decrease in VOLfc occurred when three N-fractions were applied compared to four and also +30 % treatments had a slightly higher VOLfc. Effects of the separate factors were however insignificant ($p = 0.229$ and $p = 0.680$ for fractionation and dosage rate respectively). In addition, for 'Cellule' and 'RGT Mondio', N0 resulted in equally high loaf volumes compared to the other treatments whereas for 'Evina', breads from flour without receiving nitrogen fertilization had the lowest volume.

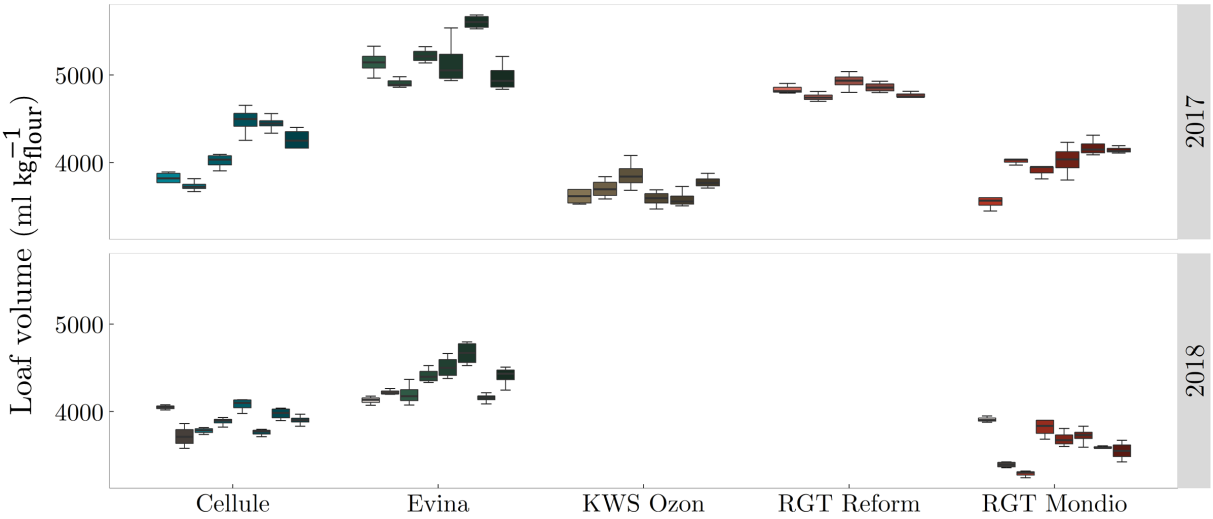

Figure 4.6: Loaf volume per kilogram flour (VOLfc) per cultivar, treatment (from left to right: *N0, N2.A,* N3.M|A|P, N4.M|A|P) and harvest year. Each boxplot is based on the value of four different breads.

Oven spring (OvnS) (both absolute and relative) was more strongly influenced by genotype. Highest OvnSs was found for 'Evina' (9.8 %) whereas 'Cellule', 'RGT Reform' and 'RGT Mondio' experienced a significantly smaller height increase during baking (respectively 5.1, 4.6 and 4.1 %). 'KWS Ozon' even had a negative OvnS and shrunk on average 1.4 % in height. The latter cultivar is typified by a high GI and AL-P which are both related to a high proportion of GLU in the flour and thus, a lower PGr. Nevertheless, based on the mean values, it could be stated that a higher N-dosage and more fractions resulted in an increase for OvnS. Although the observed effects were insignificant, equal findings were presented by Xue et al. (2016), who also observed an increase in baking quality (VOLfc) upon late N-fertilization.

In contrast to most of the previously discussed parameters, no $\mathbb{E}$-effect could be observed for the height-to-width-ratio (HtWr). Moreover, only the $\mathbb{G} \times \mathbb{E}$ and $\mathbb{G} \times \mathbb{E} \times \mathbb{M}$-interactions were significant implying that, effect of treatment was varying for both harvest years and between genotypes as is also illustrated in

Figure 4.7. Samples from 2017 show a decrease in HtWr for treatment N3.P in contrast to the increase for 2018 samples. A significant difference between results for treatment N4.A further contributes to this finding.

Besides the dimensions of the loaf, gas cell properties were examined by means of image analysis using an in-house developed script (prof. dr ir Jan Verwaeren). The absolute number of gas cells larger than 100 pix^2 was found to be influenced by genotype ('RGT Reform'>'RGT Mondio'>'Evina' and 'KWS Ozon'> 'Cellule') and environment (2017 having a significantly coarser crumb than 2018), as well as by treatment. When the latter was subdivided in its factors, N-dosage rate showed to be the main contributing factor ($p \leq 0.001$) with a decrease in the total number of gas cells larger than 100 pix^2 at higher dosage rates ($-30\,\%$ (M) $= 126 \pm 18$, suggested (A) $= 119 \pm 29$, $+30\,\%$ (P) $= 110 \pm 34$). Applying more N thus results in a finer crumb without influencing loaf volume. Moreover, a lack of interaction with $\mathbb{E}$ (*i.e.* year) also comes to expression by the similar trend for non-reference treatments as is illustrated in Figure 4.8. However, when looking at the averages split per fraction and dosage rate, the positive effect of dosage rate was lost with the finest crumb at N3.P and N4.A. In addition, effects showed to be $\mathbb{G}$-dependent (p-value for $\mathbb{G}\times\mathbb{E} = 0.013$).

> Environmental impact on protein content and composition also comes to expression in results from the breadmaking trials with a *reduced* volume and decreased height-to-width-ratio at higher protein contents. On the contrary, average crumb gas cell size is lower at increased protein contents, despite the constant loaf volumes. This might be attributed to an increased dough strength during mixing or can be the result of more stable gas cell walls during proofing, preventing them to aggregate.

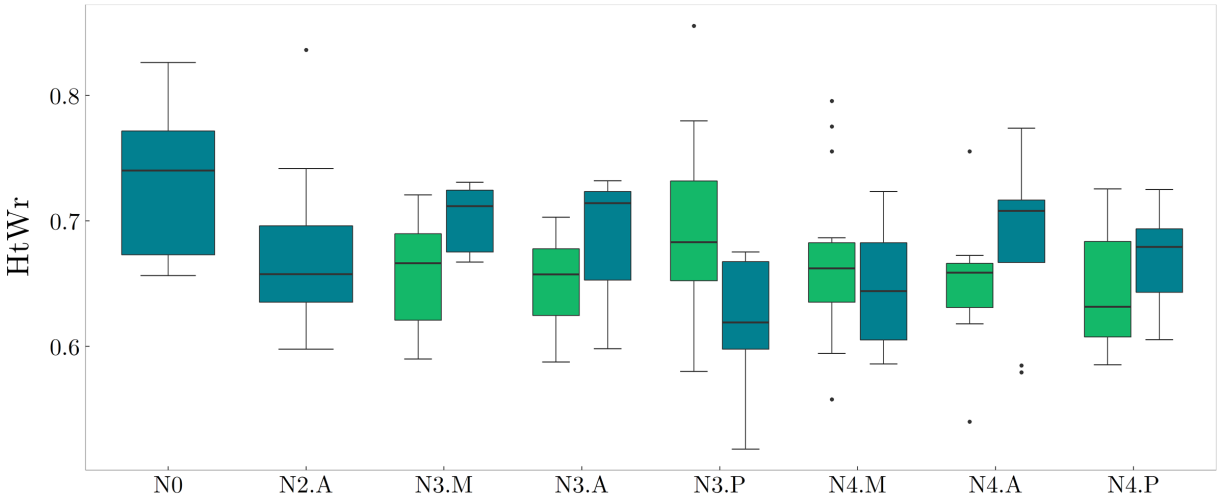

Figure 4.7: Height-to-width-ratio (HtWr) per treatment (from left to right: *N0, N2.A,* N3.M|A|P, N4.M|A|P) and harvest year. Each boxplot is based on the value of three different plate breads.

Textural parameters can be associated with starch and protein composition and functionality as well as with the water binding and its distribution in the crumb during storage. Crumb hardness (HARD) and crumb springiness (SPRING) can, in this way, provide insight in how underlying effects come to expression on a product-level thereby providing a starting point for further investigation. HARD was not significantly differing between both harvest years and also no effect of treatment could be observed despite a linear decrease at a lower N-dosage. This was different for SPRING for which a small (explaining only

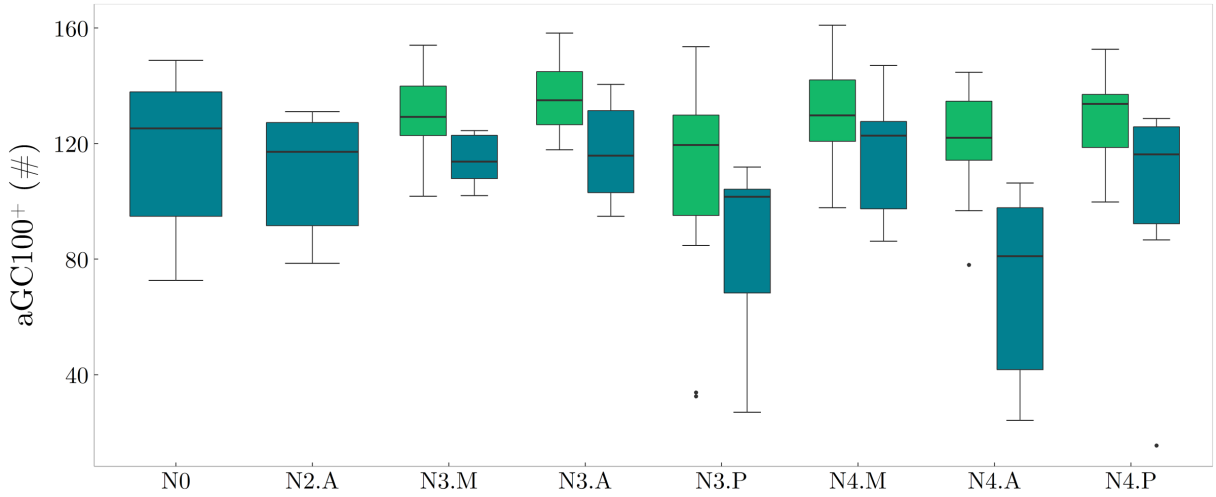

Figure 4.8: Absolute number of gas cells larger than 100 pix^2 (aGC100*) per treatment and harvest year. Each boxplot is based on the average values (4–6 slices) of two pan breads from three to five cultivars.

8.2 % of the variability) but significant $\mathbb{M}$-effect was found. However, no clear trend could be noted in the data. Contrastingly, interaction with $\mathbb{G}$ was highly significant ($p \leq 0.001$). All textural properties were mainly influenced by $\mathbb{G}$ ($H^2 \geq 50$ %, except for crumb cohesiveness (COH)) with, on one hand, 'Evina' or 'RGT Reform' and, on the other hand, 'KWS Ozon' or 'RGT Mondio' being the extremes for all texture parameters.

> **Proposition**
>
> Nitrogen fertilization only limitedly influences important quality traits such as yield and protein content compared to genotypic or environmental effects. Moreover, interaction-effects were observed frequently further tempering its status as preferred method to control wheat flour quality. Contrastingly, the application of limited amounts—in this research, 138 kg N ha−1) divided over two fractions—had markedly larger effects compared to no fertilization.
> Protein content was slightly increased which was accompanied by lower dough strength and lower loaf volumes, presumably resulting from a shift towards prolamin synthesis and thus, a higher prolamin-to-glutenin ratio. Correlation between protein content and pasting behavior were positive which contradicts earlier research and could not be explained on the basis of the current findings.

4.3.1.6 Principal component analysis

To provide additional insight in the correlations between variables, a principal component analysis (PCA) was performed on all attributes related to growth, kernel properties (morphology and composition) and functional properties. Figures 4.9 and 4.10 respectively show the loading and score plots with **A** two principal components (PC) or **B** three PC. Depending on the amount of PCs considered, the total variability explained in the analysis is 56.6 or 65.4 %.

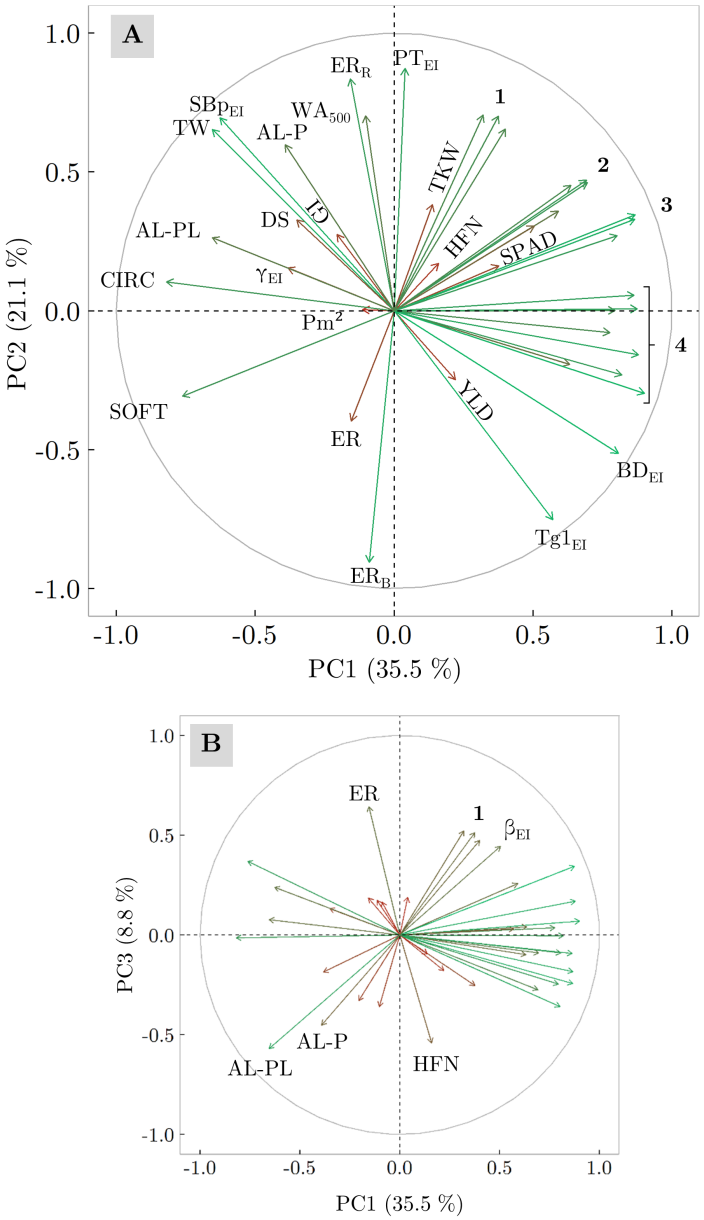

Figure 4.9: Loading plots on the basis of growth and yielding parameters, kernel characteristics (morphology and composition) and functional attributes, displaying the variability explained (in total 65.4 %) by the first three principal components (PCs) (**A** PC 1 and 2, **B** PC 1 and 3), colored following their contribution (from low ● to ●).
Group 1:holding strength (HS), final viscosity (FV), setback from trough (SBt), with enzyme-inhibition ($_{EI}$); **group 2**: Alveograph baking strength (AL-W) dough development time (DDt), Zeleny sedimentation value (ZEL), retrogradation/gelations rate (β), pasting temperature 2 (Tg2); **group 3**: Glutomatic water binding (WBwg), wet gluten content (WGC), and dry gluten content (DG); **group 4**: stability (STAB), protein concentration (PROC), Alveograph curve length (AL-L), pasting rate (α), area size (AS), and peak viscosity (PV) under $_{NEI}$- and $_{EI}$-conditions.

In the two-component model, four clusters of highly contributing variables can be distinguished. The first group comprises related rheological variables (HS, FV, SBt) which show a negative correlation with overall extraction rate (ER) and, more specifically, the breaker rolls extraction rate. This might related to the starch damage and particle size distribution in the flour, as well as with the water absorption as postulated by Hrušková et al. (2006). These attributes are also strongly correlated with other rheological parameters (retrogradation/gelations rate (β), pasting temperature (Tg)) as well as with Alveograph baking strength (AL-W), DDt and Zeleny sedimentation value (ZEL) which are represented by the second group. As discussed before, pasting properties may be influenced by protein composition and content (Ragaee and Abdel-Aal, 2006) which can also be derived from this representation. Although performed on rice starch extracts, the study of Lim et al. (1999) found proof for the positive correlation between pasting temperature 1 (Tg1) and PROC. This also comes limitedly to expression in the loading plot (quadrant IV) as well as correlations with the PTemp and α as discussed earlier. Moreover, the strong mutual correlations between α, PV and PROC in group four may indicate that under these conditions, proteins facilitate gelation upon cooling. This phenomena is attributed to an enhanced H-bond formation by the

presence of GLU and amylose (AM) during network formation (Singh et al., 2011). In contrast, Singh et al. (2011) observed a strong negative relationship between BD and the PROC which was not supported by the current findings.

Although the pasting behavior is analyzed in an excess of water, the observed effects might also originate from competition for water between gluten and starch as proposed by Dhaka and Khatkar (2015). These researchers also observed strong negative correlations between PGr on one hand and Farinograph DDt and STAB on the other hand. This can also be deducted from the current PCA following the assumption that the AL-PL is negatively correlated with PGr. When looking at the third PC (Z-space), it is seen that samples in the score plot (Figure 4.10**B**) will be further distinguished by factors correlating with the third PC. This comprises AL-L (*i.e.* dough extensibility), the overall extraction rate, pasting properties HS, FV, BD, β and the HFN.

When looking at the score plots (Figures 4.10**A** and **B**), genotypic effects, as elaborately discussed in the in the previous sections, are clearly visible. Moreover, the plot also illustrates the significant difference between samples harvested in 2017 (center ellipsoid and marks with a positive x-coordinate) and 2018 (remaining samples in quadrant II and III). Firstly, these results prove that the selected three cultivars for 2018 cover the diversity in the original set despite the wider distribution along the y-axis (second PC) of the two additional cultivars in the set of 2017 ('KWS Ozon' and 'RGT Reform') when comparing them to the other cultivars. This can be attributed to the high variation for HFN as was also discussed in Section 4.3.1.2. The three-dimensional representation in the second graph further illustrates how the mean values for the samples (different treatments) of 'RGT Reform' are separated by the third PC. In a 2D representation, both cultivars appear to be characterized by their similarity (when excluding the environment effect) for attributes which are highly correlated to PC1 and PC2 such as WA_{500}, variables in group four and the separate and overall extraction rates. In contrast, 'KWS Ozon' distinguishes itself from 'Cellule' in the third dimension on the basis of the HFN, AL-P and AL-PL. Despite loaf volume is not included in the PCA, a higher volume was obtained for 'RGT Reform' compared to 'RGT Mondio'. As both cultivars are separated following PC3, it can be postulated that loaf volume is strongly related to compositional and functional attributes represented by this PC.

4.3.2 Sulfur fertilization

For sulfur fertilization treatments, only data from a single harvest year (2018) is available. Therefore, the contribution of environment cannot be calculated on the basis of a two-way ANOVA, thus making it impossible to obtain information on the broad-sense heritability. Table 4.3 therefore displays the relative contribution of the genotype ($\mathbb{G}_{cnt}$), calculated by dividing the mean sum of squares from $\mathbb{G}$ by to total sum of means (both $\mathbb{M}$ and $\mathbb{G}$, their mutual interaction, residuals and the error term).

4.3.2.1 Yield and kernel properties

Grain yield was found to be mainly determined by genotype with a significant difference between all cultivars ('RGT Mondio' > 'Cellule' > 'Evina') whereas the dosage rate ($p = 0.012$) and timing ($p = 0.005$) were also significantly impacting YLD. However, when inspecting the latter factors separately,

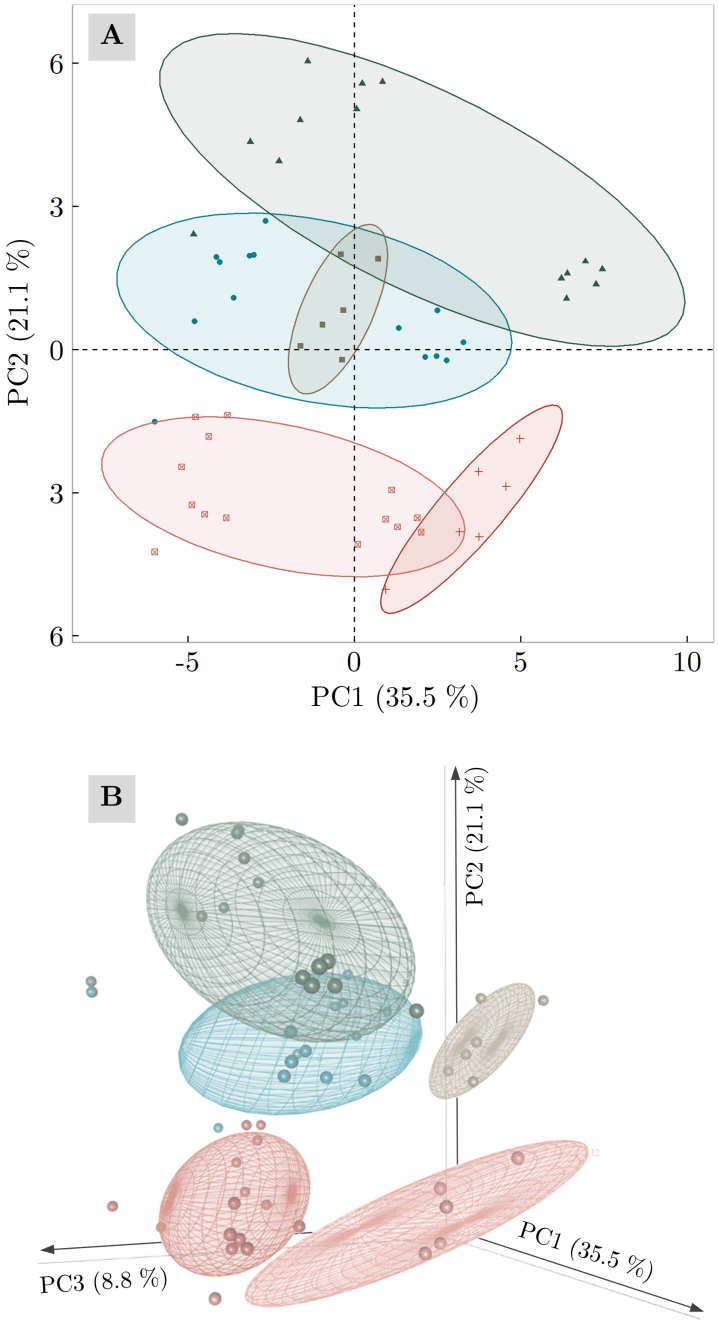

Figure 4.10: Score plots on the basis of growth and yielding parameters, kernel characteristics (morphology and composition) and functional attributes, displaying the distribution of the samples, colored by cultivar ('Cellule' ●; 'Evina' ●; 'KWS Ozon' ●; 'RGT Mondio' ●; 'RGT Reform' ●), in a 2D (**A**) and 3D (**B**) space. Data points on the left hand side of the 2D score plot are from 2018 while 2017 samples are represented by data points on the right hand side, including clusters for 'KWS Ozon' and 'RGT Reform'.

no significant differences could be observed. In general, marginally lower yields (0.225 kg ha^{-1}) were obtained when applying more S than suggested as well as when S and N were applied together. TW on the other hand was highly dependent from the applied fertilization treatment ($\mathbb{M}$ explaining 64 % of the variability) although $\mathbb{G}$ and the mutual interaction impacted the yielding property in the same magnitude (18 %).

When looking at Figure 4.11, a lowering effect of S-fertilization can be observed. While reference treatments have a mean TW varying from 86.0–87.4 kg hl^{-1}, very low to equal values were found for all S-treatments, depending on the N-dosage. This implies an effect of the S:N-ratio as was also confirmed by solely looking at the samples in which N and S were applied together (treatments denoted with an 'a'). Significant reductions in TW from 83.9 to 73.5 and, eventually, to 66.3 kg hl^{-1} were observed

when S:N-ratio was lowered from 0.505 to 0.446 and 0.354. No further reduction was observed at a S:N-ratio of 0.312. In contrast, TKW was mainly influenced by $\mathbb{G}$ with no clear effect of the applied fertilization treatments as both dosage rate ($p = 0.148$) and timing ($p = 0.064$) were insignificant when studied separately. The latter finding is in contrast with results from (Järvan et al., 2012) who observed a lowering effect of S-fertilization on TKW.

Kernel morphology, and more specifically LtWr and CIRC, was consistently more stable in case N and S were applied together. Additionally, rounder kernels were obtained when only N was applied or when treatments did not occur at the same time. With ER being related to wheat shape, spherical seeds will have the highest possible endosperm-to-bran ratio. On the basis of results from the N-treatments, CIRC was however found to be *negatively* correlated to PROC, despite no correlation with ER was found. This relation has been debated in literature. According to Williams et al. (2013), the crease that runs along the dorsal side of the seed complicates the dependency.

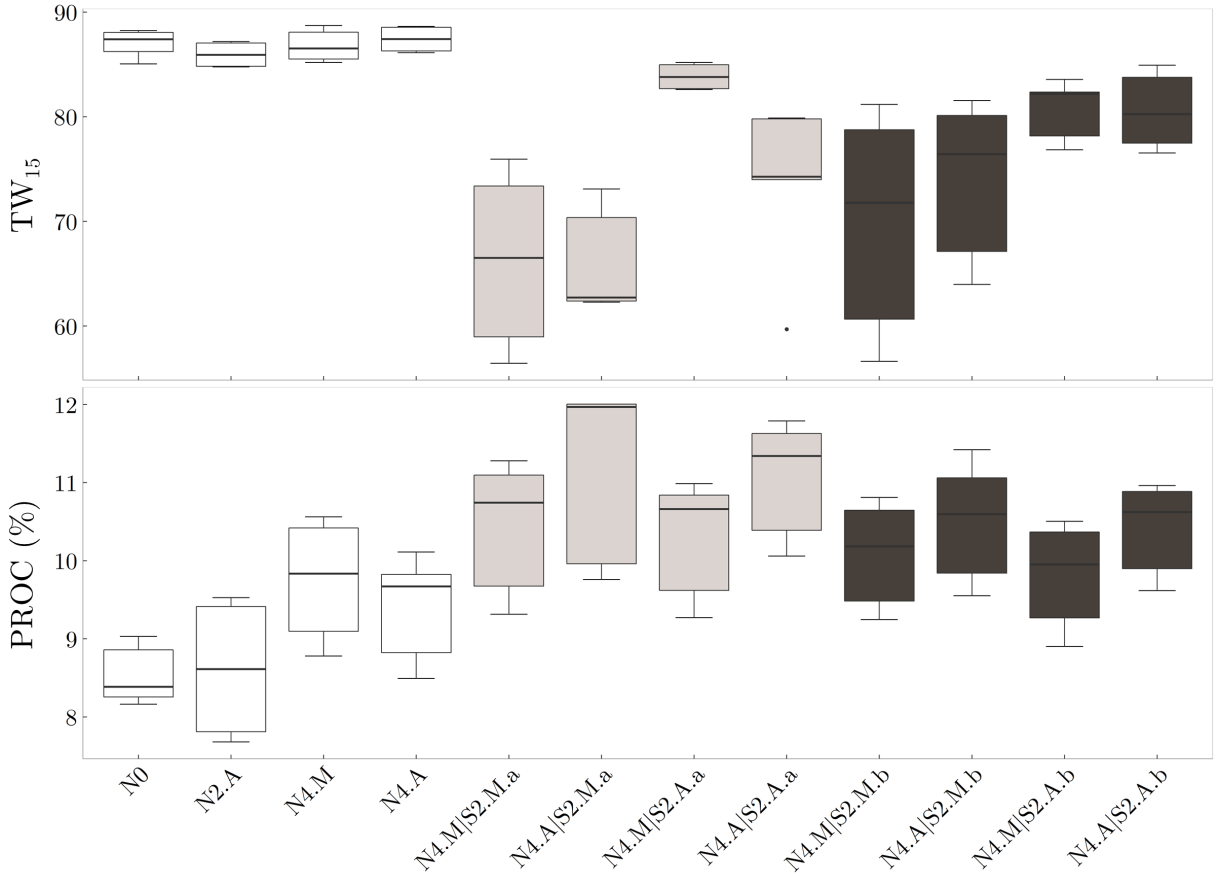

Figure 4.11: Boxplots ($n = 9$) showing the variability in test weight (TW) (after cleaning, corrected for moisture) and protein concentration (PROC) of the flour (expressed on dry matter) per treatment).

4.3.2.2 Flour composition

A minimal reduction (6.59 % to 6.38 %) of the damaged starch content could be attributed to the combination of both fertilizer treatments whereas no S or a separate application resulted in higher contents. This corresponds with the findings of Moss et al. (1981) who reported a greater kernel hardness with lower S-application. HFN was only influenced by $\mathbb{G}$ with no significant difference between the reference

treatments and S-treatments or within the latter. The development of α-amylase occurs during late stages of grain maturation ('*late maturity (endosperm) amylase*') and is probably therefore not impacted by the fertilization treatments in this study. Nevertheless, N-application tends to decrease the HFN although the driving mechanism remains unclear (Kindred et al., 2005; Liniņa et al., 2015).

Sulfur fertilization is mainly applied to improve protein composition by promoting the development of proteins rich in cystein and metheonin (γ-prolamins and low molecular weight glutelin-subunits (LMW-GS)) (Wieser et al., 2004). However, as was found earlier, protein *content* can also be increased by the application of S during grain filling. Current results support this finding with a significant absolute increase in the PROC of 1.14 % when applying S together with N or with 0.63 % in case S and N are applied separately. S:N-ratio and S-dosage rate were however not significantly impacting the protein concentration. According to Duncan et al. (2018), interaction between N and S does not markedly increases the PROC but rather lowers the protein dilution effect upon yield increases.

> Yield is marginally impacted by S-fertilization whereas protein content is markedly higher when S and N are applied together or as separate treatments. Yielding property TW could be related to S:N-ratio implying a difference in the nitrogen use efficiency.

As is illustrated in Figure 4.11, a negative correlation between the PROC and the TW was present in the data. This may possibly result from a relatively higher protein accumulation in the kernel compared to starch deposition during grain filling. However, the correlation coefficient was low ($r = -0.483$, $p \leq 0.05$) which is attributed to interactions with genotype (Figure 4.12) or more specific, source-sink relations (Yan et al., 2010; Xie et al., 2015). Other researchers correlated both variables but reported a positive correlation (Schuler et al., 1995).

By providing a basic insight in compositional differences, ZEL indicates the presence of a significant effect of S-fertilization on the amount of gluten-forming proteins. By applying S, an increase of 5 to 7 units (ml) can be attained. Large $\mathbb{G}$-effects, with a range from 30 ± 2 to 49 ± 4 ml for 'RGT Mondio' and 'Evina' respectively could be noted. As the correlations between ZEL and parameters obtained from the Glutomatic analysis (WGC, DG, WBwg) were very strong, analogue results for both analysis were found through two-way ANOVA. As illustrated in Figure 4.13, a primary difference between four (in different dosages), two or no N-fractions can be observed with a clear increase as a result of increased N-fertilization. However, applying S together with N further increased the WGC by 5 to 10 %. Similar effects were seen for DG and consequently, WBwg. Also, a systematically higher protein quality was obtained when more sulfur was applied (suggested dosage rate compared to suggested -30 %). This clearly demonstrates the impact of S-fertilization on protein composition.

4.3.2.3 Dough properties

Farinograph analysis further revealed the effects of S-fertilization on protein composition as the functionality (*i.e.* dough rheological properties) was significantly altered. Upon comparison with the reference treatments receiving 4 N-fractions, a relatively limited increase in the WA_{500} from 1.23 to 1.37 % was noted. Significantly higher Farinograph DDts and STABs were however obtained by introducing S-

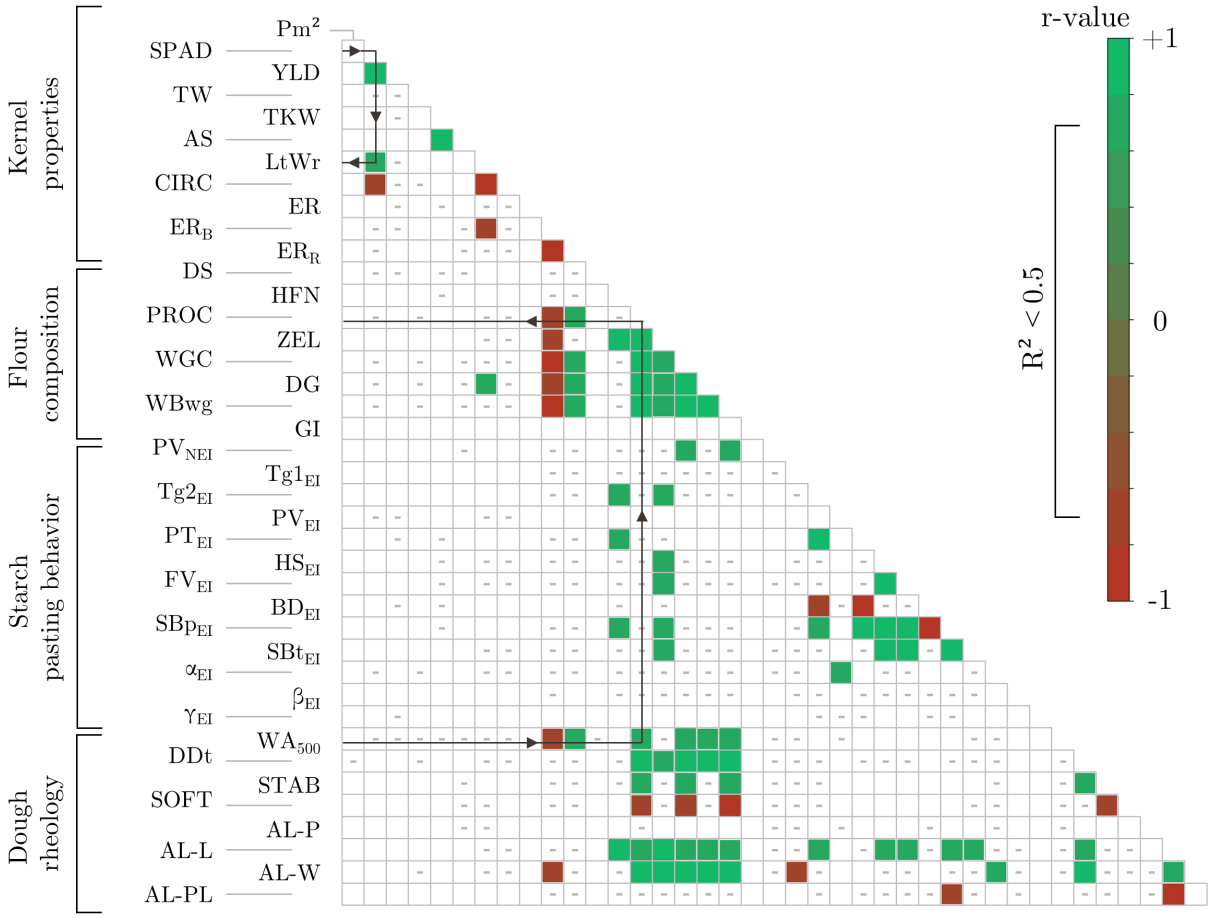

Figure 4.12: Lower correlation matrix displaying the mutual one-to-one correlations (Pearson correlation coefficient) between growth and yielding parameters, kernel characteristics (morphology and composition) and functional attributes on the basis of the mean values from the samples from the reference treatments (N0 and N2.A) and S-related fertilization treatments.
Insignificant correlations ($p > 0.05$) were excluded (blank cells) whereas R^2-values smaller than 0.5 (absolute correlation coefficient (r) < 0.707) are denoted with a '-'. Colors indicate a negative (●) or positive (●) correlation. Two examples for significant, strong correlations are marked with lines.

fertilization. Due to large standard deviations (as a result of the prominent $\mathbb{G}$-effect), no significant effects for the treatments was seen in variance analysis. However, mean values for the N+S-treatments were ranked higher than means of N|S-treatments. They are in turn also differing from no S-fertilization. Moreover, timing was found to be significantly impacting DDt (one-way ANOVA) with on average 0.5 min higher mixing times before reaching the maximum dough consistency in case both treatments were applied together.

> Both protein content and composition are significantly altered by S-fertilization, mainly when applied together with N. This can possibly be attributed to and increased nitrogen use efficiency or a generally enhanced plant health. Improved development of S-rich proteins (HMW-GS and γ-prolamins) upon S application will not only impact the total gluten content, also its composition (PGr and the HMW:LMWr) will be shifted towards stronger gluten-forming proteins and, thus, dough.

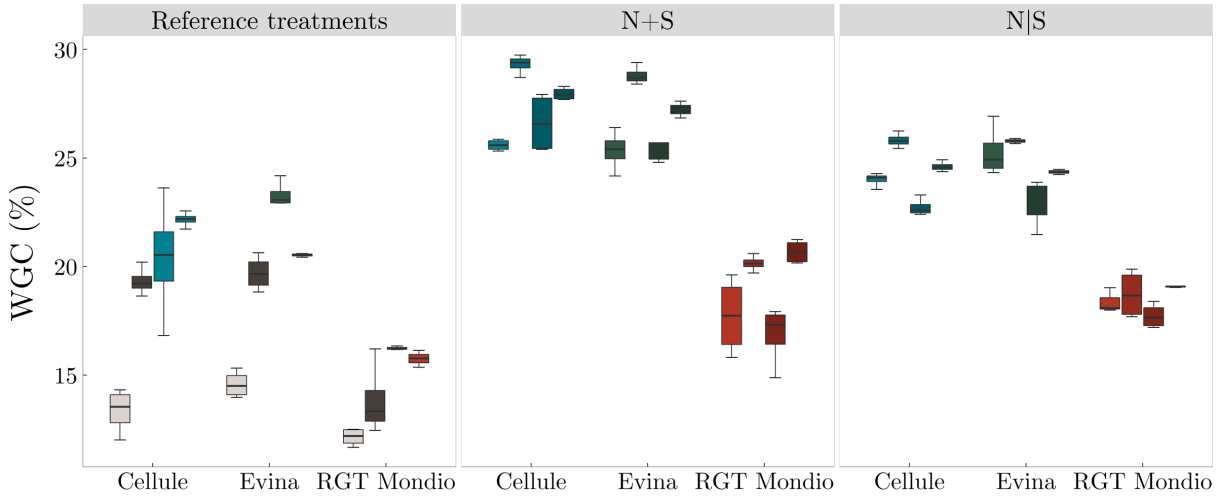

Figure 4.13: Overview of the Glutomatic wet gluten content (WGC) per cultivar and S-fertilization timing. Reference treatments include, from left to right, N0, N2.A, N4.M and N4.A whereas N+S (combined) and N|S (separate) include treatments N4.**M**|S2.M, N4.**A**|S2.M, N4.M|S2.**A**, and N4.M|S2.A.
Plants per squared meter (Pm^2).

Alveograph-values were inconsistent with mainly $\mathbb{G}$-effects and a minor effect of S-application in general. However, no clear influence of dosage or timing could be observed for AL-P and AL-L. Results for AL-PL, showed how a combined application differs from the separate application and how this also interacts with the genotype. For both 'Evina' and 'RGT Mondio', an increase can be noted for the S|N-treatment whereas a stagnation or decrease is seen for the combined application. 'Cellule' is hardly influenced by any of these management practices. However, AL-PL-values are lowered for all cultivars when comparing them with the reference treatments. S-fertilization thus lowers the elasticity of the gluten network making it more extensible. These findings are in accordance with research from Wieser et al. (2004) who attributed the effect to an increase in high molecular weight glutelin-subunits (HMW-GS) and indirectly, elasticity, in case of S-deficiency. Hence, PGr decreases and stronger, less extensible doughs were obtained. Visually, it can be deducted from Figure 4.14 that S-fertilization also reduces the within sample variability which is a direct result of a more stable AL-L-value. This implies that the bursting of the dough bubble occurs at a more similar time.

4.3.2.4 Pasting behavior

The increased protein content and the significant alterations in its composition by application of S-fertilizers during kernel filling may affect pasting properties by impacting the grain starch concentration and water binding properties. The latter may be of importance in systems where water is limiting, such as bread dough, and would be of less importance during pasting measurements as these are performed in an excess of water. However, Figure 4.12 illustrates that numerous strong, positive correlations between pasting properties, obtained in both native and enzyme inhibited conditions, and protein attributes are present in the data. As ZEL and WBwg are both related to the water binding capacity of the protein, it can be hypothesized that this plays a role in the viscosity of the starch paste. In contrast with the positive correlation between PROC and PV (under $_{EI}$ conditions) as discussed in Section 4.3.1.4, a significantly higher viscosity was obtained for samples which received N- and S-fertilization at different timings. Shen et al. (2006) and Li et al. (2013) reported the lowering effect of S-fertilization on AM-content which is

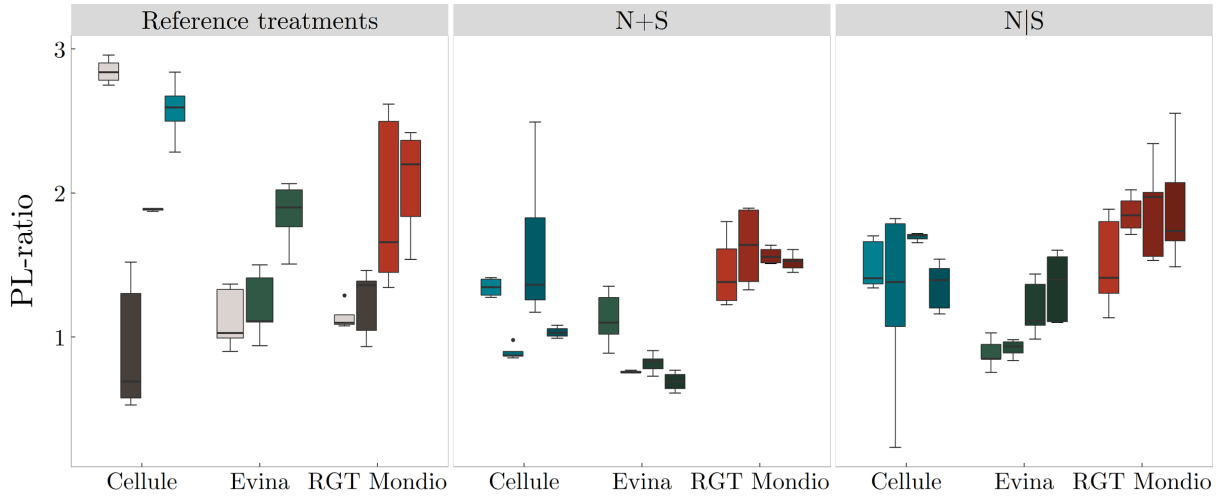

Figure 4.14: Overview of the Alveograph configuration ratio (AL-PL) per cultivar and S-fertilization timing (n = 5). Reference treatments include, from left to right, N0, N2.A, N4.M and N4.A whereas N+S (combined) and N|S (separate) include treatments N4.**M**|S2.M, N4.**A**|S2.M, N4.M|S2.**A**, and N4.M|S2.A.

generally accepted to increase the PV. Despite the marginal differences, lower PTemps for N|S-treatments were noted which is also in accordance with findings from Li et al. (2013). They attributed this lowering effect to an increased accumulation of B-type starch granules (containing less AM) through S-fertilization.

In addition, the temperature at which pasting occurs and at which the maximum viscosity is reached is dependent from the HFN despite α-amylase activity was inhibited by the addition of $AgNO_3$. A possible explanation for this correlation might be that HFN is also a viscosity based measurement and that enzyme inhibition resulted in a similar shift for all samples. As only a single measurement per sample was conducted, no two-way ANOVA could be performed. However, when studying single effects, a primary influence of the genotype was seen.

> Impact on protein composition might also influence pasting properties of the flour as seen for N-fertilization. However, the presence of a lowered amylose content and an increased concentration of B-type starch granules could be postulated on the basis of the pasting behavior.

4.3.2.5 Bread quality

VOLfc was significantly higher for all samples which received S-fertilization, independently from the timing at which this was applied. Nevertheless, timing itself was also found to be the second most important factor (with $\mathbb{G}$ being the first) which significantly influenced the VOLfc. A relative mean increase in loaf volume of respectively 6.1 % and 10.8 % for separate or combined fertilization could be noted. Furthermore, interaction between timing and genotype is illustrated by the differences when comparing the N|S- and N+S-treatments. A decrease was observed for 'RGT Mondio' (−1.5 % from 3963 to 3902 ml kg^{-1} flour) whereas a relative increase was seen for 'Cellule' (10.4 %) and 'Evina' (3.6 %) (Figure 4.15). Despite no significant differences were seen for S:N-ratio, a markedly higher value of 4683 ± 429 ml kg^{-1} flour was found at the lowest ratio (0.312) compared to almost equal values (4330–4379 ml kg^{-1} flour) at higher ratios.

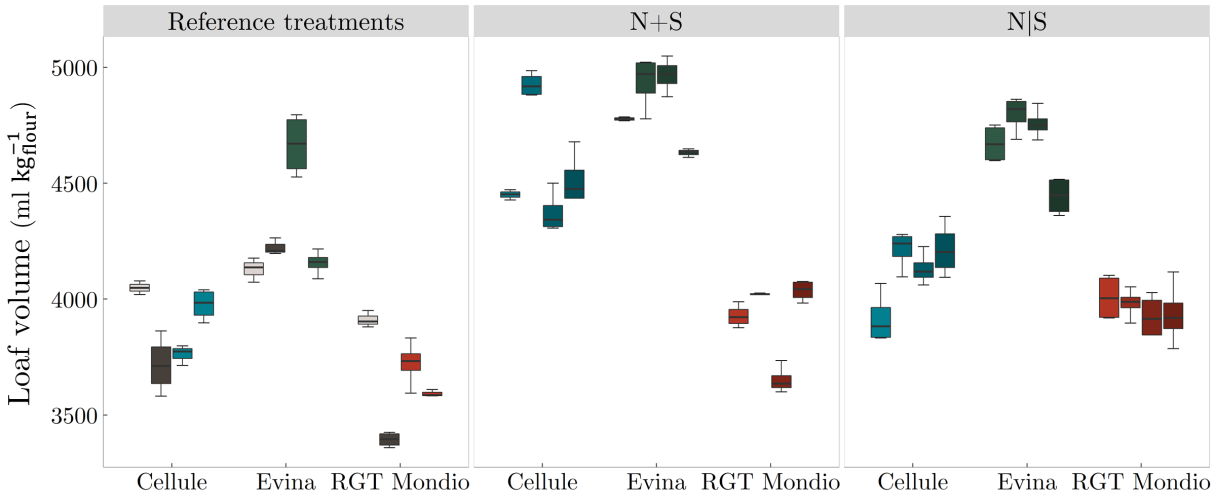

Figure 4.15: Overview of the loaf volume (per kilogram flour) (VOLfc) per cultivar and S-fertilization timing (n = 4). Reference treatments include, from left to right, N0, N2.A, N4.M and N4.A whereas N+S (combined) and N|S (separate) include treatments N4.**M**|S2.M, N4.**A**|S2.M, N4.M|S2.**A**, and N4.M|S2.A.

Some minor shifts in the OvnS could be noted with a generally higher value for the combined S-treatment (13.3 ± 6.1 %) compared to both the separate (10.7 ± 9.6 %) and reference treatments (6.6 ± 6.8 %). Again, 'RGT Mondio' shows to be the least affected whereas 'Evina' is characterized by an averagely lager increase. For this cultivar, two samples (combined application: N4.A|S2.A, separate application: N4.A|S2.M) had extremely high OvnSs of 26.8 ± 1.8 and 38.0 ± 2.0 %. In contrast to the high impact of $\mathbb{M}$ on the VOLfc and OvnS, a merely significant ($p = 0.036$) impact was observed for HtWr. This finding contradicts with earlier results which indicated that differences in protein composition are present on a compositional and functional level which were expected to come to expression in the end-product. HtWr provides a basic insight in the ability of the dough to keep its shape during baking which is thought to be impacted significantly by dough rheology and the strength of the gluten network. Use of fixed mixing times may have lowered these effects. An equally low impact was seen for the absolute number of gas cells larger than 100 pix^2 for which only a $\mathbb{G}$-effect could be noted ($p \leq 0.005$). Interaction with $\mathbb{M}$ was however also significant with increasing values at higher S-concentrations in case of a separate application and a decrease for combined application or at higher N-rates.

> Solely bread quality attribute volume is significantly impacted by S-fertilization as a result of higher oven springs. Although not supported by variation in the height-to-width-ratio, a possibly optimized balance between dough extensibility and gluten network strength lies at the basis of this effect. Hence, alterations in the composition of the gluten-forming protein—mainly an increase in the LMW-GS and a decreased PGr—can be postulated.

Despite S-fertilization clearly decreased crumb hardness (HARD) measured on the first day after baking, no significant differences or linear trends were observed for S:N-ratio, S- or N-concentration separately or the timing of the applications. Although closed-pan bread was used for the texture profile analysis, thereby normally excluding the impact of volume on these measurements, a strong negative correlation between both variables was obtained ($r = -0.732$, $p \leq 0.001$). However, when looking at the correlation, this was only found to be valid between 4000 and 5000 ml kg^{-1} flour and not for 'RGT Mondio' which, despite

its high variation in HARD, had an al most equal VOLfc (with exception of two samples). SPRING was marginally ($p = 0.0142$) impacted by $\mathbb{M}$ although this could not be attributed to one of the factors (timing, S- or N-concentration and their ratio).

Table 4.3: Simplified output (significances) of the various multivariate analysis of variance performed on growth, compositional, functional and end-product properties of the samples from N- and S-fertilizer trials, including an estimation of the broad-sense heritability (H^2) (for N-treatments) or the contribution of $\mathbb{G}$ to the variation (for S-treatments).

		Nitrogen fertilization								**Sulfur fertilization**			
	Variable	$\mathbb{E}$	$\mathbb{G}$	$\mathbb{M}$	$\mathbb{G}*\mathbb{E}$	$\mathbb{E}*\mathbb{M}$	$\mathbb{M}*\mathbb{G}$	$\mathbb{E}*\mathbb{G}*\mathbb{M}$	$\mathbf{H}^2$ (%)	$\mathbb{G}$	$\mathbb{M}$	$\mathbb{G}*\mathbb{M}$	$\mathbb{G}_{cnt}$ (%)
Kernel attributes	YLD	n.s.	***	n.s.	n.s.	n.s.	n.s.	n.s.	59	***	**	n.s.	92
	TW	***	***	n.s.	***	*	n.s.	n.s.	8	n.s.	n.s.	n.s.	4
	TKW	***	***	***	n.s.	***	*	n.s.	73	***	***	***	95
	AS	***	***	***	***	***	***	***	16	***	***	***	95
	LtWr	***	***	***	**	***	***	***	24	***	***	***	67
	CIRC	***	***	***	***	***	***	***	14	***	***	***	68
Flour composition	DS	***	***	n.s.	n.s.	**	*	n.s.	7	***	***	***	91
	HFN	***	***	***	***	***	***	***	0	***	***	***	93
	PROC	***	***	***	***	***	***	***	4	***	***	***	90
	ZEL	***	***	***	***	***	***	***	58	***	***	***	98
	WGC	***	***	***	***	***	***	***	31	***	***	***	94
	DG	***	***	***	***	***	***	***	23	***	***	***	93
	WBwg	***	***	***	***	***	***	***	33	***	***	***	94
	GI	***	***	*	***	n.s.	**	n.s.	21	***	***	***	77
Dough	AL-P	***	***	***	***	***	***	***	40	***	***	***	79
	AL-L	***	***	***	***	**	***	**	33	***	***	***	88
	AL-W	***	***	***	***	**	***	*	45	***	***	***	90
	AL-PL	***	***	*	n.s.	***	***	***	38	***	***	*	87
Bread quality	VOLfc	***	***	***	***	***	***	***	59	***	***	***	92
	OvnS	***	***	*	***	*	***	***	65	***	***	***	71
	HtWr	n.s.	***	n.s.	n.s.	***	n.s.	***	32	n.s.	n.s.	n.s.	23
	aGC100+	***	***	***	**	*	*	n.s.	12	**	n.s.	n.s.	70
	HARD	***	***	***	***	***	**	**	74	***	***	***	81
	SPRING	n.s.	***	*	n.s.	*	***	n.s.	52	n.s.	*	n.s.	38
	COH	***	***	n.s.	***	*	n.s.	n.s.	26	***	***	***	78
	CHEW	n.s.	***	**	***	***	**	*	81	***	***	***	82
	RES	*	***	n.s.	*	n.s.	n.s.	n.s.	50	***	***	***	66

Not significant (n.s.), $p > 0.05$; *, $0.01 < p \leq 0.05$; **, $0.001 < p \leq 0.01$; ***, $p \leq 0.001$.
Broad-sense heritability (H^2) for N is defined as the ratio of genotypic variance to the total variance (mean squared values) whereas for S, only genotypic variance and the variance due to management practices (fertilization), as well as their mutual interaction ($\mathbb{G}\times\mathbb{M}$) and the residuals are included.
Absolute number of gas cells larger than 100 pix^2 ($aGC100^+$), crumb chewiness (CHEW).

4.4 Conclusion

Fertilization is one of the crop husbandry practices applied most to steer wheat quality. Besides the use of nitrogen (N) as a fertilizer to promote plant health, it is administered in relatively high concentrations as it may increase protein synthesis. As this remains one of the main quality traits in wheat (which also determines the price), European farmers are worried as legislation on N-application gets ever more stringent. However, as emphasized in the previous chapter, protein *composition* is of equally high importance. Therefore, it was also investigated if the number of fractions in which N is applied could be used to alter protein composition. Moreover, with an increasing interest in sulfur (S) fertilization, this was also investigated as an alternative approach for improving wheat quality.

Results from the two-year N-fertilization trial indicated the overall limited effects of N-application, even at dosages above the currently allowed maximum values. However, compared to a zero treatment, significant increases were observed in both the protein content and grain yield. Contrastingly, no clear trends were seen between three or four fractions or the total N-dosage. Mainly for protein content, environment had a significantly higher impact whereas other factors were highly genotype-dependent. Furthermore, the observed increase in the protein content was accompanied by a shift in its composition resulting in lower loaf volumes and less rigid doughs. Although results are not conclusive, this finding again emphasizes that increasing protein contents are not necessarily linked with a higher end-product quality.

While estimates for the contribution of main factors may be under- or overestimated as samples from only two harvest years were analyzed, the obtained values correspond with the findings from the previous chapter. Enlargement in terms of both locations and harvest years is however recommended for future research without losing the focus on quality screening from kernel to end-product. Only in this way, a more nuanced insight in the possible limitations of the legislative measures with regard to fertilization can be obtained. Current results however showed that the Nitrates Directive does not have to be restrictive for cultivating quality wheat in Flanders. Nonetheless, a primary step remains the shift from high protein contents to an optimal (protein) composition. Moreover, alternative fertilization strategies have to be explored.

Effects from S-fertilization were far more significant and indicated a clear shift in the protein composition when both N and S were administered together. Moreover, all S-applications increased the protein content significantly compared to treatments in which solely N was applied. Increases were again accompanied by a more extensible dough (lower Alveograph configuration ratio), however, also resulting in a significantly higher loaf volume. This was observed for all samples that received S. Results imply the high potential of using S-fertilization to improve wheat quality and accentuates the importance of combined application with N. The current approach unfortunately does not allow to fully elucidate on the mechanism behind the advantages of a simultaneous application. It can also be postulated that an early application, before the grain filling has commenced, is beneficial for enhancing bread quality.

Apart from protein composition and dough rheology, pasting behavior was also influenced indirectly by N-fertilization. Although a lowering effect was expected at increasing protein contents, peak viscosity was found to be positively correlated with the protein content. Also the test weight was negatively correlated with peak viscosity. On the basis of observed differences, it was also postulated that starch composition

itself was altered by the fertilization treatments with an increased proportion B-type starch granules and thus, a lowered amylose content. Alternatively, flour particle size distribution itself may provide an explanation for the relation between dough rheology, pasting and the protein content. Furthermore, the impact of the flour extraction step and possible differences in the spatial distribution of proteins have to be taken into account when this is investigated further.

Despite both N- and S-fertilization treatments have shown to be relevant for improving possibly determinative quality attributes of wheat, complex interactions with environment and genotype complicate the translation to directly applicable recommendations. A possible strategy to reduce these environmental influences may also be the use of other types of fertilizers such as slow-release fertilizers. In light of climate change and when considering the contribution of timing (beneficial effect of 4^{th} N-fraction), this may provide valuable alternatives. However, it is also highly recommendable to focus on the combination of N- and S-fertilization while reducing the total amount of N.

Highlights

- N-application results in a significant increase for both yield and protein content compared to no or limited N fertilization (2 fractions). Four fractions are preferred over three thereby enabling the use of less N for obtaining the same quality.
- Effects of S-fertilization were markedly larger in case it was administered together with the 3^{rd} N-fraction instead of during kernel filling (between two N fractions).
- Increasing protein contents are accompanied by a shift in their composition. For N-fertilization, this resulted in weaker doughs whereas N+S-fertilization increased consistency as well as the dough extensibility.
- Pasting behavior cannot be considered solely starch-related as consistently, correlations with the protein content were observed. Understanding of the interaction between protein and starch in a flour matrix and the impact on viscosity development in function of water content, shear and temperature has to be improved.
- Legislative restrictions for N-fertilization (Nitrates Directive) which are currently in practice do not necessarily impede the production of high quality wheat in Flanders.

CHAPTER 5

Soil and climate as environmental parameters

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5.1 Introduction

Climate change is defined by a decrease in precipitation during summer and a substantial increase in temperature and also the frequency and magnitude of extreme weather events. Yielding parameters of wheat crops are found to be highly sensitive to such environmental and climatic variations. Salinity, water stress (both drought and waterlogging) and heat stress may give rise to a number of negative effects such as reduced photosynthesis, pollen sterility, kernel abortion and earlier senescence. These stresses directly result in a lower grain number which lead to considerable yield losses (Siebert et al., 2017). Heat stress around susceptible stages of wheat development—microsporogenesis, anther dehiscence and just after fertilization, thus, mainly during anthesis and grain filling (Semenov et al., 2014; Stratonovitch and Semenov, 2015)—is generally identified as the main threat for maintaining stable yields which are comparable or higher than the current levels (Stratonovitch and Semenov, 2015). Apart from timing, controversy exists in literature about the magnitude of the impact of drought and extreme heat events on wheat production in Europe (Semenov and Shewry, 2011; Vicente-Serrano et al., 2014). According to Zampieri et al. (2017), the relative importance of heat stress and drought in determining the yield anomalies, depends on the region. This is also confirmed by results from Baumbach et al. (2017) who revealed that the vulnerability to heat stress (particularly between May and June) is very heterogeneously distributed over Europe. Part of the contradicting findings may result from the use of the ambient temperature instead of the crop canopy temperature which can be more than 10 °C lower (Siebert et al., 2017). Moreover, these researchers also found a correlation with the water availability in the soil, thereby emphasizing the interaction between drought and heat (Webber et al., 2016). It has also been shown that water *excess* affects wheat production more than drought (Zampieri et al., 2017).

Water deficiency results in a relative increase in the grain protein content due to a shortened kernel filling period, a lowered starch accumulation and, *in extremis*, a decreased yield (Konopka et al., 2007; Flagella et al., 2010). Moreover, single kernel weight and diameter also decreases in case of water stress. This phenomenon is also confirmed by Saint Pierre et al. (2008) and Zhao et al. (2009) who noted a decrease in the flour protein content by dilution of nitrogen with carbohydrates upon irrigation. Ashraf (2014) reported that drought-induced reduction in AGPase activity was the main cause of stagnation in grain growth under severe water deficit conditions. Even though absolute increases in the protein concentration (PROC) of 12–18 % were observed, Flagella et al. (2010) found that the genotype ($\mathbb{G}$)-effect alone accounted for more variations in end-use quality than genotype $\times$ irrigation interactions (Konopka et al., 2007; Ashraf, 2014).

> An increase in the protein content is no guarantee for an improved quality of the end-product. Depending on the driving factor (*e.g.* different (mineral) fertilizers and fertilization practices), the prolamin-to-glutelin-ratio or the proportion high and low-molecular-weight glutelin-subunits may vary.

The increase in PROC was also accompanied by an increased accumulation rate of monomeric (Konopka et al., 2007; Saint Pierre et al., 2008; Ashraf, 2014) or polymeric (Flagella et al., 2010) proteins thus resulting in a respectively higher or lower prolamin-to-glutelin-ratio (PGr). In addition, Saint Pierre et al.

(2008) found that, when drought stress occurs after anthesis, the rate of accumulation of prolamin (PRO) and glutelin (GLU) did not change. Contradicting observations were made by (Zhao et al., 2009) who noted a significant increase in the PRO accumulation when high-temperature conditions were present at all grain filling stages. The content of both types of GLU (high molecular weight glutelin-subunits (HMW-GS) and low molecular weight glutelin-subunits (LMW-GS)) consistently decreased. According to Altenbach et al. (2002), the observed variations in the protein composition were also related to the relatively shorter period in which transcripts of gluten appeared (8 days after anthesis). Generally, these findings emphasize the pivotal role of the time at which the stress occurs. Besides starch and protein, it was also observed that mineral contents in the grains of winter wheat, particularly with regard to the major minerals P, K, Ca and Mg, were greatly affected by drought stress (Zhao et al., 2009).

One of the reasons why wheat can be grown under a variety of cultivation conditions, including a broad diversity in soil types, is its root architecture. This allows an efficient water uptake in both loose (*e.g.* coarse sand or sandy loam) and compacted (*e.g.* clay) soil types. Besides a genetic component, this is gravely determined by an environmental component (Manschadi et al., 2008). Despite the scarce number of research articles on this topic, clear differences in the length of the roots and the rooting density of wheat were observed (Gajri and Prihar, 1985; Rich and Watt, 2013). Nevertheless, a general consensus exists on the contribution of the extent and depth of root proliferation to water uptake, however, it has not been proven that this is the best strategy for adapting wheat to water-limited environments (Palta et al., 2011). Palta et al. (2011) even concluded that there is an increased risk of depleting soil water in environments where crops are reliant on stored soil water instead of seasonal rainfall when having a vigorous root system. In this way, water-limiting conditions before completion of the grain filling period may result in water stress and thus, a yield deprivation. Besides lacking information on how soil conditions regulate the root architecture (Rich and Watt, 2013), the water retention capacities of the soil will also vary largely providing an extra dimension when studying root-soil-water-temperature-interactions.

In most of the literature, wheat simulation models combined with local-scale climate scenarios (*e.g.* Sirius (Jamieson et al., 1998) with the 'CMIP3' multi-model ensemble and 'LARS-WG' weather generator (Semenov et al., 2010)) are used to study the impact of climate change. Mainly crop development and yielding parameters are discussed in these research outcomes as crop models are typically restricted to predicting average grain size and grain N content. Only the 'SiriusQuality' model accounts for the major storage proteins but leaves more detailed compositional attributes of both protein and starch unattended (Nuttall et al., 2017). However, by performing greenhouse trials, the desired (extreme) climate conditions can be introduced to empirically study separate or combined effects without having to rely on the prediction of extreme situations and their possible impact on complex quality traits through crop modeling. Therefore, it was chosen to study the effect of soil type on the starch and protein properties of winter wheat using a small-scale greenhouse trial. Subsequently, it was investigated how the soil humidity interacts with reduced irrigation and, due to the passively controlled ambient temperature, heat stress.

5.2 Material and methods

5.2.1 Setup of the greenhouse experiment

Coarse sand and heavy clay, as determined by means of palpitation by the National soil service of Belgium (Bodemkundige dienst van België) (BDB), was collected from two fields in Vladslo (West-Flanders, Belgium) and was transported to a greenhouse in Ghent (Belgium) for further use. Soil characteristics were priorly determined by the BDB (see Table 5.1) to calculate the fertilization requirements using the Nitrogen Index method. After homogenizing each soil, forty eight plastic tubes (H = 1.10 m and D = 0.15 m) were filled to the same distance from the top (0.2 m), 4 days prior to sowing. In order to prevent excessive moisture loss throughout the trial, the bottom of the tubes was covered using root cloth. Prior to filling, a layer of clay kernels (Argex, Zwijndrecht, Belgium) was added to level the soil to the desired height.

A fully randomized design with four replicates per combination of genotype ('Gedser', 'KWS Ozon' and 'WaxyDie'), soil type (sand and clay) and irrigation treatment (fully irrigated and limited irrigation) was constructed. Tubes were positioned in a 12 × 4 grid, standing approximately 20 cm apart from each other. Eight tubes (solid gray fill in Figure 5.1) were equipped with moisture sensors at three depths (20, 50 and 80 cm under the soil level) in the tube whereas soil temperature was separately measured in eight different tubes (dotted pattern). The genotypes were selected for their differences in protein and starch composition (based on results from earlier field trials). The former was also confirmed by SDS-PAGE analysis (Section 2.2.2). 'Gedser' showed to have the null allele, 7+9 and 2+12 alleles for the *Glu-A1*, *-B1* and *-D1*-loci whereas 'KWS Ozon' has 1, 7+9 and 5+10 and 'WaxyDie' contained alleles 2^*, 7+8 and 5+10.

Using a mesh, 15 hand-picked seeds, priorly treated with a fungicide, were carefully put in 2 cm deep holes and were again covered using some loose soil. To limit evaporation and to keep the top soil layer humid during germination, the tubes were covered with plastic foil. The latter was again removed 18 days after sowing (sowing date: 15/12/2017). On the 58th day, plant density was reduced to 6 plats per tube to obtain a sowing density comparable to field conditions (300 kernels m^{-2}. During growth, a broad spectrum fungicide treatment (Evora Xpro, 1.25 l ha^{-1}, active compounds: bixafen (75 g l^{-1}), prothioconazole (100 g l^{-1}) and tebuconazole (100 g l^{-1})) was applied according to the disease pressure. A single application of growth regulator Meteor (chloormequat (0.368 kg l^{-1}) and Imazaquin (0.8 g l^{-1})) (BASF, Belgium) was performed during stem elongation (Zadoks growth stage (Zadoks G.S.) 30) on 06/04/2018.

Irrigation of each tube was done using a pressurized hand-held sprayer on a regular basis to ensure a controlled and accurate water addition. From 13th January to 5th May, all tubes received equal amounts of tap water. In order to obtain drought stress at kernel filling stage, the limited irrigation regime was installed in half of the tubes at ear emergence (Zadoks G.S. 50). During the first 10-day period, tubes under drought conditions did not receive any water. From 16th May to 14th June, half of the water given to the fully irrigated plots was applied to the tubes under drought conditions. This resulted in an overall water reduction of 66 % compared to the fully irrigated plots. Harvesting was done by hand on 2nd July 2018. Ears and above-ground material were collected separately in hermetically sealed high-density

Table 5.1: Overview of the soil characteristics, as determined by the Belgian soil services, and basic compositional features of the applied soil types.

	Coarse sand			Heavy clay		
	Measured	*Suggested*	*Applied* (kg ha^{-1})	*Measured*	*Suggested*	*Applied* (kg ha^{-1})
pH-KCl	6.0	5.3–5.7	-	6.8	6.5–7.1	-
Total organic carbon (%)	1.24	1.8–2.8	-	2.6	1.2–1.6	-
Phosphorus (mg kg^{-1})	3.4	1.1–1.7	30	2.3	1.4–2.2	60
Potassium (mg kg^{-1})	0.70	1.1–1.7	150	2.8	1.9–3.1	50
Magnesium (mg kg^{-1})	1.4	0.6–1.0	20	4.4	2.0–3.1	0
Calcium (mg kg^{-1})	9.1	6.6–13.3	-	62.9	42.5–91.2	-
Sodium (mg kg^{-1})	< 0.094	0.29–0.57	0	0.4	0.37–0.73	0
Sulfur (mg kg^{-1})	1.62	2.6–3.6	80	0.43	3.8–5.2	25
Nitrogen	-[1]	173 (52–51–70)	173 (52–51–70)[2]	-[1]	145 (43–40–62)	145 (43–40–62)[2]
		Measured			*Measured*	
Field capacity (%)		26.9 $\pm$ 1.3			34.6 $\pm$ 1.7	
Soil density (kg m^{-3})		1248			834	
Initial moisture content (%)		11.37 $\pm$ 0.03			29.07 $\pm$ 0.16	
		Drought	*Irrigated*		*Drought*	*Irrigated*
Final moisture content[3] (%)	Top (20 cm)	8.56	8.15		13.46	13.67
	Middle (50 cm)	8.06	8.47		12.92	17.37
	Bottom (80 cm)	6.81	8.23		10.86	19.72

[1] Nitrogen index of coarse sand was 101 (very low) and of heavy clay 117 (very low).
[2] Dates for the N-applications are 15/03/2018, 09/05/2018 and 05/06/2018, basic fertilization (P, K, Mg, Ca) was applied on 22/03/2018.
[3] Values may differ from final value in Figure 5.2C due to the 20th order polynomial fit applied to the data.

polyethylene containers and resealable polypropylene bags, respectively. Threshing of the wheat was done using a Wintersteiger Id180 st4 (Ried im Innkreis, Austria).

5.2.2 Temperature and humidity of soil and environment

Throughout the experiment (starting from 29th January), soil moisture content and temperature, as well as the environmental conditions, were registered automatically using a tailor made monitoring system. Air temperature and humidity were measured using two DHT22 digital temperature-humidity sensors (accuracy: $\pm$ 0.5 °C and $\pm$ 2.0 % relative humidity) connected to a Raspberry Pi model 3b (Raspberry Pi Foundation, Cambridge, UK). For measuring the soil moisture content, 24 modbus RS485 soil moisture sensors (Catnip electronics, Lithuania) were linked to a second Raspberry Pi (model 3b) using a single RJ45-to-USB-converter in combination with a LogiLink NP0036 24-port network switch (illustrated in Figure 5.1). Using a Python script, sensor readings were collected every 30 min and saved in a text file. At the end of the experiment, the text files were downloaded and imported in Excel for further processing.

Based on a preliminary experiment, it was concluded that the values obtained from the soil moisture sensors (arbitrary unit) were linear with the soil moisture content. Prior to installing the sensors in the dedicated tubes, a small sample was taken from each tube (two per soil type) to measure the actual moisture content. This was done by accurately weighing 3.00 g of material in pre-weighed crucibles and reweighing them after drying for 24 hours (in two stages, at 50 °C for 8 hours and at 105 °C for another 16 hours). The initial moisture content was multiplied by the standardized reading from each soil moisture sensor to obtain the estimated soil humidity. Curves shown in Figure 5.2**C** are the 20th polynomial fit to the mean moisture values of the two sensors per soil type $\times$ irrigation.

Besides soil moisture content, both soil and environmental temperatures were monitored throughout the experiment. Air temperature readings from a nearby weather station (Bottelare, Ghent) were used as a comparison (Figure 5.2**A**). In addition, the amount of precipitation and the number of rainy days were

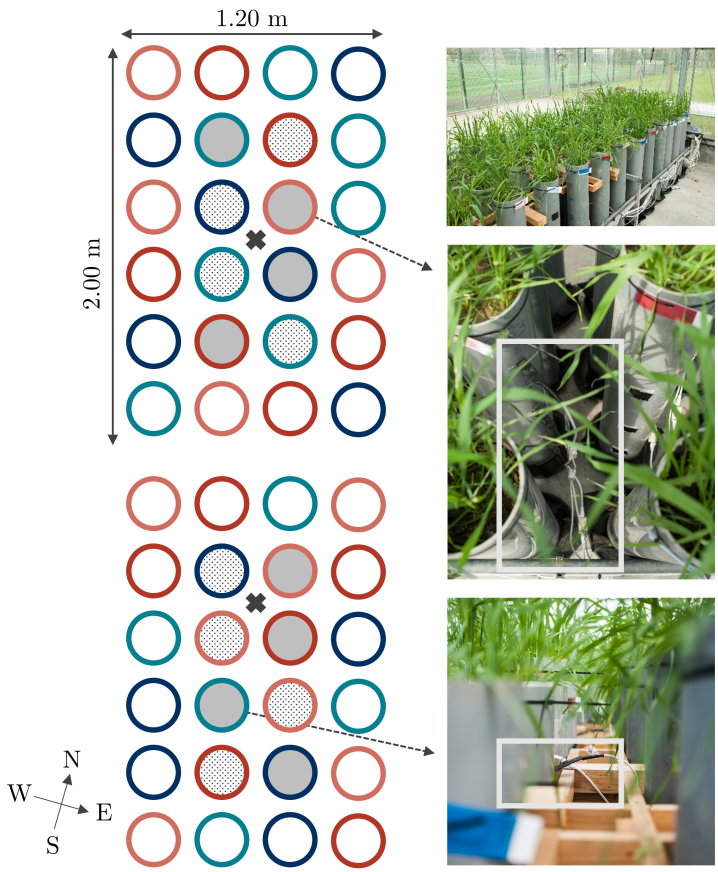

Figure 5.1: A Overview of the trial setup comprising 4 replicates per sample, positioned in a 4 by 12 grid. Soil humidity (solid gray) and soil temperature (dotted pattern) were measured in 8 tubes, environmental (*i.e.* air) temperature and humidity were measured in duplicate using a DHT22 sensor (cross).
Treatments are represented by the different colors: sand-irrigated; sand-drought; clay-irrigated; clay-drought.

compared with the amount of water given through irrigation and the frequency (Figure 5.2**B**). Although the soil temperature was measured separately using commercially available EL-USB-1 sensors (Lascar electronics, Wiltshire, UK), it was chosen to use the temperature readings from the soil-moisture sensors. Values obtained from the commercial sensors were used as validation. As can be seen from Figure 5.2**A**, the average daily temperature of the soil and environment were largely the same with a slightly reduced fluctuation in the soil temperature. On average, ambient temperature in the greenhouse was 4 °C higher than outside temperatures with a maximum difference of 8.9 °C.

The estimated soil moisture graph (Figure 5.2**C**) shows a clear difference in the initial soil moisture between clay (29.1 ± 0.2 %) and sand (11.4 ± 0.1 %). During the pre-drought stage (before May), a significant decrease in the soil moisture content was observed for the clay soil at 80 cm (bottom measurement). From the end of March until mid-April, the top and middle layer of the clay soil started to dry out as a result of the increased ambient temperatures (arrow in Figure 5.2**A**). In irrigated conditions, the decrease in soil humidity is repressed for both the middle (50 cm) and lowest (80 cm) part of the tubes whereas in drought conditions, a continued decrease in the soil humidity was observed at these depths. Interestingly, at 20 cm below the surface (top measurement), irrigation did not result in an increased soil moisture content. For sandy soils, a lower variation in the moisture content at all measuring depths is observed throughout the entire growth period with also a minor long-term decrease. Mostly the bottom part of the sandy soil had a notably lower moisture content under drought conditions.

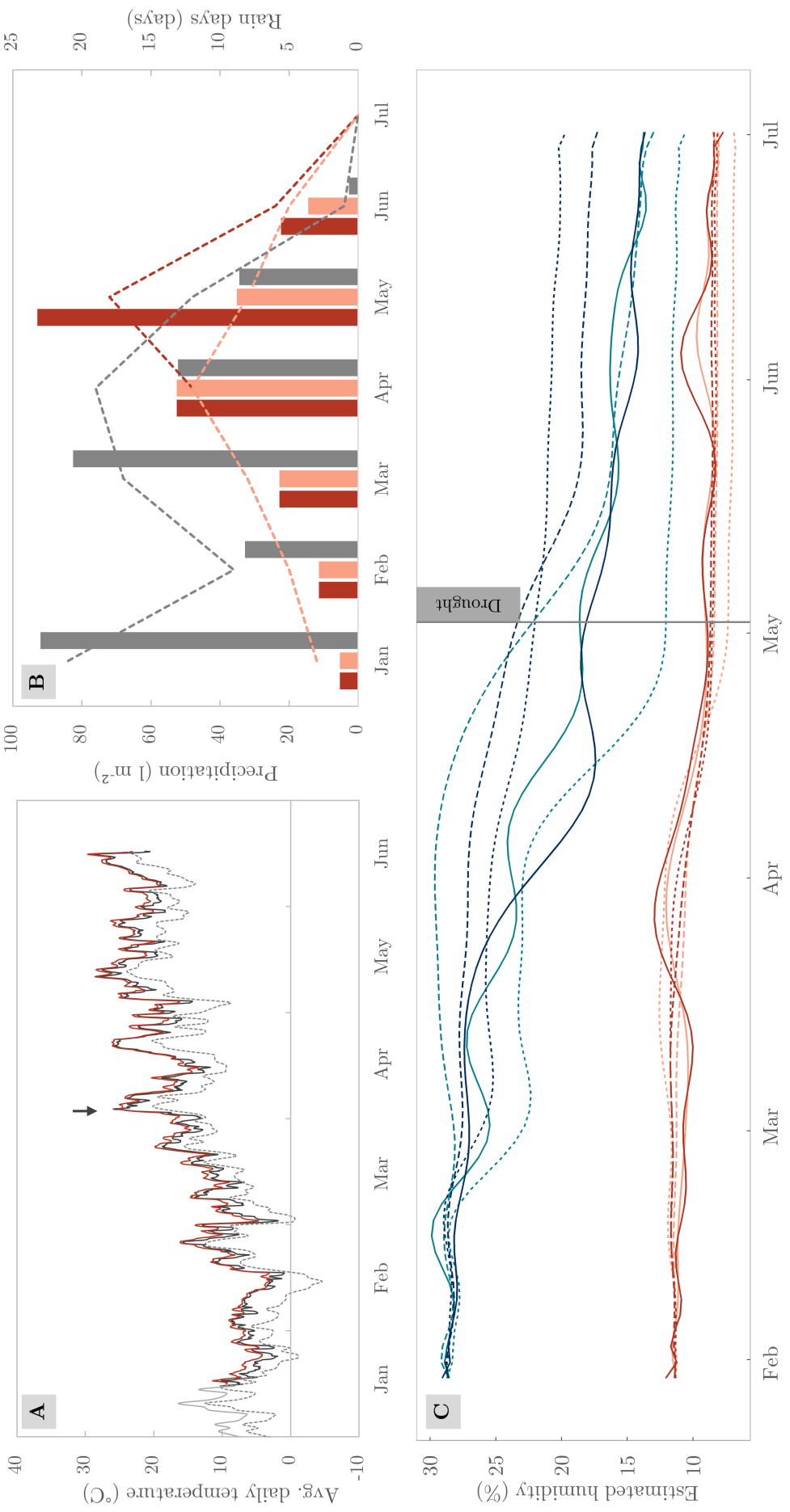

Figure 5.2: A Comparison of the outside temperature - - with inside — and soil — temperatures. **B** Comparison of the amount (bars) of precipitation ▬ and irrigation for both the fully irrigated ▬ and drought ▬ conditions as well as the number of rainy days (dotted lines). **C** Average soil moisture content for top —, middle - and bottom ···· sensors in irrigated (sand — and clay;—) and drought (sand — and clay —) treatments.

5.2.3 Plant growth and development

At seven occasions during the growth period, starting at day 36 until the day of harvest, plant height was measured from the soil surface to the top of the plant (node of the flag leaf or top of the emerged flag leaf) on the main shoot using a roll meter. Each time, all plants from every plot were measured. During height measurements, the number of tillers per plant was also noted. The number of ears and their rate of emergence were counted from ear emergence (Zadoks G.S. 50) and was classified following the Zadoks G.S.. A distinction was made between 'tip just visible', 'quarter', 'half', 'three quarters' or 'fully' emerged ears. The final number of ears was compared to the harvested number of ears as a control. Chlorophyll values were measured on the flag leaves of three to five plants from each plot using a Konica Minolta SPAD-520Plus (Konica Minolta, Tokyo, Japan). Values obtained were already averages of three measurements.

At harvest, ears were cut just underneath the first spikelet to separate them from the straw which was, on its turn, cut just above the soil level. Prior to drying (48 h, 60 °C), plant material was weighed and put in perforated plastic bags. From the weight difference before and after drying, the relative moisture content (MC) of the straw (ears and kernels excluded) was calculated. After threshing the ears, the total number of collected kernels was divided by the number of harvested ears and tillers. In this way, the average number of kernels per ear was obtained. Kernels were also weighed after counting using a Pfeuffer Contador seed counter to calculate the thousand kernel weight (TKW).

To estimate the actual drought tolerance, the drought susceptibility index (S) was calculated according to Equation 5.1. By comparing the grain yield (YLD) under drought (Y_{ds}) and the YLD under (near) optimal conditions (Y_{no}) for a specific genotype with the grain YLD under drought and near optimal conditions for all genotypes (X_{ds} and X_{no} respectively), the reduction in YLD caused by unfavorable conditions was calculated per genotype (Denčić et al., 2000).

$$S = \frac{(1 - \left[\frac{Y_{ds}}{Y_{no}}\right])}{(1 - \left[\frac{X_{ds}}{X_{no}}\right])} \tag{5.1}$$

5.2.4 Kernel properties

Dimensional parameters (AS, length-to-width-ratio (LtWr) and circularity (CIRC)) of the kernels were determined using the SmartGrain image analysis software, version 1.2 (Tanabata et al., 2012) as described in Section 3.2.2.1 (page 101). All kernels of a single plot were scanned together. After the determination of kernel characteristics, wholemeal samples were obtained by milling the kernels using a water cooled analytical mill (M20 universal mill, IKA, Staufen, Germany). Due to the limited amount of samples, no moisture determination was performed. As alternative, samples were equilibrated for 48 hours in a desiccator to obtain similar moisture contents. Results presented in the current research are therefore not expressed on dry matter but 'as is', assuming an equal moisture content.

Subsequently, PROC and total starch (TSt) of the wholemeal was determined as described in Section 3.2.3 (page 102 and onwards). For the PROC, a single measurement per plot was performed whereas

the TSt content was at least measured in duplicate per plot. Protein composition was analyzed using the reversed-phase (RP) high-performance liquid chromatography (HPLC)-approach described in Chapter 2. PGr and the ratio of HMW-GS to LMW-GS (HMW:LMWr) was determined similar to the standard protocol with a slight adaptation of the cutoff retention time between HMW-GS and LMW-GS. Of each sample (genotype $\times$ soil type $\times$ irrigation treatment), samples from two different plots were analyzed. Technical replicates (replicate injection from same HPLC-vial) were excluded from the data processing as the variation due to extraction was found to be each time larger compared to the technical repeatability.

5.3 Results and discussion

5.3.1 Plant development and yield

Plant height of cereal crops is a crucial trait related to grain yield (YLD) and is known to be typically quantitatively inherited (Wu et al., 2010). According to Hodgkinson et al. (2017), the optimum plant height for a maximum YLD is in the range of 70–80 cm. In the current study, the final plant height varied between 37.9–51.2 cm (Table 5.2) which was significantly lower than the optimal height. Compared to the heights of wheat grown in pots under mild greenhouse conditions in research of Simón et al. (2005), current values are on average 27.8 % lower. This might be the result of an insufficient nutrient availability during stem elongation or the overall presence of abiotic stresses due to the continuously high ambient temperatures at the beginning of May (average maximum daily temperature $\approx$ 32 °C).

Differences for the final plant height could be related to both the soil type and genotype ($p < 0.001$ for both variables) in contrast to the irrigation treatment of which no significant effect was observed ($p = 0.399$). Highly significant interactions with genotype were present for both soil type and irrigation treatment. This can be attributed to 'KWS Ozon' which shows a continued growth under drought conditions (Figure 5.3) compared to an unexpected reduced growth rate on fully irrigated clay soil. Furthermore, 'WaxyDie' and 'Gedser' display a more similar behavior with a continued height increase unto their final height when grown on a clay soil while a clear stagnation in their growth was observed for sandy soil treatments.

These $\mathbb{G} \times \mathbb{E}$-effects were reported earlier by various researchers including Eid (2009) who also found a relatively low broad-sense heritability (H^2) for plant height in both control (29.2 %) and drought-stress (16.3 %) conditions. This is partially in accordance with findings from Wu et al. (2010) who—although observing considerable additive and epistatic genetic effects—found clear interactions with the environment. Moreover, these effects were influenced by the growth stage with specific quantitative trait loci's being distinctly more adaptable to well-watered conditions in earlier growth stages whereas others were found to be more expressed during the entire growth period.

Besides plant height, the total number of ears and the rate of ear emergence are important developmental features which are in turn also related to yielding parameters (Eid, 2009). Moreover, a strong correlation ($\rho = 0.917$, $p \leq 0.001$) between the number of tillers and the number of ears was found. When comparing the ratio of ears to tillers (*i.e.* the number of productive tillers), a higher value is obtained for plants grown on clay soil (92 ± 4 %) compared to sandy soil plants (70 ± 12.5 %). When considering interaction effects (using a generalized linear model with Poisson regression), a primary effect of solely soil type on the total

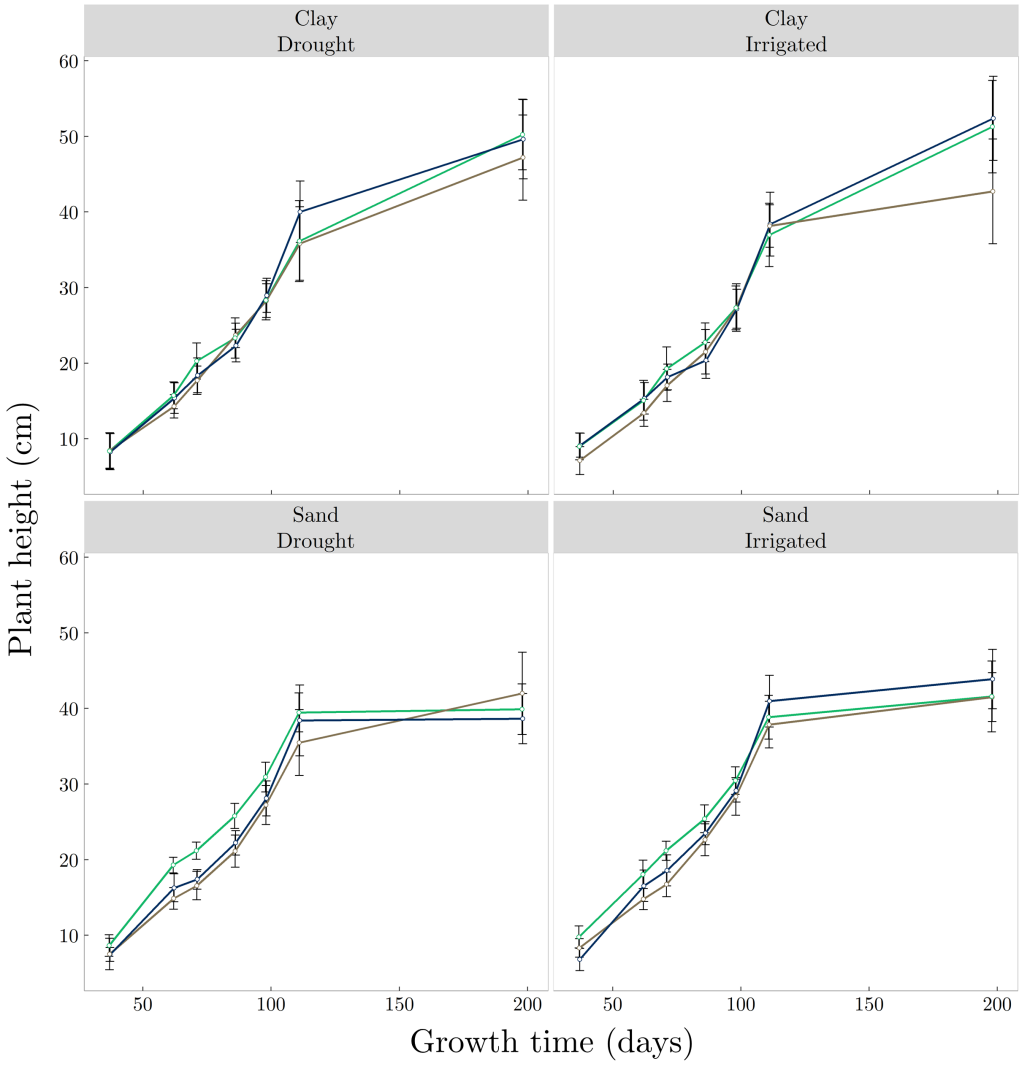

Figure 5.3: Plant height in function of growth time (expressed in days) per cultivar ('Gedser' —, 'KWS Ozon' —, 'WaxyDie' —) and treatment (soil type and irrigation treatment). Error bars represent the standard deviation over four biological repetitions.

number of emerged ears can be noted (z[47] = -4.857, $p \leq 0.001$). For all genotypes, a significantly lower amount of ears per plot (6 plants) was obtained when cultivars were grown on a sandy soil (5.5 ± 1.3 ears per plot). However, also when grown on clay soils, a low average number of ears per plot (9.6 ± 1.2 ears per plot) was obtained. This might be a direct result of the prevailing greenhouse conditions although comparable values (expressed in ears per *square meter*) were reported by Rebetzke et al. (2016) and Kilic and Yağbasanlar (2010). Under field conditions, the number of ears is generally higher (1.46–3.73 ears *per plant*) but this was also found to be dependent from the sowing density with a decreasing number of ears when sowing densities increased (Li et al., 2016c). The relatively high greenhouse temperatures during vernalization (January, February) may have lowered the number of tillers (Steinfort et al., 2017) although this is also $\mathbb{G}$-dependent and influenced by day length. As is illustrated in Figure 5.4**B**, the $\mathbb{G}$-effect showed however to be insignificant ($p = 0.151$) with 'Gedser' having an only slightly lower number of ears per plot compared to 'KWS Ozon' and 'WaxyDie'. Despite a notably lowering effect was observed at limited irrigation (8.2 *vs.* 7.1 ears per plot), the difference was insignificant.

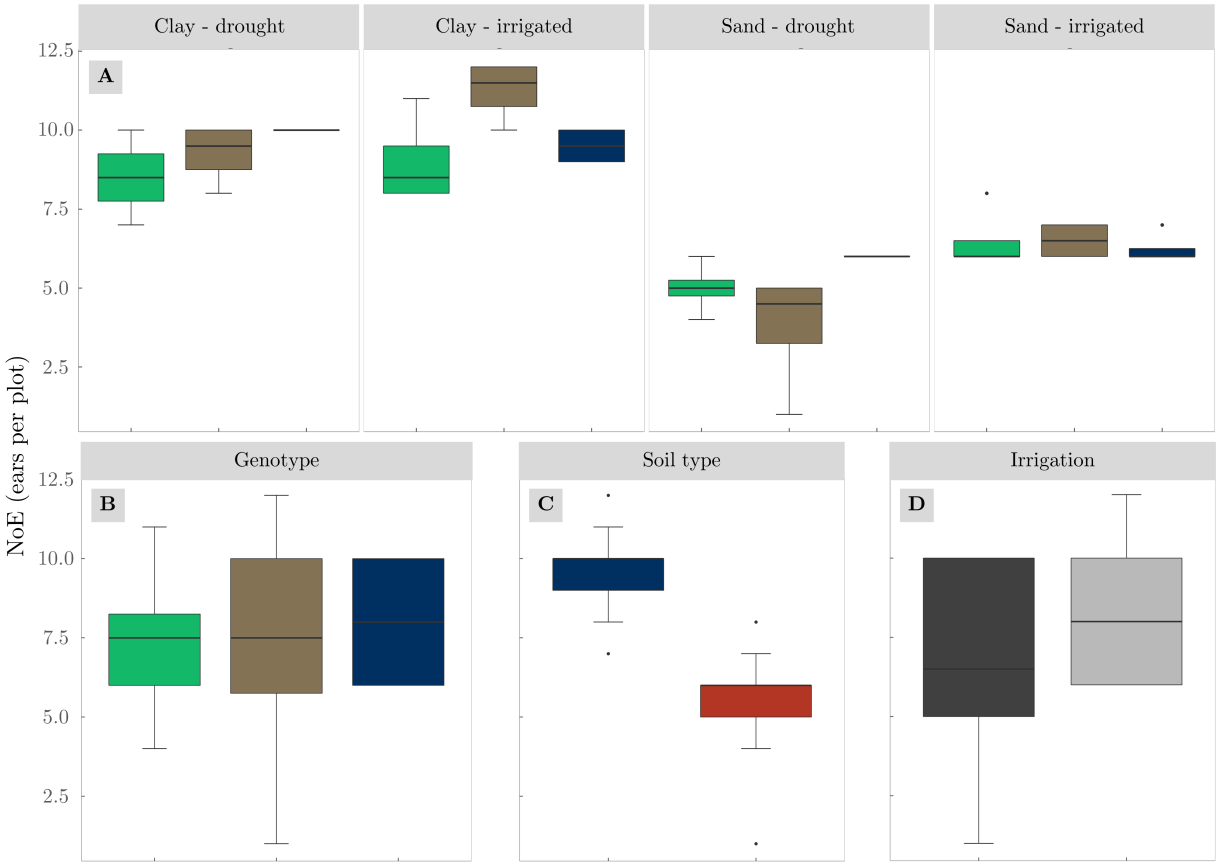

Figure 5.4: Ears per plant (NoE) **A** per sample (genotype × soil type × irrigation treatment), **B** cross-treatment (per genotype) ('Gedser' —, 'KWS Ozon' —, 'WaxyDie' —), **C** per soil type (clay —, sand —) and **D** per irrigation treatment (fully irrigated —, limited irrigation —)

> Plant growth (height and number of (productive) tillers) was subjected to an interaction between genotype and environment. General decreases in plant productivity were observed at increased abiotic stress, partially following from the treatment (drought-stress) but also from the prevailing greenhouse conditions (shortened vernalization, high mean temperatures). A significantly higher number of ears were noted for clay soil.

Soil Plant Analysis Development (SPAD)-values provide an indication of the chlorophyll content in the flag leaf of the plant and thus, the overall plant health. Measurements on the flag leaf of the main culm were performed at three moments during plant development, with a first measurement at 6th May 2018 to measure genotypic differences or variation solely attributable to the soil type. Although plants grown on clay had notably higher SPAD-values than sand (40.09 ± 2.83 *vs.* 38.44 ± 2.66), differences were insignificant. At the third measuring date (30th May, approximately one month after installing the drought treatment), leaf senescence was already present for all cultivars grown on a sandy soil under limited irrigation conditions. Relative to the second measurements (8th May), SPAD-values for 'WaxyDie' showed to have decreased most (37.6 %) compared to 'Gedser' (26.1 %) and 'KWS Ozon' (20.2 %). Interestingly, upon subdividing the treatments, it was found that SPAD-values increased for 'Gedser' and 'WaxyDie' (2.8–7.9 %) on all irrigated soils whereas only minor a decrease was observed for these cultivars on a non-irrigated clay soil (-0.4 and -3.3 % respectively). SPAD-values for 'KWS Ozon' decreased under all

Table 5.2: Overview of the developmental attributes of the plant and yielding parameters.

			Plant development				Biomass				Yielding parameters	
Soil	**Irrigation**	**Cultivar**	$plant_{height}$ (cm)	*SPAD*	*NoT* (#)	*NoE* (#)	$plant_{weight}$ (g)	*MC* (%)	*NoK* (#)	*KpE* (#)	*TKW* (g)	*YLD* (t ha^{-1})
Clay		Gedser	49.8 ± 3.0	47.8 ± 1.1	9.0 ± 1.4	8.0 ± 1.2	15.8 ± 0.5	1.6 ± 1.1	235 ± 39	29.5 ± 4.7	18.1 ± 2.5	2.37 ± 0.30
	Drought	KWS Ozon	41.8 ± 3.3	52.8 ± 2.4	10.8 ± 1.3	9.3 ± 1.0	16.4 ± 0.4	4.0 ± 2.8	200 ± 17	21.6 ± 1.3	20.9 ± 1.8	2.36 ± 0.23
		WaxyDie	51.2 ± 5.2	46.6 ± 1.8	10.3 ± 0.5	9.8 ± 1.0	16.7 ± 0.7	2.7 ± 0.5	293 ± 54	30.1 ± 5.0	14.6 ± 1.8	2.45 ± 0.68
		Gedser	41.3 ± 3.2	53.2 ± 6.3	9.0 ± 1.4	8.8 ± 1.5	16.6 ± 1.0	3.3 ± 1.6	303 ± 43	34.8 ± 2.4	21.2 ± 1.8	3.61 ± 0.30
	Irrigated	KWS Ozon	40.3 ± 0.2	56.1 ± 1.5	11.8 ± 1.0	10.8 ± 1.0	17.1 ± 0.4	2.1 ± 0.3	259 ± 60	24.0 ± 5.4	22.2 ± 2.3	3.20 ± 0.56
		WaxyDie	43.5 ± 0.7	51.2 ± 2.1	10.3 ± 0.5	9.5 ± 1.0	17.7 ± 0.7	2.6 ± 0.4	348 ± 55	37.0 ± 7.5	21.0 ± 2.4	4.08 ± 0.26
Sand		Gedser	49.6 ± 2.7	47.4 ± 2.5	7.0 ± 0.8	4.8 ± 1.0	13.9 ± 1.4	4.1 ± 0.8	33 ± 17	7.3 ± 4.2	26.1 ± 8.8	0.43 ± 0.13
	Drought	KWS Ozon	45.9 ± 2.3	50.1 ± 0.9	7.5 ± 1.0	3.5 ± 1.7	12.5 ± 0.2	6.0 ± 2.3	11 ± 8	2.5 ± 2.0	30.1 ± 15.4	0.17 ± 0.13
		WaxyDie	49.2 ± 2.1	45.7 ± 2.1	8.0 ± 0.8	6.3 ± 1.0	13.9 ± 0.9	3.0 ± 0.3	48 ± 14	7.7 ± 2.2	19.8 ± 1.4	0.53 ± 0.12
		Gedser	39.2 ± 1.2	57.3 ± 5.5	9.5 ± 0.6	6.5 ± 1.0	14.5 ± 0.8	2.7 ± 0.3	177 ± 28	27.5 ± 4.9	27.2 ± 7.7	2.63 ± 0.23
	Irrigated	KWS Ozon	41.8 ± 2.1	57.0 ± 1.8	8.0 ± 0.8	6.5 ± 0.6	14.8 ± 1.3	2.8 ± 0.2	181 ± 26	28.1 ± 4.7	19.9 ± 3.0	2.03 ± 0.29
		WaxyDie	37.9 ± 2.7	57.9 ± 5.5	8.3 ± 1.5	6.3 ± 0.5	15.8 ± 0.3	2.6 ± 0.3	195 ± 33	31.3 ± 5.7	20.8 ± 2.4	2.27 ± 0.32

Plant height ($plant_{height}$); number of tillers (NoT); number of ears (NoE); plant weight ($plant_{weight}$); number of kernels (NoK); kernels per ear (KpE).

Table 5.3: Overview of the morphological and compositional attributes of the kernel per treatment and cultivar.

			Kernel morphology			Protein			Starch
Soil	**Irrigation**	**Cultivar**	*AS* (mm^2)	*LtWr*	*CIRC*	*PROC* (%)	*PGr*	*HMW:LMWr*	*TSt* (%)
Clay		Gedser	10.18 ± 0.85	2.15 ± 0.05	0.704 ± 0.008	22.4 ± 1.2	1.15 ± 0.08	0.58 ± 0.01	55.9 ± 0.8
	Drought	KWS Ozon	11.17 ± 0.86	2.05 ± 0.07	0.723 ± 0.014	24.1 ± 0.7	1.03 ± 0.02	0.76 ± 0.03	56.2 ± 0.6
		WaxyDie	8.58 ± 0.43	1.95 ± 0.05	0.747 ± 0.010	22.0 ± 1.3	0.98 ± 0.10	0.76 ± 0.08	58.5 ± 4.6
		Gedser	11.29 ± 0.49	2.12 ± 0.02	0.711 ± 0.005	18.9 ± 0.6	1.22 ± 0.08	0.54 ± 0.04	58.8 ± 3.6
	Irrigated	KWS Ozon	11.23 ± 0.73	2.04 ± 0.07	0.724 ± 0.010	20.8 ± 1.1	0.86 ± 0.04	0.68 ± 0.00	56.5 ± 0.9
		WaxyDie	10.03 ± 0.21	1.78 ± 0.05	0.775 ± 0.006	17.4 ± 1.0	0.95 ± 0.07	0.63 ± 0.02	60.3 ± 3.4
Sand		Gedser	12.33 ± 2.28	1.93 ± 0.14	0.739 ± 0.023	25.2 ± 1.0	1.66 ± 0.08	0.59 ± 0.01	58.0 ± 4.2
	Drought	KWS Ozon	14.03 ± 7.45	2.02 ± 1.02	0.728 ± 0.365	24.8 ± 12.7	1.16 ± 0.09	0.60 ± 0.07	53.2 ± 2.6
		WaxyDie	10.00 ± 0.29	1.94 ± 0.04	0.743 ± 0.007	24.8 ± 0.4	1.12 ± 0.03	0.79 ± 0.05	53.4 ± 3.2
		Gedser	12.30 ± 1.97	2.03 ± 0.08	0.726 ± 0.011	21.1 ± 0.9	1.50 ± 0.17	0.54 ± 0.02	55.6 ± 1.0
	Irrigated	KWS Ozon	10.72 ± 1.24	2.22 ± 0.06	0.692 ± 0.011	25.9 ± 1.0	0.96 ± 0.01	0.70 ± 0.06	51.4 ± 3.7
		WaxyDie	9.30 ± 0.99	1.87 ± 0.07	0.757 ± 0.011	21.3 ± 0.8	0.94 ± 0.12	0.67 ± 0.03	55.9 ± 2.3

conditions when comparing the two last measurements (-1.5 to -5.7 %). Under drought conditions on sandy soil, SPAD-values for 'WaxyDie' decreased remarkably more (-37.6 %). The immediate decrease for 'KWS Ozon' may indicate that N-translocation to the kernel was promoted earlier.

Kilic and Yağbasanlar (2010) found that the number of grains/kernels per ear is the yield component (besides the number of fertile tillers and the grain weight) that is the most sensitive to high temperatures and drought. It is also argued that a $\mathbb{G}$-effect for this trait was present (Denčić et al., 2000). In both studies, a significant relative decrease in the number of kernels (16.6 to 19.8 %) was observed for plants grown under drought conditions as a result of a poor assimilation and reduced translocation of photosynthates to the grain. Similar effects for plants grown on a clay soil were observed in the current research with relative reductions between 18.8 % for 'WaxyDie' to 10.0 % for 'KWS Ozon'. However, looking at sandy soils, a very low number of kernels per ear could be noted for drought conditions (Table 5.2).

Irrigation on its own had no significant effect ($p = 0.710$) on the thousand kernel weight (TKW) whereas soil type and genotype both had a high impact ($p \leq 0.008$). Interactions between irrigation and soil type or genotype were also found to be significant. 'WaxyDie' had the lowest TKW (19.0 g) compared to 'KWS Ozon' and 'Gedser'. These findings are in contrast with literature in which a reduction of 21.7 to 12.0 % for TKW was reported for drought treatments compared to the marginally lower decrease (2.3 %) observed in the current study (Denčić et al., 2000; Kilic and Yağbasanlar, 2010; Mwadzingeni et al., 2016). Values are however generally low (14.6–30.1 g compared to ≥ 40 g) which may be attributed to the shortened grain filling period resulting from the overall high temperatures under greenhouse conditions.

Grain YLD strongly varies from 0.19 to 4.33 t ha^{-1} with a highly significant influence of all variables ($p \leq 0.010$). Nevertheless, no significant differences in the mean values per cultivar could be observed ('Gedser' = 2.26 t ha^{-1}, 'KWS Ozon' = 2.07 t ha^{-1} and 'WaxyDie' = 2.34 t ha^{-1}). Moreover, as illustrated in Figure 5.5, a treatment-specific (soil type × irrigation) correlation with the TKW was observed.

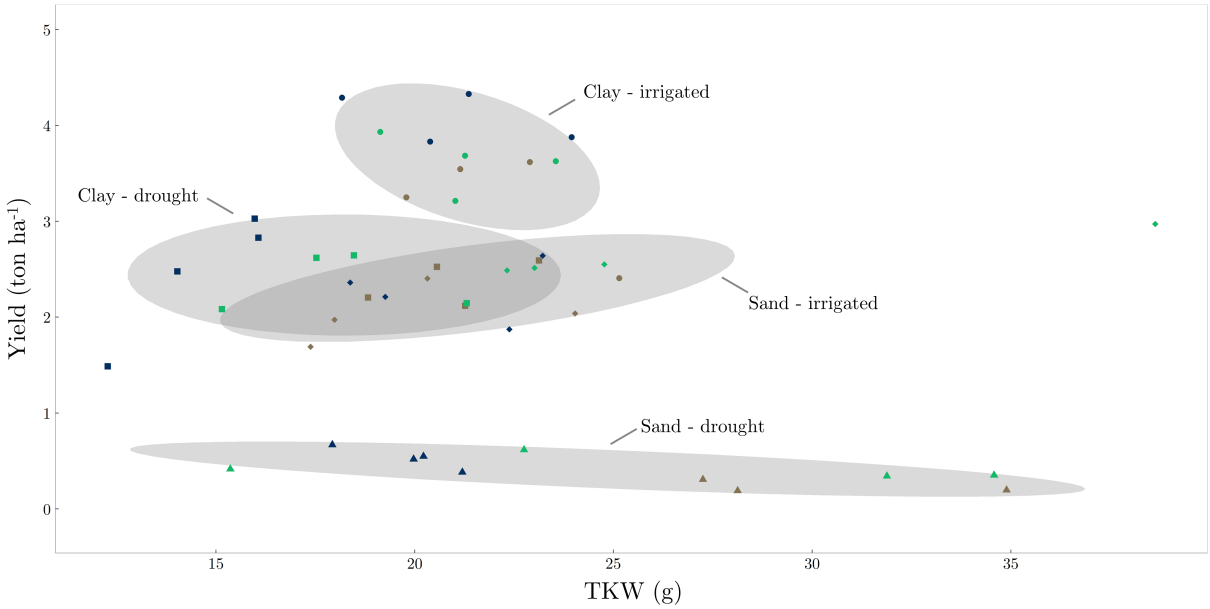

Figure 5.5: Relation between thousand kernel weight (TKW) and yield per plot for the different treatments (sand and clay; irrigated and drought conditions).

To further study the $\mathbb{G}$-dependency, the drought susceptibility index (S) can be determined. A higher S indicates an increased YLD reduction due to drought stress. For clay, the lowest S was found for 'KWS Ozon' (0.77) while 'WaxyDie' and 'Gedser' were more susceptible to drought (resp. 1.01 and 1.17). The opposite was found when genotypes were cultivated on a sandy soil as 'KWS Ozon' had a S-value of 1.07 while 'Gedser' and 'WaxyDie' were slightly more tolerant (resp. 1.01 and 0.93).

YLD is frequently correlated with plant height, however, correlations are gravely varying between studies as well as between cultivation conditions. As wheat yield is defined by three components (productive spikes per unit area, number of grains per spike and kernel weight), Eid (2009) separately investigated correlations with plant height. In heir research, a considerably higher r-value was found for plant height and TKW under stressed conditions ($r = 0.858$) compared to the control treatment ($r = 0.424$). In contrast, research by Kilic and Yağbasanlar (2010) could not support these findings. They did not observe a correlation between TKW and YLD ($r = 0.059$) in a study on durum wheat (*Triticum turgidum* D.) whereas a highly significant negative correlation between YLD and plant height ($r = -0.53$) existed under drought conditions. Hence, no correlation between plant height and TKW was noted. In drought stress conditions, increased plant height may be beneficial for exploiting soil moisture at depth and for enabling mechanical harvesting, therefore leading to increased biological as well as grain YLD. (Wu et al., 2010). No general or treatment-specific correlations between yielding parameters and plant height were observed in the current research (Table 5.4).

> A significant yield decrease was noted for plants grown under drought conditions despite equal values for the thousand kernel weight between treatments. Smaller plants were accompanied by less productive tillers with less kernels (with a similar weight) per ear and thus, a lower overall yield.

5.3.2 Kernel morphology and composition

Although morphological characteristics such as area size (AS), perimeter, length and width (or circularity (CIRC)) of grains are often studied, no consensus on the relationship with YLD or compositional properties exists (as discussed in Section 1.2.3.1, page 16). However, as assimilate translocation lays at the basis of the kernel size, a difference can be expected as drought stress may severely impede these processes. A primary effect of the genotype was observed with 'WaxyDie' having a notably lower average AS of 9.48 ± 0.80 mm^2 compared to 'KWS Ozon' and 'Gedser' which both had an AS of approximately 11.58 $\pm$ 1.75 mm^2 (Table 5.3). This $\mathbb{G}$-effect was not influenced by interactions with soil type or irrigation treatment. Soil type itself, however, had a significant effect on AS with approximately 10 % larger kernels when grown on sand (11.33 ± 2.22 mm^2 *versus* 10.41 ± 1.14 mm^2). For the length-to-width-ratio (LtWr) and CIRC, no effect of soil type or irrigation condition was observed, only a highly significant genotype effect ($p \leq 0.001$) was perceived. The smaller AS of the kernels from 'WaxyDie' was accompanied by a higher CIRC compared to the other two cultivars.

As described in literature, drought stress increases the relative protein concentration (PROC) of wheat as a result of a decreased carbohydrate accumulation during the kernel filling stage. In the current study, extremely high protein contents ranging from 16.5 to 28.5 % were obtained. When the protein contents are compared with the values noted for these cultivars grown under field conditions ('KWS Ozon' = 10.3

$\pm$ 0.8 %, 'Gedser' = 10.0 $\pm$ 0.9 % and 'WaxyDie' = 8.5 $\pm$ 0.1 %), more than a doubling of the content was observed. These increases are in the same order as the values reported by Flagella et al. (2010).

A significant effect of all factors on the PROC was found. Samples grown on a sandy soil had an average PROC of 23.8 $\pm$ 2.3 % which was 2.9 % (absolute) higher than for samples grown on a clay soil. A similar effect was observed when looking at the irrigation treatment with fully irrigated samples having a lower PROC compared to drought treated samples (20.9 $\pm$ 2.8 % versus 23.9 $\pm$ 1.8 %). On one hand, this shows the entanglement of both variables, soil type and irrigation treatment, implying a higher water retention capacity of the clay soil. On the other hand, this supports the finding that drought stress results in an increased grain PROC. The lack of a significant interaction term for soil type and genotype illustrates that all genotypes were affected in a similar magnitude as a result of the irrigation treatment (or soil type). The main $\mathbb{E}$-effect also comes to expression by the relatively low H^2 of 16 % whereas soil type and irrigation explained 28 and 30 % of the variance respectively.

The robust negative correlation between the protein and starch content—which has been reported previously by various researchers—was also observed in these experiments (r = -0.539, p $\leq$ 0.001). However, from Figure 5.6, in which the protein and starch content are set out against the grain YLD, it can be seen that the relation is not linear over the entire range. From this correlation plot, it can be seen that a group of samples with very low yields ($\leq$ 1 t ha^{-1}) have a similar starch content as samples for which higher yields were observed (1.5–3.0 t ha^{-1}). From this point on, a more linear trend is present. A similar effect can be noted for the protein content in relation to the yield. At very low yields, extremely high protein contents are found (sandy soil, drought treatment). However, equally high contents were found at higher yields. These findings are also supported by the outcome of the analysis of variance which showed a sole effect of the soil type on the total starch (TSt). Moreover, only a weak correlation between the TSt and the YLD was established (r = 0.416, p = 0.005), implying a point at which both variables scale independently from each other.

> Strongly reduced yields were accompanied by extremely high protein contents although a non-linear relation was observed. Despite significant differences between soil types were observed, the differences were attributed to drought stress. This also implied the entanglement of both factors (irrigation and soil type) in the development of drought stress.

PROC showed to be negatively correlated with biomass (*i.e.* number of ears, number of kernels per ear, plant weight) implying that an increased use of nutrients for plant development results in a decreased protein assimilation in the kernel. Mainly the YLD and the number of kernels (which is a part of the yield) are strongly correlated with PROC. Moreover, a negative relationship between protein and starch content was present. In contrast with literature, no evidence for a relationship between protein content and composition (higher prolamin (PRO) content) was found in the data. The correlation analysis also showed a strong positive correlation between TKW and the AS of the kernels. This indicates that shrunken and shriveled kernels (with a low AS) are low in TSt. Also, no correlation between AS and PROC was observed in contrast to the positive relationship between AS and plant moisture content (MC).

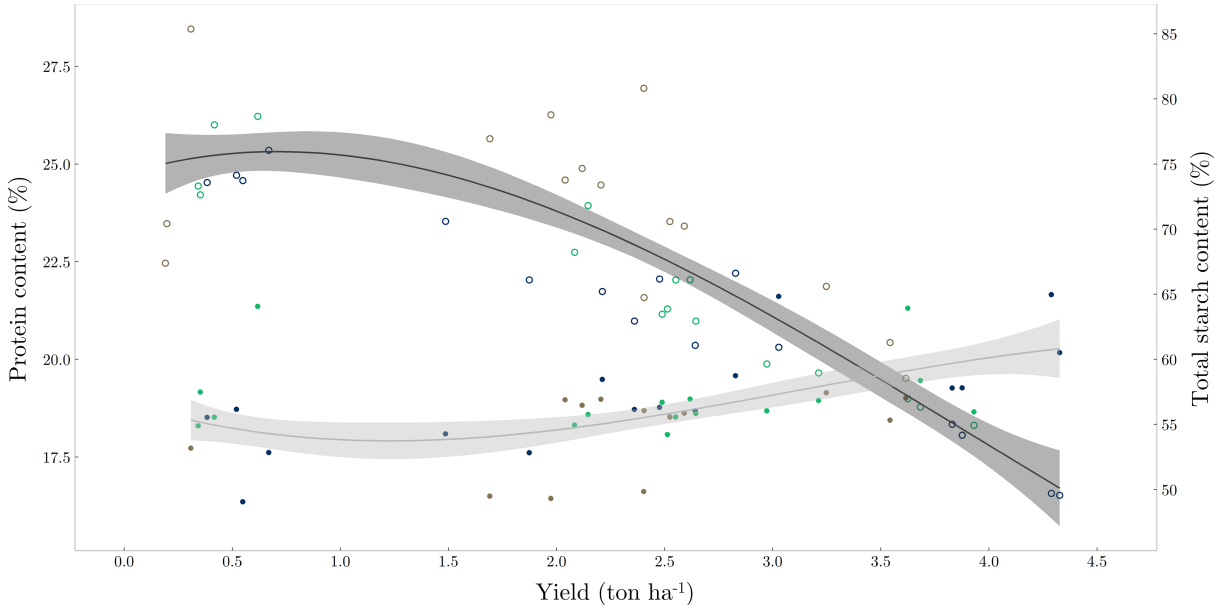

Figure 5.6: Correlation plot illustrating the non-linear relationship between grain yield and starch content ●, as well as protein content ○ for the three cultivars ('Gedser' ●, 'KWS Ozon' ●, 'WaxyDie' ●). Grey rulers show the 95 % confidence intervals.

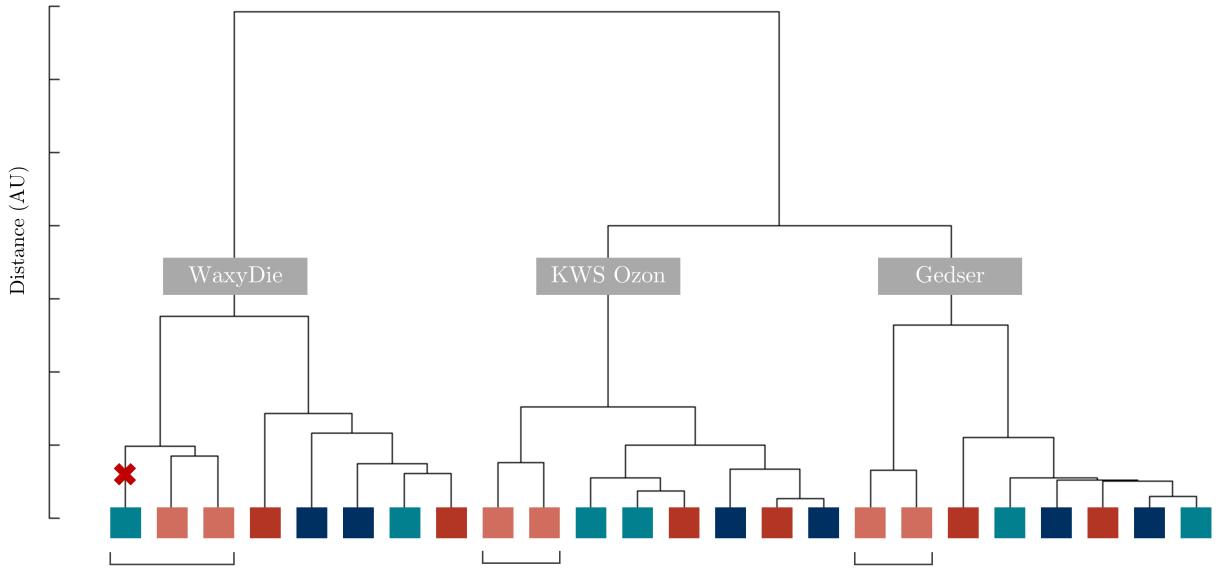

Figure 5.7: Dendrogram of gluten protein (prolamin and glutelin) using agglomerative hierarchical clustering (Ward's D2-method) displaying the diverging protein composition (clay-irrigated —, clay-drought — ; sand-irrigated — ; sand-drought —) of samples grown on a sandy soil under drought conditions.

5.3.2.1 Protein composition

Protein composition of the wholemeal samples was screened by means of high-performance liquid chromatography (HPLC) (Chapter 2). The dendrogram based on the chromatographic data of the gluten-forming proteins (PRO and glutelin (GLU)), is shown in Figure 5.7. A primary distinction between the three cultivars was observed with 'WaxyDie' being more distinct from 'KWS Ozon' and 'Gedser'. Within each of these clusters, two main groups can be distinguished. Firstly, a group containing samples from both irrigated clay and sandy soils as well as samples which are grown on a clay soil under drought

conditions can be found. It is assumed that the latter group did not experience drought stress to a full extent. This also corresponds with the soil moisture measurements (Figure 5.2) from which it can be derived that clay under drought conditions had a slightly higher moisture content compared to irrigated sandy soil (10.86 *vs.* 8.23 %). To enlarge the effect of the different soil types, all tubes could have been brought to the same initial moisture content.

Secondly, the two biological repetitions of samples grown on sandy soils with limited irrigation are grouped separately. Within the former groups, no clear distinction between subgroups can be made as the similarity between biological repetitions is lower than the similarity between different treatments. A single sample for clay soil under drought conditions was found in the sand-drought group for 'WaxyDie'. This is probably a misclassification due to a not-correctable retention shift in the raw chromatograms.

These results clearly show the effect of severe drought stress, mainly present on a sandy soil which may be attributed to a reduced water retention capacity, on the composition of the gluten-forming proteins. Although this data is not presented here, the discussed distinction was found for both PRO and GLU implying that the effect occurred in both protein fractions. Even more, for the GLU fraction, samples from 'KWS Ozon' grown under drought stress conditions on a sandy soil were clustered together with 'Gedser'.

In contrast to the output from the dendrogram, no correlations were found between protein compositional attributes and plant developmental characteristics (Table 5.4). This might be a result of the treatment-specific relationships with the protein composition resulting in weak overall correlations. Furthermore, no information on the functional properties could be predicted on the basis of the chromatograms using the partial least squares (PLS) regression-models described in Chapters 2 and 3. The chromatographic data was however unsuitable due to uncorrectable shifts in these measurements compared to the chromatograms in the training set. Moreover, compositional differences resulting from the extreme environmental conditions may supersede conventional assumptions which are not (yet) enclosed in the models.

> The composition of the gluten-forming protein was affected by drought-stress again emphasizing the mitigating effect of clay soil on the severity. Genotypic effects were however larger compared to treatment effects although for glutelin, a more significant shift was observed.

Table 5.4: Correlations (Pearson correlation coefficients) of the plant developmental and compositional variables.

	Plant development										Kernel morphology and composition						
Variable	$plant_{height}$	SPAD	NoT	NoE	$plant_{weight}$	MC	NoK	KpE	TKW	YLD	AS	LtWr	CIRC	PROC	PGr	HMW:LMWr	TSt
$plant_{height}$	1																
SPAD	−0.95 ***	1															
NoT	n.s	n.s	1														
NoE	n.s	n.s	0.91***	1													
$plant_{weight}$	n.s	n.s	0.79**	0.93***	1												
MC	n.s	n.s	n.s	−0.61 *	−0.64 *	1											
NoK	n.s	n.s	0.72**	0.86***	0.94***	−0.65 *	1										
KpE	n.s	n.s	n.s	0.68*	0.83***	−0.71 *	0.93***	1									
TKW	n.s	n.s	n.s	−0.65 *	−0.64 *	0.66*	−0.60 *	n.s	1								
YLD	n.s	n.s	0.73**	0.81**	0.90***	−0.61 *	0.96***	0.92***	n.s	1							
AS	n.s	n.s	n.s	n.s	−0.62 *	0.71**	n.s	−0.59 *	0.93***	n.s	1						
LtWr	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	1					
CIRC	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	−0.99 ***	1				
PROC	n.s	n.s	n.s	−0.60 *	−0.74 **	n.s	−0.80 **	−0.74 **	n.s	−0.86 ***	n.s	n.s	n.s	1			
PGr	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	1		
HMW:LMWr	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	−0.59 *	1	
TSt	n.s	n.s	n.s	n.s	0.67*	n.s	0.61*	n.s	n.s	0.58*	n.s	n.s	n.s	−0.74 **	n.s	n.s	1

$p \leq 0.001 = ^{***}$; $p \leq 0.01 = ^{**}$; $p \leq 0.5 = ^{*}$.
Plant height ($plant_{height}$); number of tillers (NoT); number of ears (NoE); plant weight ($plant_{weight}$); number of kernels (NoK); kernels per ear (KpE).

5.4 Conclusion

This experimental setup allows to study plant growth and grain composition in relation to the soil type in interaction with drought stress. To estimate the magnitude of the stress, soil humidity was monitored thereby providing insight in the prevailing conditions under which the plant development took place. Moreover, this has given an indication of the different ways in which the soil moisture content and distribution evolves under the investigated treatments. The inclusion of compositional attributes further elucidated how both variables (soil type and irrigation conditions) contribute to changes in the protein content and its composition and the total starch content. Inclusion of these attributes can be considered compelling when studying wheat quality and its relation with cultivation conditions. With the primary focus on grain yield, these quality traits are however frequently overlooked.

The use of moisture sensors has provided interesting information on how both soil types react differently under the same environmental conditions. Without a relation with the availability of water for the crop, this could not be correlated well with the observed effects. Moreover, by including the factor irrigation (*i.e.* drought), the impact of soil type was partially reduced due to their high mutual interaction. Clay soil has an increased water retention capacity which extends the time before drought stress occurs. It also influences root architecture and water uptake efficiency, thereby further increasing the complexity the comparison.

A major effect of drought stress was observed for the number of kernels which also resulted in a strongly varying yield. Besides the strong genetic effect, a significant impact of treatment was also noted. Interestingly, thousand kernel weight was only marginally reduced which is different from earlier research. Also, the frequently reported correlation between thousand kernel weight and grain yield could only be confirmed for the irrigated treatments although correlation coefficients were low. The susceptibility of different wheat cultivars to drought stress was emphasized, although the magnitude of this effect also varied according to the soil type. On clay soil, the impact was limited which may imply that and increased water retention capacity may be beneficial for counteracting drought and heat stress in wheat cultivation. This is in accordance with earlier research from Nobel (2012) who has shown the broader range in soil moisture contents usable for plant growth in clay soil.

Grain yield has also been correlated with the total starch content and protein content although this was only linear at yields higher than 1.5 t ha^{-1}. In case of drought treatments, a stagnation in the concentration of both components was observed. For the drought treatment on sandy soils, protein contents were extremely high. Nevertheless, the detrimental effects on yield as a result of drought stress will surpass the possibly positive effects of increased protein contents. Moreover, significant differences in the protein composition, mainly in the glutelin fraction, were clearly shown using the RP-HPLC-approach. Insight in changes on a functional level are however lacking due to limited sample availability. This supports the future development of approaches which can handle small amounts of samples, thereby enabling the use of greenhouse trials for comprehensive (from kernel to end-product) research. In addition, it is advisable to control environmental conditions in a more excellent manner in future experiments in terms of temperature, daylight and relative humidity. While such experiments provide essential information, field trials remains the golden standard and are recommended to validate key outcomes.

Highlights

- Yield reduction mainly resulted from a decrease in the number of productive tillers and number of kernels per ear whereas thousand kernel weight was unaffected.
- Protein content significantly increased as a result of the lowered yields and starch contents.
- Clay soil mitigates the effects of drought stress, presumably due to its higher water retention capacity.
- Protein composition, mainly from the glutelin, is significantly altered by drought stress. No impact of soil type was observed.
- The high resolution of the RP-HPLC-based technique has shown to be able to reveal treatment-related effects.

PART II

STARCH-GLUTEN INTERPLAY AND ITS CONTRIBUTION TO EXTRUDATE AND BREAD QUALITY

> "An important problem in the preparation of theories and the making of models is the identification of important quantities that determine the essential forces at every organizational level of a system."
> GEOFFREY WEST, Scale: the universal laws of life, growth, and death in organisms, cities, and companies

CHAPTER 6

Contribution of gluten *content* and *composition* to the quality of wheat flour based ready-to-eat extrudates

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6.1 Introduction

Emerging technologies such as extrusion cooking can be used to produce an entire new assortment of foods in a versatile, low-cost and very efficient way. This comprises expanded ready-to-eat snacks or breakfast cereals, as well as baby foods (Oliveira et al., 2015). Moreover, extrusion is being used progressively in industrial feed production—with approximately 95 % of the dry pet food being extruded—as it allows to functional improve, detoxify, sterilize and texturize a large variety of food commodities and food ingredients through the molecular transformations and chemical reactions which take place during extrusion (Tran et al., 2008). The potential of wheat derivatives to be applied in such products, allows a further diversification in the currently used raw materials and end-products. However, its use may encompass additional difficulties, depending on the flour or wholemeal composition.

Extrusion processing for the production of ready-to-eat snacks using cereal flours combines low water contents with a high temperature, shear rate and pressure. Thereby, the beneficial functional effects of gluten proteins as required during breadmaking are partially abolished. Research from both Wang et al. (2017a) and Pietsch et al. (2018) has shown that, on a molecular level, the strong disulfide bond formation and hydrophobic interactions during twin-screw extrusion induce an intense polymerization of the wheat gluten. This prevents a proper expansion, thus resulting in dense and chewy instead of light and crisp extrudates. Although findings are sometimes contradictory, increasing protein concentrations generally tend to decrease quality attributes such as the expansion rate, the water absorption and the hardness (Ding et al., 2006). Contrastingly, the contribution of *compositional* differences to these characteristics is largely unknown.

Therefore, low quality (*i.e.* lower protein content) wheat cultivars are preferred for extrusion processing as the use of protein-rich wheat flour might lead to deficit end-products or production line downtime. As gluten proteins bind vast amounts of water, the melt viscosity will rapidly increase which may result in high motor torques and possibly, ceasing of the extruder. Furthermore, the protein-water interactions taking place during starch pasting may also severely impact determinative processing conditions. This triangular relationship with the feed moisture content (related to the applied water feed rate (WFR)) is also limitedly investigated due to its complexity.

Apart from the aforementioned application, vital gluten (pure or in combination with starches) remain popular for the production of protein enriched foods or meat analogs. An opposing trend is however noticed on a consumer level with a recent trend towards lower gluten consumption and an increased prevalence for gluten sensitivity or intolerance (Caio et al., 2019). Nevertheless, by using new technologies, wheat gluten can be modified, thereby providing a range of functional properties at a more modest price than its competitors such as milk and soy proteins (Day, 2011). Upon increasing the proportion of gluten-forming proteins in the formulation, their composition also becomes progressively important.

In light of enhanced diversification of wheat and the optimization of quality attributes for specific fields of application, this chapter focuses on how protein content and composition affect the use of wheat for producing (protein enriched) expanded ready-to-eat extrudates, thereby enclosing variation in the feed moisture content. Differences in the starch composition are separately discussed in Chapter 7.

6.2 Materials and methods

6.2.1 Experimental design

6.2.1.1 Raw materials and composite flours

Low-protein wheat flour (LPWF) (*'export flour'*) was bought from Paniflower (Zwijnaarde, Belgium). Based on a preliminary study, three differing types of vital gluten were collected: Tereos gluten (TEG) from Tereos (Aalst, Belgium), Biowanze gluten (BWG) from Beneo (Wanze, Belgium) and DAFA gluten (DAG) (distributed by Limagrain) which were kindly supplied by Dossche Mills (Deinze, Belgium). Prior to preparing the blends of the LPWF and the different vital gluten, the moisture and protein content of all raw materials was determined (Section 6.2.2) in order to calculate the mixing ratios. Blends of LPWF with protein concentrations (PROCs) of 11.0, 16.0, and 30.0 % for TEG and 16.0 and 30.0 % for the two other types of vital gluten (BWG and DAG) were prepared one day prior to the extrusion experiments. Raw materials were mixed vigorously for 8 min using a concrete mixer. A small sample of each blend was collected in a hermetically sealed high-density polyethylene container. Larger quantities were stored in polypropylene bags at room temperature until further use.

6.2.1.2 Extrusion experiments

The expanded cereals were produced using a Clextral BC45, equipped with an automated water dosage system (tap water) and gravimetric feeder. This co-rotating twin screw extruder has an electric heated and actively cooled barrel divided in five zones (Figure 6.1). Die temperature was not actively controlled. However, before starting the extrusion experiments, the die was preheated by the transfer heat of the fifth barrel zone. Barrel diameter D was 55.5 mm and barrel length L 1000 mm (L/D-ratio = 18.02). A circular die (D = 3.2 mm) was used in combination with a single knife rotating at an average speed of 800 min^{-1}.

Results from preliminary trials were used to select suitable extruder operating conditions. Feed rate (23.5 kg h^{-1}), screw speed (150 min^{-1}), and temperatures of zones 1–5 (40, 80, 100, 110, and 120 °C respectively) were kept constant during all trials. As shown in Figure 6.1, a conventional screw configuration was used. Seven conveying parts (denoted with a 'C') with varying windings (indicated by the line density), three kneading parts ('K') and another four conveying parts were installed on a 1.00 m long screw. During the first set of trials (LPWF and TEG-blends) water feed rate (WFR) of 4.2 to 0.6 kg h^{-1} with 0.6 kg h^{-1} increments were used. In a second series of trials (BWG- and DAG-blends), WFRs of 4.2 and 3.6 kg h^{-1} were excluded. Therefore, results for WFRs outside the range of 0.6 to 3.0 kg h^{-1} were excluded for data analysis.

Sampling was done after setting an equilibration time of at least 5 min after changing the feed material or WFR. Both spherical extrudates and strands were produced during each trial and were allowed to cool before packing them separately in hermetically sealed high-density polyethylene containers or polypropylene bags. For the determination of the moisture content, spherical extrudates were collected at the die outlet directly in high-density polyethylene containers without prior cooling. Within 6 hours after production, the moisture content was determined as described in Section 6.2.3.1.

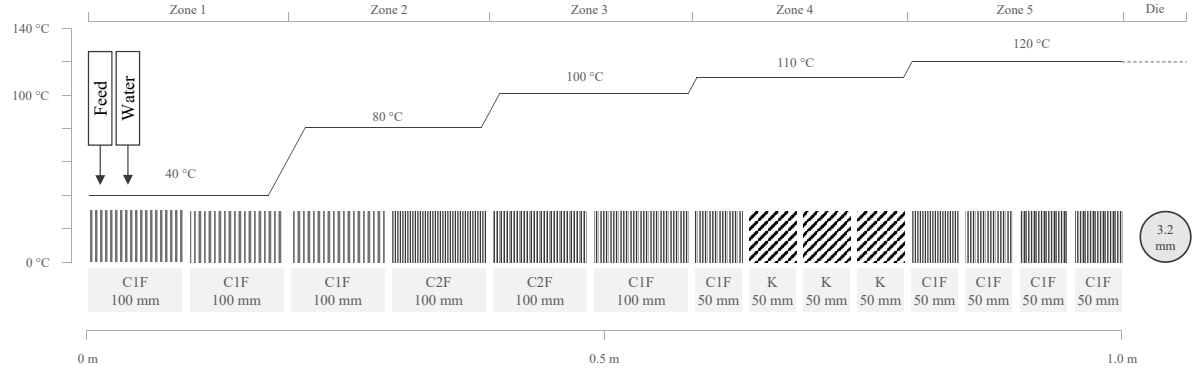

Figure 6.1: Schematic overview of the selected screw configuration for all extrusion trials.
C: conveying part, K: kneading part; 1/2 = number of revolutions per length unit.

6.2.2 Compositional and functional analysis of raw materials

6.2.2.1 Protein content

A VarioMax C/N (Elementar Analysesystemen, Langenselbold, Germany) was used to determine the nitrogen content of the raw materials. Four hundred milligrams of each sample was accurately (precision of 0.1 mg) weighed in a crucible and placed in the autosampler. A conversion factor of 5.7 was used to calculate the PROC. Using the moisture content of the raw material, determined following AACC Method 44-15.02 (AACC International., 2000b), the PROC was translated to the content on dry matter.

6.2.2.2 Protein composition

The protein composition of the vital gluten and the LPWF was screened using the high-performance liquid chromatography (HPLC)-technique described in Chapter 2. Using this technique, insight in both the prolamin-to-glutelin-ratio (PGr) and the ratio of HMW-GS to LMW-GS (HMW:LMWr) of the samples was obtained.

6.2.2.3 Viscoelastic properties

The viscoelastic behavior of the vital gluten was studied using the plate-plate module on an Anton Paar MCR 102 stress-controlled rheometer (Anton Paar, Ostfildern, Austria). After mixing 3.00 grams of vital gluten with 3.00 ml demineralized water for 1 min using the Glutomatic (Perten Instruments, Hägersten, Sweden) mixer, the gluten ball was transfered to the rheometer. The gap was set at 1.00 mm and paraffin oil was applied to prevent moisture evaporation during the measurement. Sample relaxation (20 min) was followed by a 5 min creep-phase at a stress of 250 Pa. Recovery lasted for 10 min. From the obtained curves, the maximum deformation was derived (maximum value in the creep-phase) whereas the elastic recovery ($\%\mathrm{REC}_{el}$) (expressed relatively) was calculated through Equation 6.1.

$$\%REC_{el} = \frac{([maximum\ deformation] - [final\ deformation])}{[maximum\ deformation]} \times 100 \tag{6.1}$$

The water addition for preparing the vital gluten was chosen on the basis of preliminary trials. No homogeneous gluten ball was formed at higher hydration rates (120 and 150 %) for BWG. Despite gluten did not fully develop using the selected hydration (100 % of sample weight), characteristic differences may come to expression in the measurements. Furthermore, shear applied under extrusion conditions differs largely from the stress used during the measurements.

6.2.3 Extrudate properties

6.2.3.1 Moisture content and average extrudate weight

Moisture content (MC) of the extrudates was determined in threefold using samples which were collected directly at the extruder outlet. Five to thirty extrudates were counted and weighed in aluminum crucibles and were dried in two stages: at 50 °C for 24 h and at 130 °C for another 16 h. After cooling to room temperature, the crucibles were again weighed on an analytical balance. From the relative weight difference, the moisture content (MC) was calculated. By comparing this with the MC of the melt (calculated using the initial feed MC and the WFR), the moisture loss (ML) was determined. By dividing the dry weight of the extrudates by the number of extrudates in the crucible, the average weight per kernel (WpK) was obtained.

6.2.3.2 Dimensions and morphology

Expansion index expansion index (EI) was measured by dividing the diameter of the extrudate strands, determined in tenfold with a caliper (precision 0.01 mm) by the diameter of the die outlet (3.2 mm).

Morphology Dimensional parameters (area size (AS), length-to-width-ratio (LtWr) and circularity (CIRC)) of the spherical extrudates were determined using the SmartGrain image analysis software, version 1.2 (Tanabata et al., 2012). Scans of 50 to 200 spherical extrudates were made using an Epson Perfection 2580 flatbed scanner (Seiko Epson, Nagano, Japan) against a blue paper background. After equally distributing the extrudates on the scanner surface, the lid was carefully laid on top of the extrudates and a scan was made at 300 DPI. Raw, non-optimized, images were stored in the .jpeg file format with a resolution of 2550 $\times$ 3510 pix. After loading the files in the software, five random background areas and extrudate areas were selected for automated thresholding. As this software was originally developed for grain kernel analysis, the option to include the beard of the kernels was switched off.

6.2.3.3 X-ray micro-tomography

Internal structure of extrudates was expected to differ largely as a result of differences in the composition of the raw materials and the feed. To obtain a representative and quantitative insight in the gas cell size distribution as well as in the thickness of the cell walls, X-ray micro-tomography (X-ray µCT) was applied. By including these parameters, it was also attempted to elaborate on the driving factors of sensory attributes hardness and brittleness which are both determinative for extrudate quality (Chevallier et al., 2018).

A selection of extrudates was scanned by X-ray μCT at the Centre of X-ray Tomography of Ghent University (UGCT) using the HECTOR system (Masschaele et al., 2013). Prior to scanning, the extrudate strands were dried overnight at 40 °C. Fifteen millimeter long pieces were used for the actual scan. High voltage on X-ray source was set at 70 kV and power on target was 10 W. For each scan, 2401 projections were taken over an angle of 360° with an exposure time of 1000 ms per projection. Additionally, 80 flat field and 40 dark field (offsets) images were also taken. The in-house developed Octopus Reconstruction software (distributed by XRE, Ghent, Belgium) was used to reconstruct the raw data to a 16-bit dataset of 2000 × 2000 × 1700 cubic voxels with an 11.00 μm voxel pitch.

Image analysis Image analysis was done using the Octopus Analysis software (Vlassenbroeck et al., 2007). After loading the images, data was converted to 8-bits after which air was thresholded. Subsequently, the total volume and gas cells were segmented using binary operations (iteratively closing and filling holes in 2D and 3D) to obtain a satisfying volume of interest. Objects were labeled and separated using a distance map and rejoined using the 'smart rejoin' function. After classifying the obtained volumes on size, the data was exported to an Excel worksheet and the labeled objects were saved.

3D visualization The internal structure of a selection of the extrudates was visualized in 3D using VGStudio. The labeled objects were loaded in the software and colored based on their gray value (representing their size). A threshold was set to exclude the smallest volumes.

6.2.3.4 Water absorption

By determining the water absorption index (WAI) of (intact) extrudates, knowledge of the ability of the matrix to bind water is acquired. This parameter has been related earlier with the molecular degradation of both protein and starch (molecules) (Ye et al., 2018). Therefore, it may possibly provide a valuable insight in the effect of processing conditions on molecular degradation for which otherwise complex analytical techniques are required (*e.g.* (2D) size exclusion chromatography, fluorophore assisted carbohydrate electrophoresis, high-performance anion-exchange chromatography with pulsed amperometric detection).

From both the intact and ground extrudates, the WAI was determined using the method of Anderson et al. (1970) with minor modifications. Two grams of sample (intact, spherical extrudates or powder) was accurately weighed in a 50 ml centrifuge tube and was mixed for 5–6 s using a vortex mixer after adding 20 ml of distilled water. Subsequently, the tube was left undisturbed for 20 min in a water bath at 25 °C with intermittent shaking for 2–3 s every 5 min. The solution was centrifuged (4,000 × g) for 20 min, after which the supernatant was carefully decanted. After weighing the sediment, the WAI was determined by dividing the resulted weight by the weight of the dry solid. Uniform extrudate powder was obtained by grounding the sample for 20 s using a water-cooled blender (M20 universal mill, IKA, Staufen, Germany) and by sieving the obtained powder over a 500 μm sieve.

6.2.3.5 Texture

Extrudate hardness was measured using a TX.XTplus Texture Analyzer (Stable Microsystems, Godalming, United Kingdom), mounted with a 50 kg load-cell and aluminum circular probe (P/36R, D = 36 mm). Extrudates were compressed at 2.00 ml s^{-1} for a distance of 5.000 mm. Pre- and post-test speeds were set at 12.000 ml s^{-1} and a trigger force of 0.300 kg (2.94 N) was used to initiate data recording. Per sample, 20 randomly selected spherical extrudates were measured separately after positioning them in the center of the test platform. Using an in-house developed R-script (version 3.4.3) (R Core Team, 2018), the breaking strength of the spherical extrudates—defined as the maximum force of the first peak which showed a 15 % loss in measured force over a 0.16 s interval (80 data points)—was calculated.

6.3 Results

6.3.1 Raw material composition and functionality

Both raw materials, the low-protein wheat flour (LPWF) and the vital gluten, were screened on their protein concentration (PROC), protein composition and basic functional behavior. This allowed basic insight in the diversity of the samples and the therefrom resulting composite flours. PROCs of the vital gluten were on average 73.5 % with only 1.3 % difference between the samples with the highest (DAFA gluten (DAG)) and lowest (Tereos gluten (TEG)) PROC (Table 6.1). The flour had a low PROC of 9.3 $\pm$ 0.1 %.

The prolamin-to-glutelin-ratio (PGr) of the LPWF (1.16) was similar to the ratio found for TEG (1.17). Biowanze gluten (BWG) and DAG showed significantly lower values (0.63 and 0.64 respectively), indicating that, relatively, higher amounts of GLU were present in these samples. This difference may be attributed to the industrial extraction process in which conventionally no solvents are used. A less pronounced distinction between raw materials was also noted for the ratio of HMW-GS to LMW-GS (HMW:LMWr). While TEG and BWG have an equal ratio, DAG appears to contain more low molecular weight glutelin-subunits (LMW-GS). Compared to the vital gluten, the LPWF has a higher ratio. Both ratios (PGr and HMW:LMWr) are in accordance with values reported in literature (Uthayakumaran et al., 1999; Vigni et al., 2013; Barak et al., 2013).

By looking at the dendrogram based on the chromatographic data of prolamin (PRO) and glutelin (GLU) (Figure 6.2), clear compositional differences between the vital gluten samples can be observed. A major resemblance between DAG and BWG is noted as these form a separate cluster from TEG and LPWF which are mutually less similar. Although differences in both protein groups are visible, mainly the GLU composition was found to vary.

> Extrusion processing partially remains a black-box as the conditions under which transformations take place (short time and high shear, pressure and temperature) are difficult to reproduce simultaneously using fundamental analytical techniques. Off-line empirical testing of melt properties, particularly its

Table 6.1: Overview of the compositional and functional characteristics of the low protein wheat flour and vital gluten used for preparing the composite flours.

Material	Code	Protein content (%)	PGr	HMW:LMWr	DEF_{max} (%)	$\%REC_{el}$ (%)
Export flour	LPWF	9.3 ± 0.1	1.16 ± 0.00	0.38 ± 0.03	na	na
Tereos gluten	TEG	72.8 ± 0.3	1.17 ± 0.04	0.33 ± 0.01	23.8 ± 1.8	73.2 ± 1.5
Biowanze gluten	BWG	73.6 ± 0.2	0.63 ± 0.03	0.33 ± 0.00	24.3 ± 0.4	68.6 ± 3.2
DAFA Gluten	DAG	74.1 ± 0.4	0.64 ± 0.02	0.29 ± 0.00	25.3 ± 3.5	75.0 ± 1.2

viscoelastic behavior, thus requires tailor made equipment. On-line measurements however have to be performed separately as measuring equipment does not allow the production of proper extrudates.

By means of creep-recovery measurements, the viscoelastic behavior of the vital gluten was studied in order to estimate the magnitude of variance between the samples on a functional level. Despite DAG showed the least resistance against deformation, it also had the highest elastic recovery (75.0 %). TEG were most stiff but had a higher recovery than BWG (73.2 % versus 68.6 %). The observed differences, however, were small compared to the variation in the composition of the vital gluten which could not be correlated due to the low number of samples. In contrast with findings from Barak et al. (2013), the vital gluten sample with the highest PGr (TEG) was the least extensible. A trend between PGr and elastic deformation can be noted.

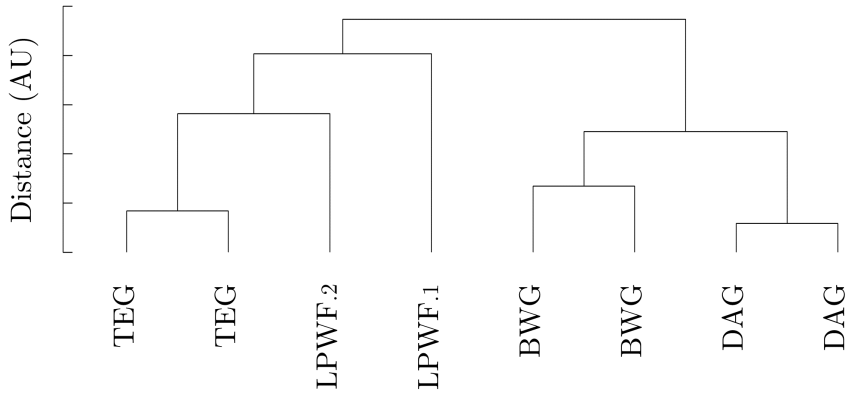

Figure 6.2: Dendrogram of the gluten proteins (prolamin and glutelin) for the two batches of low-protein wheat flour (LPWF) (denoted with 1 and 2) and both technical repetitions of the vital gluten (Tereos gluten, Biowanze gluten, DAFA gluten). The dendrogram was constructed using agglomerative hierarchical clustering using the Euclidean distance and complete linkage.

6.3.2 Extrudate properties

6.3.2.1 Expansion index and morphology

Expansion at die emergence occurs due to the pressure difference before and after the die causing the moisture in the viscous melt to evaporate. This allows gas cell formation in the extrudate during the solidification of the melt. The size and number of gas cells depends on the viscosity of the melt, the ability of the network to withstand rupture during the flash evaporation and the strength of the material

to prevent collapse afterwards. The formation of a starch-gluten network can aid in providing structure but may also prevent expansion due to a high elasticity. Hence, both the composition and content of the (gluten-forming) protein can be determining. Moreover, the feed moisture content and by consequence, the water feed rate (WFR) can impact expansion.

A strong significant effect of all three factors, WFR, PROC and type of vital gluten, and their mutual interactions on the expansion index (EI) was observed. As illustrated in Figure 6.3, a decrease in the WFR from 3 to 1.2 kg h^{-1} resulted for all experiments in an increased EI (Table 6.2). For the TEG and DAG a significant decrease in the EI was observed at a protein content of 30 %. Moreover, the EI from samples containing 30 % protein was always significantly lower compared to samples containing 16 %, 11 % of 9.3 % protein. This illustrates the negative effects of an increased gluten network formation on the ability of extrudates to expand. In addition, the water binding capacity of gluten protein may lead to an increased melt viscosity which may further limit the expansion. In contrast to TEG and DAG, extrudates containing BWG showed a steadily increasing EI, even at 30 % protein and a WFR of 0.6 kg h^{-1}.

Extrudate morphology can provide additional insight in the effects of protein content and gluten network formation on expansion. The area size (AS) was significantly correlated ($p \leq 0.001$) with the EI whereas the R^2-value of 0.64 may imply an interaction with circularity (CIRC) and length-to-width-ratio (LtWr). PROC had a significant effect on the AS resulting in a decreased value at higher concentrations. However, the highest AS was found for samples containing 16 % protein. The type of vital gluten also significantly affected the extrudate morphology. The addition of DAG ensued an increase compared to pure LPWF whereas adding BWG and TEG decreased the AS. A consistent decrease was found at an increasing WFR. Although all factors significantly affected both CIRC and LtWr, effects were more limited compared to the impact on AS. CIRC for samples containing less than 16 % protein was significantly higher compared to 30 % protein. CIRC reduced for DAG and BWG compared to pure LPWF or LPWF+TEG. No clear trend of WFR on circularity could be observed. However, the highest and lowest values were obtained at 4.2 kg h^{-1} and 0.6 kg h^{-1} respectively. LtWr, on the contrary, was only affected by the PROC with no significant influence of the type of vital gluten.

The expansion index (EI), diameter and morphological attributes of the extrudates are a result of both the number and size of the gas cells within the extrudate. Samples containing no additional gluten (pure LPWF) or TEG were analyzed for their internal structure using X-ray µCT. The number of gas cells follows the same tendency as the EI with an increasing number of cells as extrudates were more expanded. However, extrudates containing 30 % protein produced at a WFR of 1.2 kg h^{-1} had a markedly higher number of gas cells with a significantly smaller equivalent diameter despite its low EI (4.47 versus 5.35–5.70). For this sample, approximately 5000 gas cells could be distinguished in comparison to 2069–2535 gas cells for samples with less to no added gluten at the same WFR. This also resulted in a significantly (32 %) smaller equivalent diameter of the gas cells. Although no quantitative data is included in this research, Figure 6.4 illustrates how the cell walls showed to have a lower thickness at higher PROCs. Image analysis however did not yield representative values for the cell wall thickness with a large heterogeneity throughout the scanned slices. Equivalent diameter and sphericity of the gas cells was influenced by the PROC ($p < 0.01$) and WFR ($p < 0.01$) as well as their mutual interaction. Both the highest and lowest PROCs resulted in the least spherical gas cell structure whereas 16 and 11 % protein had the highest sphericity.

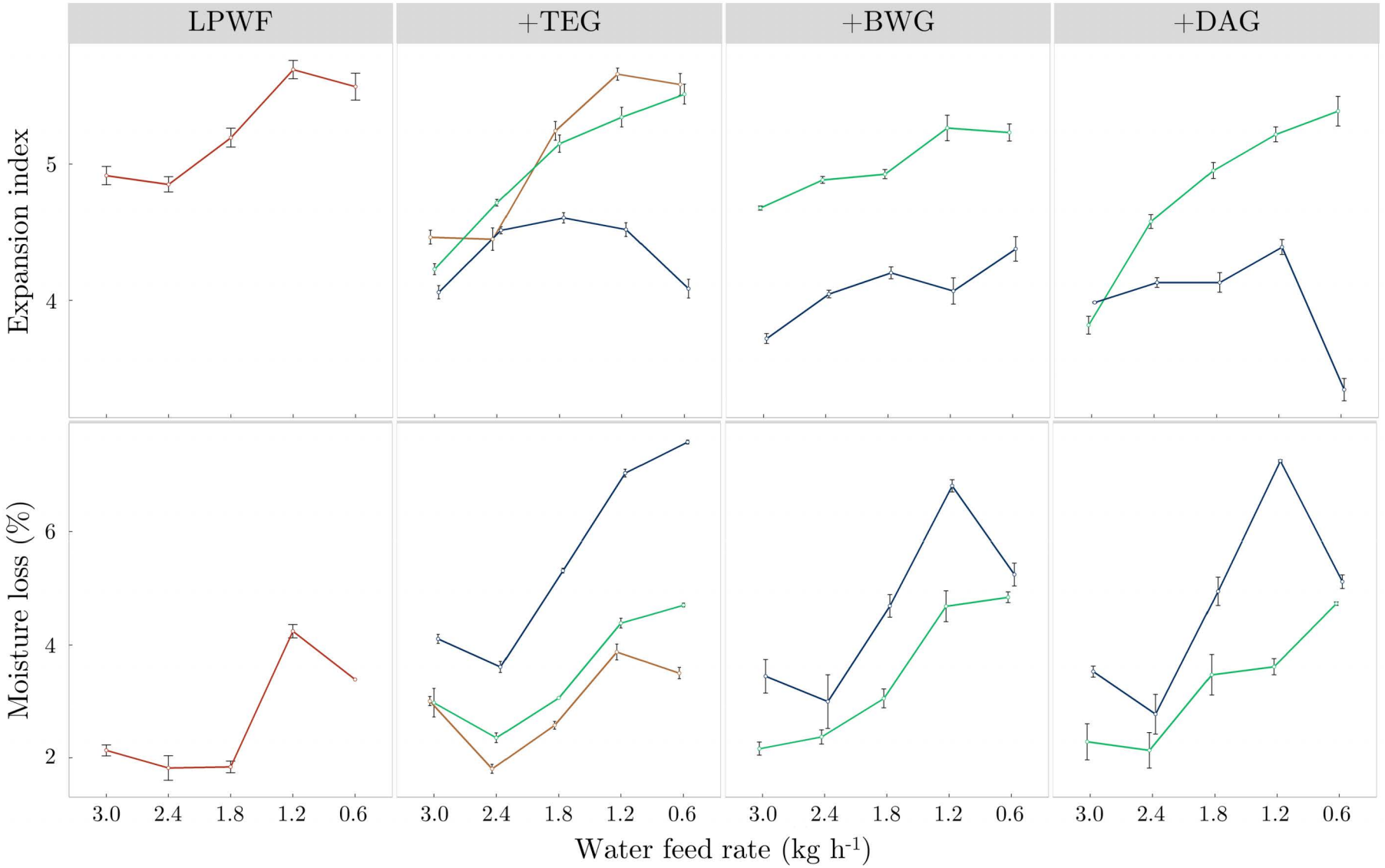

Figure 6.3: Expansion index and moisture loss of the extrudates on the basis of low-protein wheat flour (LPWF, 9.3 % protein —) mixed with various types of vital gluten (TEG, BWG, DAG) to different protein contents (11 % —, 16 % —, 30 % —) at decreasing water feed rates of 3.0 to 0.6 kg h^{-1}.

Increased expansion can result from larger gas cells or a higher number of smaller gas cells. In the latter situation, gas cell wall *thickness* will be lower thereby affecting sensorial attributes of the extrudates. To obtain such a thin layer—which is able to expand, solidify and retain structure without collapsing—a melt with optimal viscoelastic properties is required.

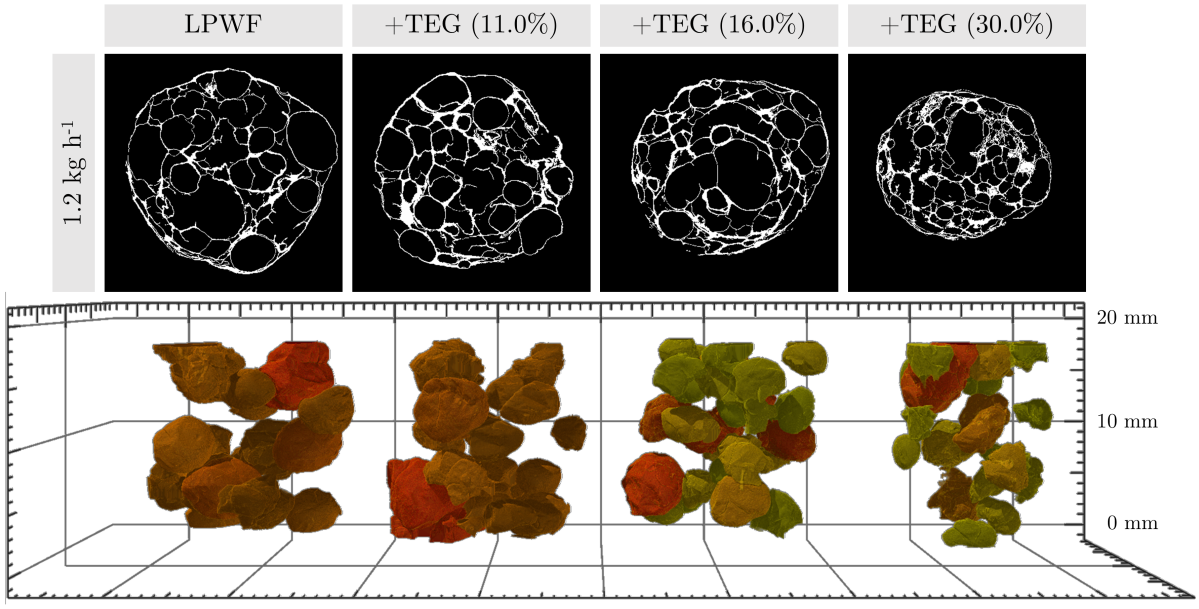

Figure 6.4: 2D and 3D illustration of the internal structure of extrudates on the basis of low protein wheat flour (LPWF, 9.3 % protein) mixed with Tereos gluten (TEG) to different protein contents (11 %, 16 % and 30 %) at a water feed rate of 1.2 kg h^{-1} using X-ray µCT imaging.

6.3.2.2 Moisture loss

The remaining moisture content of the extrudates provides an indication of the amount of water that evaporates at die emergence. By subtracting the remaining moisture content from the theoretical feed moisture content, the moisture loss (ML) was calculated. As is illustrated in Figure 6.3, the extrudates based on LPWF have a relatively constant ML of approximately 2 % at WFRs $\geq$ 1.8 kg h^{-1}. Upon lowering the WFR, a steep increase in the ML occurs for all types of vital gluten at all PROCs. At a WFR of 0.6 kg h^{-1}, the ML decreases again although it remains higher than the initial value. Similar phenomena were found for extrudates containing 11 % (TEG) or 30 % (BWG and DAG) protein. However, for the latter samples, an increase in the ML is already present at a WFR of 1.8 kg h^{-1}. Furthermore, at a PROC of 16 %, the ML keeps on increasing when the WFR is lowered to 0.6 kg h^{-1}.

Although the effect of the WFR on the ML is not linear, a clear decrease in the ML can be observed for samples prepared with higher WFRs ($\geq$ 1.8 kg h^{-1}). Similar to the CIRC, the type of vital gluten has no significant effect with only a slightly lower ML for samples prepared with TEG compared to BWG and DAG (3.79 and 4.01 %). PROC on the other hand had a very strong effect on the ML with a markedly lower value at 16 % protein compared to 30 % (3.35 $\pm$ 1.02 % versus 4.87 $\pm$ 1.51 %). Further lowering the PROC resulted in a consistent decrease in MLs (11 % protein = 2.78 $\pm$ 0.74 % and 9.3 % = 2.50 $\pm$ 0.95 %).

Moisture loss is only partially negatively correlated with the expansion of the extrudate and is strongly affected by the protein content. When the melt contains high protein contents and a low moisture content (*i.e.* low water feed rate), the moisture loss and expansion index show a varying behavior. Possible causes are the variations in water binding, competition for water by (degraded) starch molecules and/or the formation and rupturing of gas cell walls during expansion.

6.3.2.3 Water absorption index

Milled and intact extrudates were analyzed to relate both compositional and morphological attributes to their water absorption index (WAI) (Figure 6.5). For the WAI of the intact (spherical) extrudates (WAI_s), significant increases at WFRs of 1.8 or 1.2 kg h^{-1} (for BWG and DAG *vs.* TEG and LPWF respectively) were found. No clear effect of the PROC on this observation could be noted. Contrastingly, WAI of milled samples (WAI_m) remains more constant in function of the WFR. At low protein contents (9.3, 11 and 16 %) and for all types of vital gluten, a similar course is visible for the WAI_s. This phenomena largely corresponds with the WFRs at which the EI significantly increases (1.2 kg h^{-1}) and stagnates (0.6 kg h^{-1}). For BWG and DAG, increases in the WAI_s were already visible at 1.8 kg h^{-1} although the maximum values remains at 1.2 kg h^{-1}. For samples containing 30 % protein, a generally lower WAI_s was found at WFR $\leq$ 1.8 kg h^{-1}.

The WAI_m was consistently lower for samples containing 30 % protein although only a marginally significant effect of WFR was found ($p = 0.052$). PROC ($p \leq 0.001$) and the type of vital gluten ($p = 0.015$) were however significantly influencing the WAI_m. Samples containing more than 16 % protein had a significantly lower WAI_m compared to 11 or 9.3 % protein. A minimal difference between the WAI_m of TEG (4.15 ± 0.67) on one hand and BWG (3.94 ± 0.44) or DAG (3.93 ± 0.40) on the other hand was also observed.

6.3.2.4 Texture

Hardness can be used as a measure for describing the overall texture properties of extrudates. Yet, it results from the complex interactions between (macro)molecular and morphological attributes (internal structure and dimension). On the basis of the compression curves (not presented), a shift from deformation (single peak) to breaking (multiple fracture peaks) could also be observed upon lowering WFR from 3.0 kg h^{-1} to 1.2 kg h^{-1}. As such, a main effect of the WFR on extrudate hardness was observed for all samples with a consistent significant decrease from 189 ± 59 to 47 ± 20 N. A water addition of 0.6 kg h^{-1} did not further lower the hardness (41 ± 21 N) significantly. For BWG, a high PROC (30 %) resulted in a generally higher hardness which could not be observed for the other two types of gluten (TEG and DAG) which had similar or even lower hardness at 1.8 or 1.2 kg h^{-1}. The effect of the type of vital gluten ($p \leq 0.001$), PROC ($p \leq 0.001$) and their mutual interaction ($p \leq 0.039$) was therefore also significant.

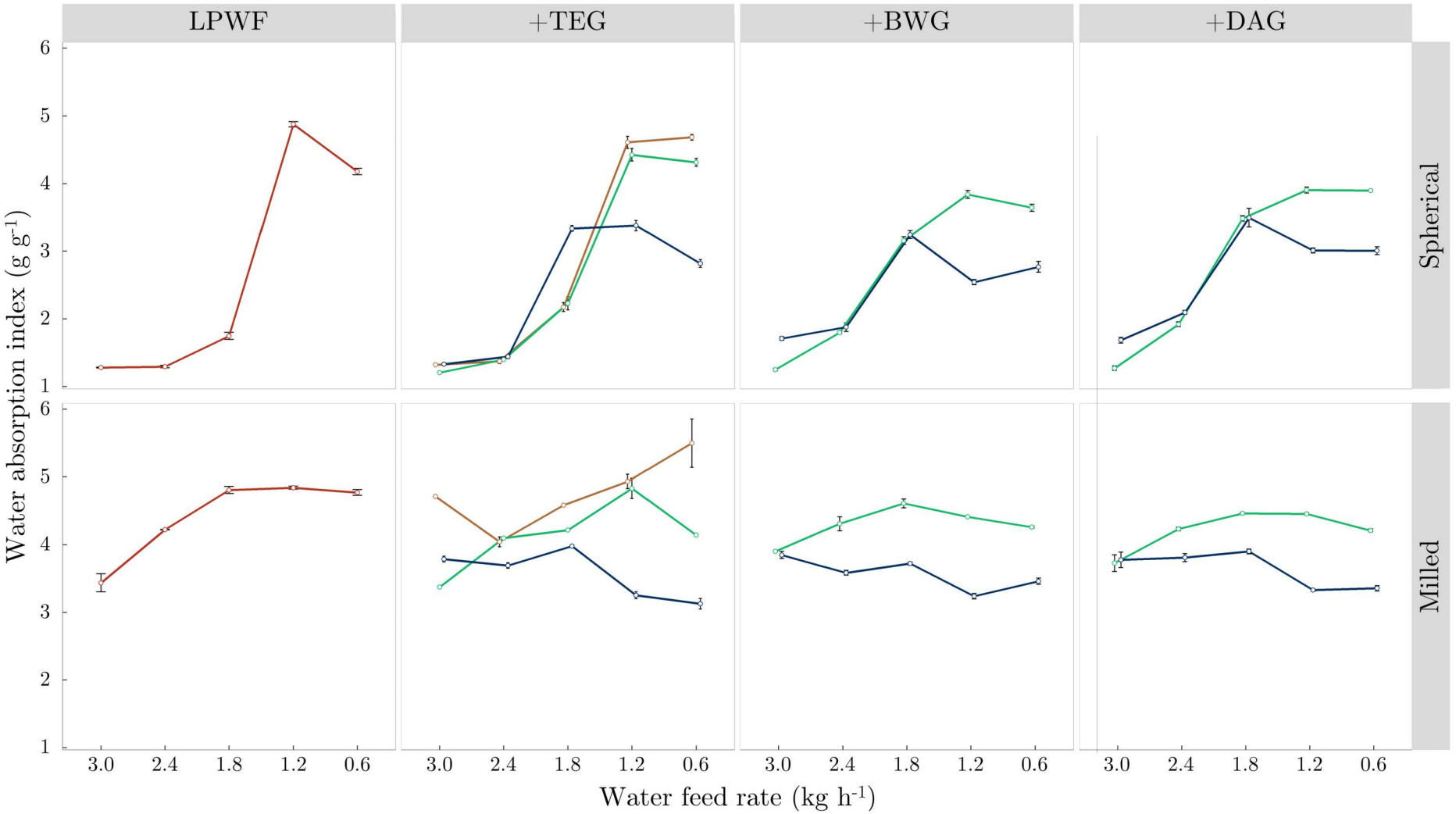

Figure 6.5: Water absorption index of spherical and milled extrudates, on the basis of low protein wheat flour (LPWF, 9.3 % protein —) mixed with various types of vital gluten (TEG, BWG, DAG) to different protein contents (11 % —, 16 % —, 30 % —) at decreasing water feed rates from 3.0 to 0.6 kg h^{-1}.

Table 6.2: Quality attributes of the extrudates produced using low-protein wheat flour (LPWF) blended with different types of vital gluten (Tereos gluten (TEG), Biowanze gluten (BWG), DAFA gluten (DAG)) to varying protein contents (11, 16 and 30 %), tested at multiple water feed rates (WFR)

Factor	ML (%)	EI	AS (mm^2)	CIRC	WAI_s (g\g)	WAI_m (g\g)	HARD (N)
LPWF	2.50 ± 0.95^{b}	4.90 ± 0.67^{a}	127 ± 41^{d}	0.829 ± 0.018^{a}	2.22 ± 1.52^{a}	4.42 ± 0.57^{a}	149 ± 135^{a}
TEG	3.79 ± 1.49^{a}	4.60 ± 0.59^{b}	113 ± 33^{b}	0.829 ± 0.020^{a}	2.42 ± 1.37^{a}	4.15 ± 0.67^{ab}	160 ± 130^{a}
BWG	4.03 ± 1.45^{a}	4.53 ± 0.55^{b}	121 ± 34^{c}	0.822 ± 0.024^{c}	2.58 ± 0.86^{a}	3.94 ± 0.44^{b}	116 ± 78^{b}
DAG	3.99 ± 1.54^{a}	4.43 ± 0.65^{b}	132 ± 32^{a}	0.824 ± 0.020^{b}	2.78 ± 0.93^{a}	3.93 ± 0.40^{b}	104 ± 59^{b}
9.3 %	2.50 ± 0.95^{c}	4.90 ± 0.67^{a}	127 ± 41^{b}	0.829 ± 0.018^{a}	2.22 ± 1.52^{a}	4.42 ± 0.57^{ab}	149 ± 135^{b}
11.0 %	2.78 ± 0.74^{bc}	4.80 ± 0.65^{a}	122 ± 39^{c}	0.829 ± 0.017^{a}	2.40 ± 1.42^{a}	4.76 ± 0.55^{a}	206 ± 160^{a}
16.0 %	3.35 ± 1.02^{b}	4.84 ± 0.54^{a}	133 ± 37^{a}	0.828 ± 0.017^{a}	2.77 ± 1.32^{a}	4.22 ± 0.36^{b}	161 ± 106^{bc}
30.0 %	4.87 ± 1.51^{a}	4.13 ± 0.34^{b}	107 ± 22^{d}	0.823 ± 0.025^{b}	2.36 ± 0.83^{a}	3.59 ± 0.28^{c}	114 ± 67^{c}
3.0 kg.h^{-1}	2.96 ± 0.74^{bc}	4.24 ± 0.43^{c}	102 ± 14^{e}	0.826 ± 0.019^{c}	1.38 ± 0.19^{c}	3.82 ± 0.41^{b}	189 ± 59^{a}
2.4 kg.h^{-1}	2.48 ± 0.70^{c}	4.51 ± 0.32^{b}	112 ± 13^{d}	0.829 ± 0.016^{b}	1.65 ± 0.30^{c}	4.00 ± 0.27^{ab}	157 ± 29^{b}
1.8 kg.h^{-1}	3.69 ± 1.24^{b}	4.80 ± 0.45^{a}	136 ± 18^{c}	0.831 ± 0.016^{ab}	2.78 ± 0.67^{b}	4.29 ± 0.38^{a}	84 ± 33^{c}
1.2 kg.h^{-1}	5.24 ± 1.47^{a}	5.02 ± 0.61^{a}	150 ± 38^{b}	0.822 ± 0.023^{d}	3.89 ± 0.76^{a}	4.16 ± 0.73^{ab}	47 ± 20^{d}
0.6 kg.h^{-1}	5.03 ± 1.21^{a}	4.99 ± 0.82^{a}	155 ± 42^{a}	0.815 ± 0.027^{e}	3.74 ± 0.69^{a}	4.10 ± 0.78^{ab}	41 ± 21^{d}

Means with the same superscript in the same column are significantly different ($p < 0.05$).
Extrudate hardness (HARD).

6.4 Discussion

For both the extrudate morphology and its internal structure (gas cell size and number), as well as the therefrom resulting parameters such as hardness and the WAI of the spherical extrudates (WAI_s), pronounced differences were observed. These can be attributed to both variations in protein composition and concentration as well as to the WFR. However, this relation is assumed to be indirect as variables contributed significantly to the EI. In this way, a larger contact surface is obtained which may be a primary attribute. It was found that mainly the PROC impacted the WAI compared to the protein composition. This finding is also visually supported by the loading plot in Figure 6.6 which displays the variance in the dataset explained by the two first principal components (75.7 %). A study of Day and Swanson (2013) has also shown that PROC and WFR are the main factors influencing extrudate properties. The narrow shape of the cluster containing samples produced using pure LPWF, results from the single PROC of the samples (9.3 %).

While significant compositional differences for the vital gluten were demonstrated by means of fundamental (*i.e.* model-system independent) techniques—despite these may have been enlarged due to the high resolution of the technique—, functionality after blending with the LPWF may be varying. Synergistic effects from blending different types of gluten (native and vital gluten) may have been overlooked (Uthayakumaran et al., 2002). Contrastingly, exclusion of starch-protein interactions may be helpful to identify the (macro)molecular attributes that lay at the basis of observed phenomena but which are at the same time determining during extrusion processing.

Extrudate hardness is one of the main factors influenced by the type of vital gluten and, in this way, the protein composition. Although a positive correlation with the prolamin-to-glutelin-ratio (PGr) can be observed, insufficient variation was present to accurately test this hypothesis. It can be postulated that an enhanced network formation through increased protein cross-linking in the presence of more GLU forms the basis of this observation. The feed moisture content (WFR) also had a significantly higher impact on impact on hardness.

From the principal component analysis (PCA), it was also concluded that the CIRC and LtWr are respectively negatively and positively correlated with the PROC. In addition, the EI shows a strong negative correlation with the PROC as was also reported by various researchers (Allen et al., 2007; Day and Swanson, 2013). This might be attributed to two effects: an increased elasticity of the melt by the development of a gluten network, or the formation of a highly viscous melt due to the altered water distribution in the feed matrix (Day and Swanson, 2013). Both effects will inhibit proper expansion at die emergence, promoting the rupture of the cell walls and a possible collapse of the structure before solidifying (Faubion and Hoseney, 1982). Gluten proteins may in opposition also have a stabilizing effect by providing structure to the gas cells, thereby enhancing their expansion (Allen et al., 2007; Draganovic et al., 2011). For samples containing TEG or DAG at levels of 30 % protein, however, an optimum in the EI was observed.

The steady increase in EI for samples with BWG at a PROC of 30 % further supports the presence of interaction with the composition of the added vital gluten. However, the PGr—which can be used as a measure for cohesiveness and elasticity (Uthayakumaran et al., 2002; Barak et al., 2015)—and the results

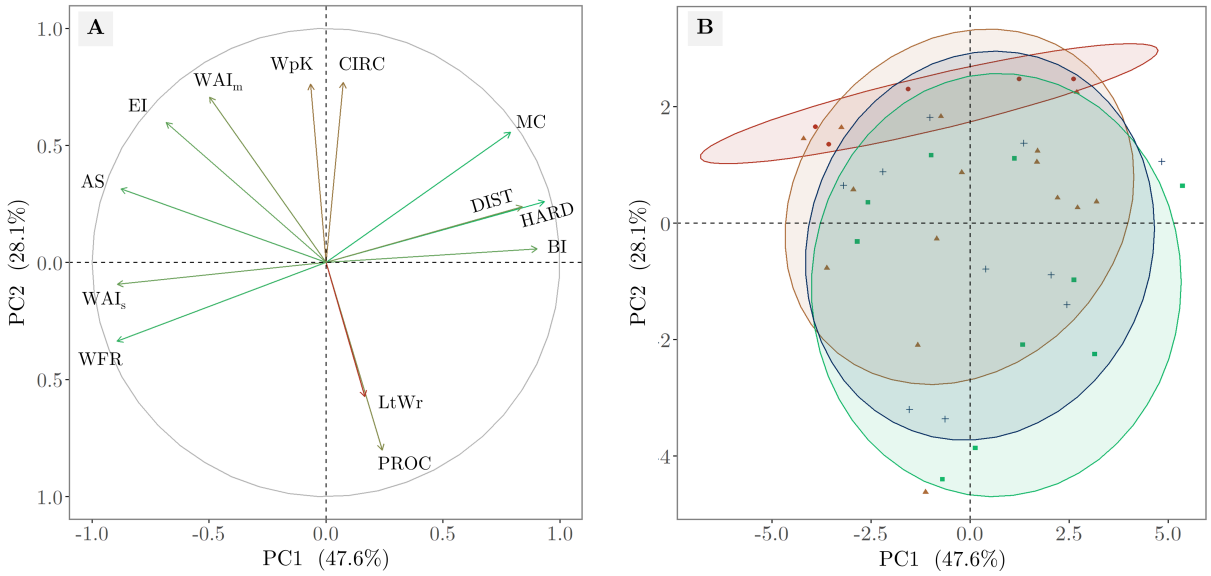

Figure 6.6: Loading (**A**) and score (**B**) plot for the first two principal components (PCs) explaining respectively 47.6 % and 28.1 % of the variability in the dataset. Ellipses in the score plot indicate the material (low-protein wheat flour (LPWF) ●; blends with: TEG ▲; BWG ■; DAG +).
Extrudate hardness (HARD); compression distance to first peak (DIST); breaking index (BI).

from the creep-recovery measurements could not be correlated with the EI. As water plays a crucial role during gluten network formation and starch gelatinization, it can be assumed that the triangular relationship between water, protein and starch majorly contributed to these results by impacting the melt viscosity. According to Robin et al. (2012), this is determining for expansion at die emergence. Moreover, static rheological measurements were performed on a matrix different than the melt at lower shear pressures which will also contribute negatively to the strength of the correlations.

> A delicate balance between viscosity, elasticity and cell-wall stability has to be achieved through combining specific starch and protein compositional properties as well as through hydration of the starch-gluten-mixture.

When looking at the WAI of the milled extrudates (WAI$_m$), no divergent trend could be observed for the BWG-samples compared to DAG-based extrudates whereas addition of TEG lowered the WAI$_m$ at 2.4 and 1.8 kg h^{-1}. PROC, however, significantly influenced WAI$_m$ with a generally lower index at higher concentrations (negative correlation). This phenomena may be attributed to a higher protein-protein and protein-starch interaction making the components less available for binding water. Alternatively, more starch damage and depolymerization of the proteins may have occurred at high shear conditions (highly viscous melts) making them bind less water (Ghumman et al., 2016). The type of protein can also play a role in this effect as Brnčić et al. (2011) and Yu et al. (2017) found that for whey protein, a *positive* correlation between PROC and the WAI exists. The current results, however, do not support this hypothesis. Moreover, analogously to the EI, an optimum in the WAI$_m$ at WFRs $\leq$ 1.8 kg h^{-1} was observed. This WFR may form a critical point at which the low feed moisture content promotes a super-linear increase of the shear stress, thus promoting molecular degradation. Consequently, adverse effects on both EI and WAI$_m$ can be expected. The latter hypothesis was also confirmed by research from Ghumman et al. (2016).

6.5 Conclusion

This research broadens the understanding of the impact of protein content and composition on quality attributes of wheat-based extrudates in relation to the applied water feed rate and thus, feed moisture content. Using fundamental techniques, differences in the protein composition and functionality of the vital gluten samples could be demonstrated. Marginal correlations existed between these compositional properties and the visco-elastic behavior of the gluten, presumably due to the low number of samples. However, composition of the gluten proteins showed to have a limited but significant effect on extrudate quality. The expansion index, water absorption index and (internal) morphology showed varying trends upon comparison of the different vital gluten samples. The magnitude of these interaction effects also increased at higher protein contents. The lack of strong correlations with properties of the raw materials was attributed to the transformations taking place during extrusion processing.

Protein content and the water feed rate were more determining for the quality attributes although relationships with the expansion index, the moisture loss at die emergence and the water absorption index were non-linear. Generally, a decrease in the expansion index was observed at increasing protein contents and lowering water feed rates. At 30 % protein, an optimum was noted for two types of vital gluten whereas a consistent increase was observed for the third type. Remarkably, an inverse effect was observed for the moisture loss. Morphology of the extrudates (sphericity) was almost solely dependent on the protein content of the blends.

The water absorption index was examined using both milled and intact (spherical) extrudates. The latter appeared to be strongly correlated with the expansion index in terms of the water feed rate at which an increase was observed, presumably related to the porosity of the extrudates. Results for the milled samples, however, showed a lowering effect related to the protein content with a similar trend for all vital gluten samples. A consistent increase or decrease in the water absorption index was respectively noted at the lowest and highest protein contents whereas, for samples containing 16 %, an optimum was observed around 1.8 kg h^{-1}. Shear degradation of the starch molecules and proteins in the matrix is expected to lay at the basis of this phenomena.

In contrast to the prior extrudate properties, hardness and brittleness (measured as the breaking index) were mainly dependent on the applied water feed rate with a less pronounced influence of the protein content. Protein composition had a minor effect with an overall higher hardness for vital gluten from Biowanze although this was only noted at an incorporation to 30 % protein.

A general observation is the high level of interaction between proteins and water in the current system and the dependency of most of the quality attributes thereon. Although results suggest an important contribution of protein composition, data was inconclusive to state which type of protein composition is desired. Moreover, impact of processing conditions has to be taken into account when further assessing these properties in future research. A general concern with regard to increasing the protein content in the feed material, is the increased water absorption and viscosity of the melt. Therefore, a more fundamental approach should be applied. However, the search for off-line viscosity measurements under comparable conditions (shear, pressure, temperature) remains a challenge. On-line viscosity measurements may

form an alternative but do not avoid the need to perform extrusion experiments which are time and labor intensive as well as poorly repeatable.

Highlights

- Protein content is a main determining factor in extrudate quality.
- Internal and external morphology of the extrudates is affected by the water feed rate, protein content and its composition.
- Interaction between protein content and water feed rate results in a shift for the turnover in the underlying mechanisms for these macroscopic effects.
- Moisture loss at die emergence was affected by both the protein composition (of the added vital gluten) and the protein content of the blend.
- Melt viscoelastic behavior should be studied to better understand underlying effects as empirical testing or black-box modeling is too labor and time intensive given the used equipment.

CHAPTER 7

Variation in amylose concentration to enhance wheat flour extrudability

This chapter is adapted from: Hellemans, T., Nekhudzhiga, H., Van Bockstaele, F., Wang, Y.J., Emmambux, M.N., & Eeckhout, M. "Variation in amylose concentration to enhance wheat flour extrudability," *Journal of Cereal Science*, *Revised version submitted*.

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7.1 Introduction

Besides protein properties, as discussed in Chapter 6, starch attributes may be employed to enhance processability and end-product quality due to the importance of both starch-starch and starch-protein interactions (Matthey and Hanna, 1997). For example, one of the main factors determining the extrudate properties is the melt viscosity (Martin et al., 2003; Robin et al., 2011) which, on its turn, has been related to starch physicochemical properties (*e.g.* AM:AMP-ratio, granule size distribution, presence of amylose-lipid complexes, *etc.*) (Launay and Lisch, 1983) and the starch pasting behavior (Kristiawan et al., 2018). Additionally, these interactions are further complexed by the competition for water between protein and starch during extrusion (Mohamed and Rayas-Duarte, 2003; Ding et al., 2006).

In practice, the concentration of amylose (AM) of conventional bread wheat is narrowly distributed between 25–28 %. Furthermore, (partial) waxy wheat flour (WWF) is relatively unknown in industry (Van Hung et al., 2006; Šárka and Dvořáček, 2017) compared to waxy maize flour which is frequently used for extrusion applications. Also high amylose wheat flour (HiAm-WF) (containing up to 50–80 % AM) has only been commercially introduced recently in North America. Starch properties of WWF may provide possibilities to counteract the potential negative effects of the presence of wheat gluten proteins as it lowers the specific mechanic energy (Kowalski et al., 2015), thereby lowering the chance of ceasing the extruder. The latter can be considered as one of the main hurdles in the field of extrusion processing. Moreover, they can also be used to obtain enhanced sensory features or to produce high-energy foods (due to the rapid digestion of extruded waxy starch).

Results on the added value of waxy (wheat) flour to produce ready-to-eat snacks are however contradictory. A main effect on the expansion of the extrudates is observed with studies showing an increase (Fleischman et al., 2016; Kowalski et al., 2015) or decrease (Baik et al., 2004; Jongsutjarittam and Charoenrein, 2014; Thakur et al., 2017) in the expansion index (EI). In Jongsutjarittam and Charoenrein (2014), who compared waxy rice and rice flour based extrudates, this contradictory effect was attributed to the collapse during cooling. In addition, an interaction with the feed moisture content was noted with a reduced collapse at high moisture contents.

Although the effect of amylose content on extrudate texture has been attributed to differences in the EI, these findings are also under discussion with researchers observing both harder (Jongsutjarittam and Charoenrein, 2014) and softer (Kowalski et al., 2015) textures at lower AM concentrations. As amylopectin (AMP) is more sensitive to molecular degradation (debranching) under extrusion conditions, waxy starches will show more short chain dextrins (low degree of polymerization (DP)). This will lower the melt viscosity (less network formation) and may therefore impact end-product properties (Van Den Einde et al., 2004; Van den Einde et al., 2003). Under high-shear conditions (at high specific mechanic energies), molecular disruption was promoted in both regular and waxy barley flour leading to an increased water absorption index (WAI) and EI (Baik et al., 2004). However, for regular flour, the presence of intact (*i.e.* not (exo)degraded) polymers was observed resulting in an enhanced network formation due to the increased entanglement of the linear amylose chains. These interactions made it more difficult to pull the network apart during expansion resulting in a decreased EI (Jongsutjarittam and Charoenrein, 2014).

7.1.1 Problem statement

The relation between the AM concentration and extrudate quality attributes has been studied extensively. This was, however, mainly done using rice or maize flour or starch (Sompong et al., 2011; González et al., 2013). Kowalski et al. (2015) stated that waxy *wheat* flours have not seen wide use in extrusion industry although other waxy flours (waxy maize and waxy barley) have been previously analyzed through extrusion. Moreover, the relationship is often studied using one or several pure waxy and regular cultivars thereby neglecting the intermediate amylose concentrations (partial waxy cultivars). These types may be interesting as, as shown in bread, a slightly reduced AM concentration increased end-product quality whereas 100 % waxy wheat significantly reduces structure and quality of the final product (Hayakawa et al., 2004; Kowalski et al., 2015).

This second part on the use of extrusion processing for wheat flour investigates the relationship between on one hand AM content and amylopectin fine structure and on the other hand end-product quality of ready-to-eat extrudates. To broaden the knowledge of the genotypic variability of WWF and how this impacts its use for producing ready-to-eat extrudates, both blending trials and genotype trials were performed. Together with the findings from Chapter 6, the current research attempts to provide a better understanding of how each component, (gluten-forming) protein and starch, contribute to extrudate quality.

7

7.2 Material and methods

7.2.1 Experimental design

7.2.1.1 Raw materials

Five waxy wheat genotypes (Table 7.1) were cultivated during 2017–2018 at the research farm of Ghent University and University College Ghent (Bottelare, Belgium). The field trial (Moortsele, Belgium, 50.96525 latitude, 3.77977 longitude) was conducted on a sandy loam soil with perennial rye grass as forefruit and was fertilized according to the advised dose rates for nitrogen (87 kg N ha^{-1}, 50 kg N ha^{-1} and 60 kg N ha^{-1} at Zadoks growth stage (Zadoks G.S.) 22, 30 and 39, respectively). The harvested and cleaned wheat was tempered overnight to 15.5 % moisture and milled to flour using a Bühler laboratory mill (as described in Section 3.2.2.2). After a two week maturation period in plastic buckets at room temperature, flour samples were packed in polyethylene bags and were shipped to Pretoria (South Africa) for extrusion experiments. Regular wheat flour (*'Export flour'*) was obtained from Paniflower (Zwijnaarde, Belgium). This low-protein wheat flour ($\leq$10 % protein) contains no additives (*e.g.* vitamin C, enzymes, *etc.*) and is used as a reference flour throughout this research.

7.2.1.2 Formulation of blends

In this paper, results of two sets of extrusion trials are presented. In the blend-trial (BT), the influence of the AM content on extrusion properties and extrudate quality is studied. Therefore, WWF (P+A) and regular wheat flour (EXP) were mixed in five different ratios—100:0 (pure waxy wheat), 75:25, 50:50,

25:75, and 0:100 (pure regular wheat flour)—to obtain AM contents of approximately 0, 5.5, 11, 16.5 and 22 %. After weighing each flour in a separate bucket, a semi-industrial Hobart mixer equipped with a whisk was used to prepare homogeneous blends by mixing at low speeds for 10 min.

Table 7.1: Overview of the wheat genotypes used in the extrusion experiments and their origin.

Genotype	Owner	Origin	Wheat type	Code
WaxyDie	Dieckmann Seeds	Research farm	Winter wheat	WxD
1154.06	Dieckmann Seeds	Research farm	Winter wheat	D11
Waxymum	Limagrain Céréales Ingrédients SAS	Research farm	Winter wheat	WxM
NX12Y48222	USDA ARS (Bob Graybosch)	Research farm	Winter wheat	NXY
Penawawa+Alpowa	Washington State University (Craig Morris)	Research farm	Spring wheat (blend)	P+A
Export flour	Paniflower nv.	Commercial flour	Winter wheat (blend)	EXP

7.2.1.3 Extrusion experiments

The ready-to-eat expanded cereals were produced using a CFAM TX-32 laboratory scale co-rotating twin-screw extruder (CFAM Technologies, Potchefstroom, South Africa). The extruder barrel consisted of five independent electrically heated and actively cooled zones. Prior to commencing the extrusion experiments, the die was preheated by the transferred heat of the fifth barrel zone. Barrel diameter D was 32.0 mm and barrel length L 500 mm (L/D-ratio = 15.65). A circular die (D = 3.0 mm) was used in combination with a single rotating knife. Results from preliminary trials were used to select suitable extruder operating conditions. Product feed rate (5 kg h^{-1}), water addition (0.28 kg h^{-1}), screw speed (150 min^{-1}), and temperature of zones 1–5 (40, 90, 100, 110, and 120 °C respectively) were kept constant during all trials. A conventional screw configuration, as shown in Figure 7.1, consisting of an initial conveying zone, two conveying and reaction zones (both ending with mixing elements), a single kneading block (for intense mixing during gelatinization) and a final conveying zone was used. All extrusion experiments were performed in duplicate in order to include processing variability in the statistical analysis.

To ensure representative and uniform sampling, an equilibration time of at least 4 min was set after changing the feed material. Both spherical extrudates and strands were produced during each trial and were allowed to cool before packing them separately in hermetically sealed high-density polyethylene containers or sealable polypropylene bags. For the determination of the moisture content, the spherical extrudates were immediately packed in airtight high-density polyethylene containers without prior cooling. A part of the collected and air-cooled extrudates was milled with an analytical mill (A11, IKA, Staufen, Germany) to pass through a 500 µm sieve. The samples were kept in polyethylene bags in an airtight container and stored at 4 °C until further analysis.

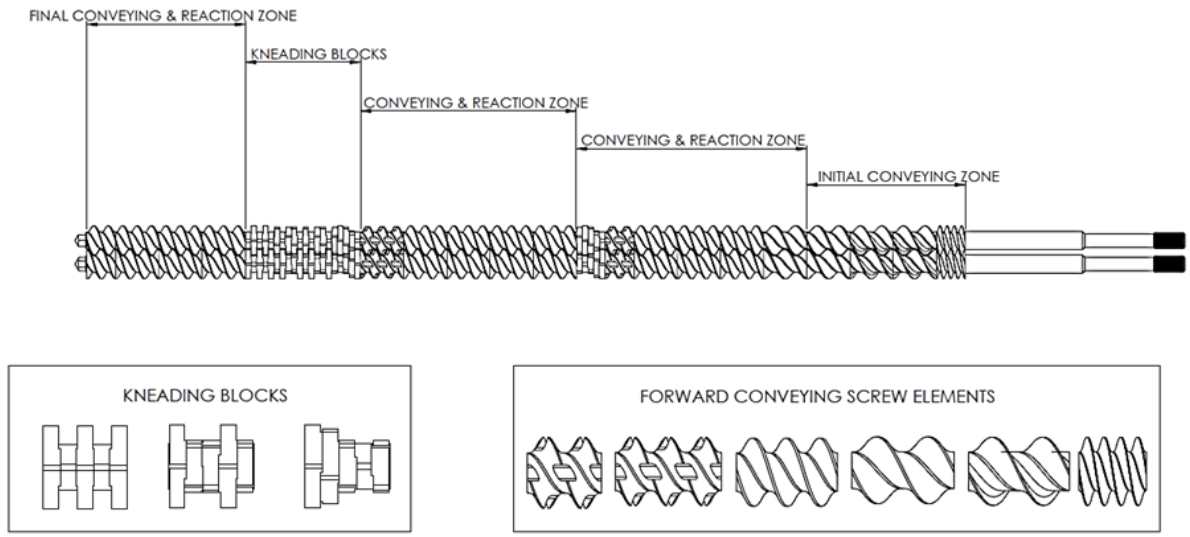

Figure 7.1: Schematic overview of the screw configuration used during all extrusion trials discussed in this chapter.

7.2.2 Compositional and functional analysis of raw materials

7.2.2.1 Protein content

Protein concentration (PROC) of the raw materials was determined as described in Section 6.2.2.1. PROC of the blends was calculated using the moisture content and protein content of the raw materials.

7.2.2.2 Starch content and composition

From the five WWFs and the regular wheat flour (EXP), the starch content and composition (AM content) as well as the amylopectin fine structure were determined following the methods described in Section 3.2.4.3 (page 104). For the latter two measurements, starch extracts were obtained by the dough-ball method. In the final step of the sample preparation, after centrifugation (4000 $\times$ g, 20 min), starch was defatted by overnight soxhlet extraction.

7.2.2.3 Starch granule size distribution

Starch granule size distribution of the starch was determined as described in Section 3.2.4.5 (page 108). Granule size is defined in terms of 10^{th} percentile (D_{10}), median (D_{50}), 90^{th} percentile (D_{90}) and the relative contribution of B-type ($\leq$10.0 µm) and A-type (10.01–35.0 µm) granules (Zhang et al., 2016c). The proportion of granules larger than 35 µm is denoted as 'A^{+}'.

7.2.2.4 Pasting behavior

Pasting behavior of the flour (both blends and the pure genotypes) and ground extrudates was measured using a Physica MCR 101 Rheometer (Anton Paar, Ostfildern, Austria) equipped with a starch pasting

cell with a six-blade vane. Prior to commencing the pasting cycle, flour was priorly mixed with 15 ml demineralized water in a 50 ml centrifuge tube whereas the ground extrudate (14 % w/v, 2.1 g on dry basis) was added directly to the measuring cup under constant stirring. This was required as otherwise aggregation and sedimentation of the sample occurred, resulting in fluctuating sample concentrations in the measured suspension.

To further enhance homogenization of the suspension, a pre-shear—a logarithmic increase of the mixing speed to 960 min^{-1} while heating to 50 °C during a period of 30 s—was directly followed by stirring at 160 min^{-1} throughout the analysis. A holding phase of 1 min at 50 °C was succeeded by a heating phase to 95 °C at 5 °C min^{-1}. Subsequently, a holding phase at 95 °C for 5 min and cooling phase (rate equal to heating phase) was completed. The measurement was ended by a final holding phase at 50 °C for 2 min. Pasting parameters were derived manually from the curves as unconventional pasting behavior was observed.

7.2.2.5 Thermal analysis

Thermal properties of both the raw material (flour) and ground extrudates was determined using a high-pressure differential scanning calorimeter (DSC) (HP DSC827e, Mettler Toledo, Greifensee, Switzerland). Approximately 10.0 $\pm$ 0.2 mg of sample (as is) was mixed with distilled water (40 mg) in 100 mg DSC-pans. Prepared samples were left at least 24 h at room temperature to equilibrate. After calibrating the equipment with Indium (Tp = 156.6 °C, 28.455 J g^{-1}), scanning was performed from 30–130 °C at a rate of 3.0 °C min^{-1}. All measurements were performed at a pressure of 4 MPa using N_2. An empty pan was used as reference.

7.2.2.6 Flour water absorption

In agreement with the method described in Section 3.2.5.2 (page 109), the flour water absorption was determined using a 50 g mixing bowl.

7.2.2.7 Viscoelastic dough properties

Dough viscoelastic properties were measured for the different genotypes by means of a frequency sweep using an Anton Paar MCR 102 rheometer (Anton Paar, Ostfildern, Austria). Prior to measurements, dough pieces were obtained by mixing 10.0 g of flour with 5.8 ml demineralized water using the Glutomatic apparatus (Perten Instruments, Hägersten, Sweden). After mixing for one minute, the dough piece was carefully transported to the rheometer and the measurement was started. Prior to the frequency sweep, a 20 min relaxation period was set. The measurement itself was conducted at 20 °C going from 0.1–20 Hz at 25 Pa. From the obtained storage (G') and loss (G") moduli, the phase shift angle ($\tan(\delta)$) and the complex shear modulus ($|G^*|$) were calculated. Values at 10 Hz were used for further analysis.

7.2.3 Extrudate properties

From the extrudates, the moisture content (MC) and kernel weight (WpK) was determined in a similar fashion as described in the previous Chapter (page 6.2.3). Also the dimensional properties EI and the morphological parameters (AS, length-to-width-ratio (LtWr) and circularity (CIRC)) as well as the extrudate texture were measured using the same methods. To calculate the EI, the diameter of the die used for these experiments (3.0 mm) was used.

7.3 Results

7.3.1 Raw materials

Sample composition was evaluated for both protein and starch characteristics in order to broaden knowledge on effects occurring during extrusion and their impact on end-product quality. Besides protein, the total concentration of starch, its composition (amylose (AM) content, amylopectin fine structure), and starch granule size distribution was studied. Furthermore, various functional properties such as the viscoelastic behavior (by means of dynamic oscillatory rheology) and the water absorption (using the Brabender Farinograph) of the flours were determined. The average values for these analysis are shown in Table 7.2.

The protein concentration (PROC) of all genotypes is remarkably low for bread wheat, varying from 9.7–11.7 %. This may be a direct result of the limited amounts of nitrogen fertilization applied during cultivation and the water shortage during grain filling period (data not shown). On the contrary, the short growing season (April to July) of spring wheat genotypes (P+A), naturally increases the PROC. The phase shift angles ($\tan(\delta)$) and the complex shear moduli ($|G^*|$) of all waxy samples, except D11, are highly comparable under the used test conditions. This indicates that the dough has comparable viscoelastic properties at 58 % hydration. On the contrary, values for the water absorption (WA) obtained using the Brabender Farinograph ranged from 62.5 to 71.6 % for D11 and NXY respectively.

Research from Ali et al. (2014) and Zhang et al. (2014) attributed a varying WA to differences in the AM concentration and the starch granule size distribution. Also the total starch (TSt) content was related to WA although it was unclear if this was directly related to the starch or if this was an indirect effect of protein dilution (Purna et al., 2011). Starch contents were not significantly differing for the waxy genotypes with exception of P+A which had a lower starch concentration (76.8 $\pm$ 2.2). The AM content of the regular wheat flour (EXP) (21.96 $\pm$ 0.73 %) is in accordance with the range reported by Van Hung et al. (2006) and concentrations in all waxy-cultivars was below 1 %. Minor variations within this group could be observed with contents ranging from 0.22 $\pm$ 0.01 % to 0.58 $\pm$ 0.04 %.

For the amylopectin (AMP) chain-length distribution (CLD), a significantly higher average chain length (aCL) (*i.e.* degree of polymerization (DP)) was seen for WxD (DP = 20.79). EXP, on the contrary, had on average shorter amylopectin chains (aCL = 20.03). This distinction can be related to significant differences in the distribution pattern with lesser DP 6–24 chains and more long (DP 25–65) branches for the waxy wheat flours (WWFs) compared to regular wheat flour (Table 7.2). Presence of A-type (DP = 6–12) and B_3^+ (37–65) AMP chains was negatively correlated and showed the largest variation. For

the A-type chains, WxD was significantly differing from D11 and P+A which were in turn significantly lower from EXP. An opposite trend was found for the B_3^+-chains. In addition, only EXP had a significantly higher proportion of B1-type chains (DP 13–24) whereas it had a lower amount of midlong (B_2-type, DP 25–36) chains. D11 and WxD had a significant higher concentration B_2-type chains. In general, an average relative proportion of 25.59, 47.55, 17.46 and 9.41 % was found for the different chain lengths (A, B_1, B_2, B_3^+-types, respectively).

Results for the starch granule size distribution were markedly different for all genotypes except WxD and D11 which showed similar trends. Besides the relation between granule size and flour water absorption (during mixing and pasting), Soh et al. (2006) found that dough extensibility increased at smaller granule sizes. However, no one-to-one correlations were observed between granule size properties and dough rheological attributes in this research. In contrast, peak temperature (PTemp) in starch pasting measurements was significantly negatively correlated with the proportion B-type granules ($r = -0.881$, $p = 0.048$) which might be related to their increased water absorption capacity as a result of the higher surface area-to-volume ratio.

> Differences in the protein composition of the waxy genotypes is less pronounced with a minimal variation for dough rheological parameters. However, with starch granule size distribution interacting with gluten-network formation, differences in functional attributes are possibly reduced. Water absorption of waxy wheat flour is higher resulting in a stiffer dough (higher $|G^*|$) in oscillatory rheological measurements performed at a fixed water addition.

7.3.1.1 Thermal properties

In addition to the aforementioned functional properties, thermal properties of the flour (starch gelatinization and protein denaturation) was studied more in-depth by using pressurized DSC (limited amount of water) and viscosity measurements (starch pasting cell under constant stirring in excess of water). As extrusion processing partially remains a *black-box*, it is advantageous to gain a broad insight in the pasting behavior under both conditions to get a basic understanding of the differences related to the presence of water.

DSC DSC-profiles of the flour from the different genotypes all showed a single endotherm within a temperature range of 53.1–67.9 °C (Figure 7.2 and Table 7.2). EXP had the lowest onset (T_o) and peak (T_p) temperature (53.1 and 59.7 °C respectively) and the second lowest endset temperature ($T_e = 65.1$ °C). However, the enthalpy (ΔH) of the sample (5.4 J g^{-1}) was equal to the values obtained for WxD and D11 which had a higher T_o and T_p. Within the four waxy flours, P+A could be distinguished on the basis of the markedly higher T_o (59.2 °C), T_p (63.8 °C), and T_e (67.9 °C). Both NXY and WxM, however, had higher enthalpies (6.9 J g^{-1} and 6.6 J g^{-1} respectively) compared to P+A (6.4 J g^{-1}).

Pasting behavior Pasting profiles of the pure waxy wheat *flours* illustrate clear differences (Figure 7.6**A**), mainly in the pasting temperature (Tg), peak viscosity (PV) and the PTemp. Genotypes WxD and D11 show very similar trends, analogously to the findings from the DSC measurements. NXY is characterized by the highest PV (3348 ± 16 mPa s), followed by P+A (2537 ± 19 mPa s), and WxD

Table 7.2: Composition and functionality of the raw materials (waxy flours and reference flour).

		WaxyDie	1154.06	Waxymum	NX12Y48222	Penawawa+Alpowa	Waxy flours[2]	Export flour
Composition	*PROC* (%dm)	9.7 ± 0.0	10.0 ± 0.0	10.2 ± 0.0	11.7 ± 0.0	11.7 ± 0.0	10.7 ± 1.0	9.7 ± 0.01
	TSt (% dm)	80.5 ± 4.2	80.8 ± 0.8	81.6 ± 2.6	81.6 ± 1.8	76.8 ± 2.2	80.3 ± 2.0	na
	AM (%)	0.54 ± 0.04	0.57 ± 0.12	0.58 ± 0.08	0.22 ± 0.01	0.34 ± 0.03	0.45 ± 0.16	21.96 ± 0.73
	AMP A (%)	24.60 ± 0.06	25.19 ± 0.19	25.77 ± 0.18	25.71 ± 0.09	25.53 ± 0.24	25.36 ± 0.46	26.73 ± 0.17
	AMP B_1 (%)	47.36 ± 0.08	47.56 ± 0.17	47.18 ± 0.25	47.48 ± 0.06	47.41 ± 0.11	47.40 ± 0.20	48.28 ± 0.08
	AMP B_2 (%)	18.37 ± 0.00	17.99 ± 0.06	17.33 ± 0.13	17.27 ± 0.20	17.41 ± 0.25	17.67 ± 0.46	16.39 ± 0.11
	AMP B_3^+ (%)	9.68 ± 0.02	9.26 ± 0.04	9.71 ± 0.06	9.53 ± 0.06	9.65 ± 0.10	9.57 ± 0.18	8.60 ± 0.02
	AMP aCL (%)	20.79 ± 0.01	20.53 ± 0.01	20.58 ± 0.02	20.53 ± 0.02	20.58 ± 0.08	20.60 ± 0.11	20.03 ± 0.01
	D_{10} (µm)	9.2 ± 0.1	1.2 ± 0.3	4.7 ± 0.1	5.4 ± 0.1	11.4 ± 0.0	8.2 ± 2.9	na
	D_{50} (µm)	20.8 ± 0.1	22.6 ± 0.3	20.5 ± 0.1	21.9 ± 0.1	20.3 ± 0.0	21.2 ± 1.0	na
	D_{90} (µm)	39.6 ± 0.2	46.6 ± 1.1	45.5 ± 0.2	47.0 ± 0.1	35.7 ± 0.0	42.9 ± 5.0	na
	B-type (%)	11.8 ± 0.2	9.7 ± 0.5	19.7 ± 0.3	15.3 ± 0.2	5.5 ± 0.0	12.4 ± 5.4	na
	A-type (%)	72.8 ± 0.1	68.5 ± 0.4	60.4 ± 0.1	63.0 ± 0.2	83.5 ± 0.0	69.7 ± 9.1	na
	A^+ (%)	15.4 ± 0.1	21.8 ± 0.9	19.8 ± 0.6	21.6 ± 0.2	11.0 ± 0.2	17.9 ± 4.66	na
Functional properties	*Water absorption*[1] (%)	65.9	62.5	65.7	71.6	63.9	65.9 ± 3.5	60.1
	tan(δ) (rad)	0.30 ± 0.01	0.33 ± 0.01	0.33 ± 0.00	0.31 ± 0.01	0.32 ± 0.01	0.32 ± 0.01	0.36 ± 0.01
	$\lvert G^* \rvert$ (MPa)	264 ± 29	164 ± 10	266 ± 62	288 ± 66	255 ± 42	247 ± 48	150 ± 9
	T_o (°C)	56.0	56.9	56.6	56.7	59.2	57.1 ± 1.2	53.1
	T_p (°C)	60.0	61.5	60.7	61.4	63.8	61.5 ± 1.4	59.7
	T_e (°C)	65.0	65.7	65.1	65.5	67.9	65.8 ± 1.2	65.1
	ΔH (Jg^{-1})	5.4	5.4	6.6	6.9	6.4	6.1 ± 0.7	5.4

[1] Water absorption at 500 BU as determined by Brabender Farinograph.

[2] Mathematical mean for all waxy cultivars (left of 'waxy flours'-column). Standard deviation is based on all values for waxy cultivars.
Amylopectin chain-length distribution classes (AMP A/B_{1-3^+}); i^{th} percentile of the starch granule size distribution (D_i); onset, peak and endset temperature ($T_{o,p,e}$); enthalpy (ΔH).

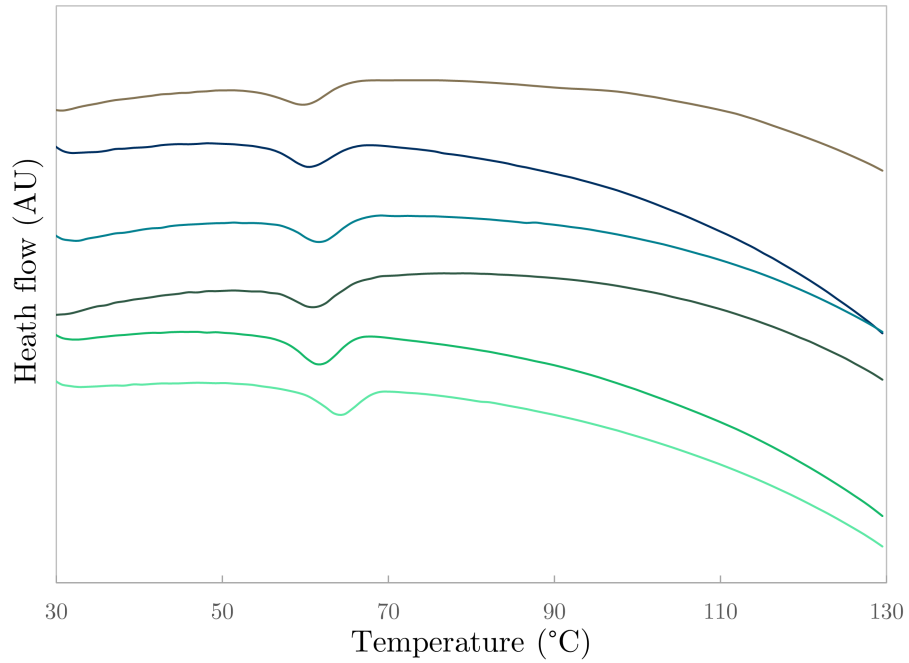

Figure 7.2: DSC curves of pure waxy wheat flours and standard export flour (WxD —, D11 —, WxM —, NXY —, P+A —, EXP —.)

and D11 (≈2123 mPa s). WxM has a significantly lower PV of 1154 ± 39 mPa s which also results in a significantly lower holding strength (HS) and final viscosity (FV) of 128 ± 3 and 190 ± 5 mPa s respectively. By the magnitude of this effect, it may be postulated that an increased α-amylase activity has resulted in a rapid degradation of the starch upon heating. However, as is shown in Section 8.3.1.2 (243), NXY (highest PV) has a higher enzyme activity. According to Dhital et al. (2011), the higher proportion of B-type granules (19.7%) may provide an alternative explanation for the effect on the PV. These authors attribute such variation to describe different mechanisms such as the space-filling effects of large granules, a decreased AMP content and an increased concentration of amylose-lipid complexes. From the current results, no final conclusion can however be drawn.

P+A has a higher T_g of 65.7 °C compared to all other waxy flours of which the pasting temperature is approximately 60 °C. This also may be attributed to the generally smaller granule size (D_{90} = 35.7 µm) which has been correlated with increased gelatinization temperatures by Soh et al. (2006). However, these authors also reported an increased water absorption during swelling for smaller B-type granules which was not found in this research. All parameters from the pasting curves can be found in Table 7.3.

Figure 7.6**B** illustrates the consequences of blending flour to obtain a specific AM content. The two distinct peaks at approximately 6 and 10 min which are mainly visible in the curves with blending ratios 75:25, 50:50 and 25:75, are from the waxy wheat and regular wheat respectively. At decreasing blending ratios (towards pure regular wheat flour), the PV of the first peak will decline while the second peak increases in height. Nevertheless, the total area under curve (AUC) for the two first peaks appears to be relatively lower for the 50:50 and 25:75 wx:std blend compared to the expected peak area. Moreover, both pure flours (P+A and EXP) have significantly higher FVs compared to the blends (586 and 678 mPa s respectively, compared to 209–289 mPa s).

A significant genotypic effect can be observed for pasting behavior and, to a lesser extent the gelatinization behavior measured through DSC. Differences are possibly resulting from a variation in the starch granule size distribution, protein content and enzyme activity. Upon blending, an uncommon pasting behavior showing two peaks can be observed whereas native partial waxy wheat flour will show only a single peak.

AM is considered crucial during network formation upon cooling as the long molecules can form a backbone in the starch-protein network. According to Singh et al. (2009a), this is however depending on the molecular weight of the molecules. Various researchers observed a positive correlation between AM content and FV although the interplay with proteins, lipids and non-starch polysaccharides contributed significantly to these effects (Sasaki et al., 2000; Blazek and Copeland, 2008). The similarly high FVs for both pure waxy and pure regular wheat flour may be attributed to the AM:AMP-ratio (Blazek and Copeland, 2008) and the ability of AMP molecules to aggregate via double helix formation in the absence of distorting elements (such as AM) (Cameron et al., 1994).

7.3.2 Extrudate characteristics

Extrudate quality was evaluated by means of their moisture content (MC) and average kernel weight, morphological attributes and texture (*i.e.* hardness). Results for both the genotype-trial (GT) and blend-trial (BT) are shown in Table 7.4.

MC of the extrudates shows to be significantly ($p < 0.001$) influenced by the genotype with D11 having the highest value (15.7 %) whereas P+A has the lowest value (13.9 %). EXP differentiates itself from the waxy-based extrudates through the very low MC of 10.6 %. This implies an effect of the AM content on moisture loss during expansion at die emergence. Results from the BT confirm this hypothesis as extrudates made from 100:0 wx:std have a significantly ($p < 0.001$) higher MC than 50:50 blends and, subsequently, regular wheat flour (13.9 %, 12.7 % and 10.6 % respectively). Despite minor variations between genotypes were recorded for the average weight per kernel (WpK)—ranging from 0.166 to 0.221 g per extrudate for D11 and NXY—no influence of AM content ($p = 0.477$) was observed.

When the melt exits the die, expansion occurs as a result of the flash evaporation of water resulting in (a) a reduction of the moisture content of the extrudate, (b) a rapid decrease of the melt temperature and, consequently, (c) a solidification of the viscous structure. By dividing the resulting diameter of the extrudate (strands) by the diameter of the die (3.0 mm), the expansion index (EI) is obtained. Although differences in the average EI were limited ($\Delta EI = 0.28$), a genotypic effect ($p < 0.01$) could be observed. P+A and D11 had the highest EIs (3.97 ± 0.12 and 3.94 ± 0.20 resp.) while NXY being the least expanded (3.71 ± 0.30). Additionally, AM contents significantly affected the EI, however, the correlation was not linear (Figure 7.3). The 25:75 wx:std blend resulted in the highest value (4.16) with a decrease towards higher incorporations of WWF in the blends. Regular wheat flour had the lowest EI when considering the BT which may be related to a higher resistance against expansion or a collapse of the gas cells upon cooling.

Based on automated image processing of scans of the spherical extrudates, the morphology could be studied. Besides area size (AS), the length-to-width-ratio (LtWr) and the circularity (CIRC) provide an

Table 7.3: Overview of the results from the starch pasting behavior of flour from pure genotypes and blends (14 % dm) using a Physica MCR 101 rheometer (Anton Paar, Ostfildern, Austria).

Sample	$\mathbf{T}_g$ (°C)	$\mathbf{PV}_1$[1] (mPa s)	$\mathbf{P}_{temp_1}$[1] (°C)	$\mathbf{PV}_2$[1] (mPa s)	$\mathbf{P}_{temp_2}$[1] (°C)	**HS** (mPa s)	**BD**[2] (mPa s)	**FV** (mPa s)	$\mathbf{SB}_{tot}$[2] (mPa s)
WaxyDie	59.3 ± 0.0	2189 ± 52	69.8 ± 0.6	-	-	664 ± 4	1525 ± 48	1120 ± 4	456 ± 8
1154.06	60.2 ± 0.0	2056 ± 10	71.2 ± 0.0	-	-	692 ± 16	1364 ± 6	1158 ± 4	466 ± 12
Waxymum	59.3 ± 0.0	1154 ± 39	69.4 ± 0.0	-	-	128 ± 3	1026 ± 36	190 ± 5	62 ± 1
NX12Y48222	59.3 ± 0.0	3348 ± 16	70.3 ± 0.0	-	-	860 ± 3	2488 ± 19	1480 ± 11	620 ± 8
100:0 wx:std	65.7 ± 0.0	2537 ± 19	73.1 ± 0.0	1271 ± 5.4	89.2 ± 0.0	732 ± 4	1804 ± 22	1318 ± 4	586 ± 8
75:25 wx:std	65.7 ± 0.0	1335 ± 10	74.0 ± 0.0	858 ± 1.4	91.5 ± 0.0	559 ± 3	777 ± 13	848 ± 7	289 ± 8
50:50 wx:std	65.7 ± 0.0	610 ± 31	74.0 ± 0.0	757 ± 40	93.3 ± 0.0	537 ± 27	219 ± 13	746 ± 7	209 ± 34
25:75 wx:std	65.7 ± 0.0	258 ± 1	74.0 ± 0.0	912 ± 24	93.3 ± 0	565 ± 0	364 ± 24	808 ± 64	243 ± 64
0:100 wx:std	80.1 ± 0.5	-	73.1 ± 0.0	1891 ± 78	93.3 ± 0.0	722 ± 14	1169 ± 65	1400 ± 131	678 ± 120

[1] Subscript for PV_i and $PTemp_i$ indicates the peak in the pasting curves for the blend trials.
[2] BD is calculated as the difference between the y-value (viscosity) of the highest peak and the HS.

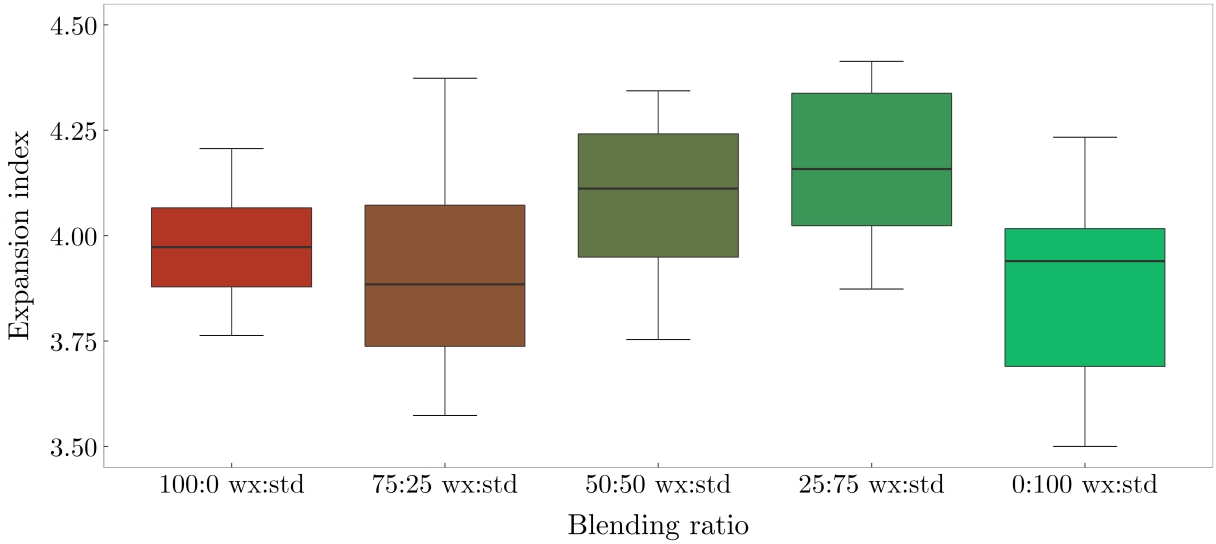

Figure 7.3: Effect of an increasing amylose concentration, from 0.34 % (●) to 21.96 % (●) by blending of waxy and regular wheat flour, on extrudate expansion (expressed as the expansion index (EI)).

additional insight in the resistance of the extrudate against collapse after expansion. All three measures are showing a significant influence of the genotype ($p<0.001$), as well as from the amylose content (*i.e.* blending rate) ($p < 0.001$). The AS is the lowest for WxD and D11 which are both significantly differing from NXY and WxM. P+A and EXP have the highest AS. Despite the significantly lower value for NXY-based extrudates compared to those of EXP and P+A (108 ± 18 mm^2 *versus* 130 ± 26 mm^2 and 126 ± 20 mm^2), the LtWr and CIRC of the former genotype is insignificantly different from EXP and P+A. Results from the BT clearly indicate an optimum for both LtWr and CIRC at a blending ratio of 75:25 wx:std. Both lower and higher amylose contents result in increased widths, thus, decreased CIRC. For the AS, no clear trend of AM content was observed.

> An optimum in the expansion index implies the presence of a trade-off between resistance to expansion and collapse upon cooling. As extrudate area sizes of both waxy and non-waxy genotypes are similar, interaction with gluten proteins are expected.

Extrudate texture (*i.e.* crispness) is an important quality attribute for expanded ready-to-eat snacks as it is typical for this type of products. Although crispness, as a sensorial parameter, can be defined in various different ways (Saeleaw and Schleining, 2011), hardness and compression distance at which the outer layer of the extrudate breaks are known to be a good indicators.

Clear genotypic effects for both the hardness and compression distance are observed ($p < 0.001$) with WxM and EXP having an average higher breaking strength (25.997 ± 9.575 N and 25.732 ± 6.141 N respectively) compared to all other genotypes ranging from 18.688 ± 6.043 to 21.396 ± 11.085 N). However, for the compression distance, EXP is significantly differing from all waxy genotypes. This means that, before breaking occurs, extrudates from EXP have to be compressed to a larger extent (0.76 ± 0.34 mm compared to 0.46 ± 0.24 mm), indicating that extrudates made from waxy wheat flour are more brittle.

Table 7.4: Quality attributes of extrudates from genotype-trial (GT) and blend-trial (BT).

Genotype blending ratio	MC (%)	WpK (g)	EI	AS (mm^2)	LtWr	CIRC	HARD (N)	Distance (mm)	BI[1] (%)
WaxyDie	14.9 ± 0.6^{ab}	0.188 ± 0.008^{b}	3.85 ± 0.24^{ab}	94 ± 15^{b}	1.20 ± 0.13^{d}	0.814 ± 0.050^{a}	21.396 ± 11.085^{a}	0.51 ± 0.25^{a}	3.8
1154.06	15.7 ± 0.1^{a}	0.166 ± 0.014^{c}	3.94 ± 0.22^{a}	84 ± 17^{c}	1.26 ± 0.19^{c}	0.789 ± 0.085^{ab}	21.141 ± 9.879^{a}	0.48 ± 0.25^{a}	3.9
Waxymum	14.8 ± 0.4^{bc}	0.197 ± 0.005^{b}	3.84 ± 0.17^{ab}	112 ± 20^{a}	1.31 ± 0.13^{b}	0.804 ± 0.048^{b}	25.997 ± 11.085^{a}	0.46 ± 0.28^{a}	4.3
NX12Y48222	14.5 ± 0.6^{bc}	0.220 ± 0.004^{a}	3.71 ± 0.30^{b}	108 ± 19^{a}	1.36 ± 0.15^{a}	0.781 ± 0.065^{c}	20.297 ± 10.379^{a}	0.46 ± 0.23^{a}	3.8
Waxy flours[2]	14.9 ± 0.5	0.193 ± 0.022	3.84 ± 0.11	99 ± 13	1.28 ± 0.07	0.797 ± 0.015	23.052 ± 1.798	0.455 ± 0.034	3.9 ± 0.2
100:0 wx:std	13.9 ± 0.4^{a}	0.218 ± 0.010^{a}	3.97 ± 0.12^{bc}	126 ± 205^{bc}	1.37 ± 0.14^{a}	0.793 ± 0.052^{b}	18.688 ± 6.043^{c}	0.39 ± 0.19^{c}	2.8
75:25 wx:std	13.7 ± 0.3^{a}	0.213 ± 0.019^{a}	3.92 ± 0.24^{bc}	116 ± 205^{bc}	1.25 ± 0.20^{c}	0.790 ± 0.086^{a}	19.846 ± 7.956^{bc}	0.40 ± 0.22^{c}	3.1
50:50 wx:std	12.7 ± 0.5^{b}	0.212 ± 0.008^{a}	4.09 ± 0.18^{ab}	136 ± 205^{ab}	1.27 ± 0.14^{b}	0.797 ± 0.063^{ab}	20.120 ± 6.690^{bc}	0.51 ± 0.26^{bc}	4.1
25:75 wx:std	12.3 ± 0.7^{b}	0.221 ± 0.004^{a}	4.16 ± 0.18^{a}	131 ± 195^{a}	1.31 ± 0.17^{b}	0.778 ± 0.090^{ab}	24.142 ± 8.907^{ab}	0.58 ± 0.24^{b}	4.9
0:100 wx:std	10.6 ± 0.0^{c}	0.209 ± 0.008^{a}	3.85 ± 0.24^{c}	130 ± 275^{c}	1.39 ± 0.15^{a}	0.756 ± 0.091^{c}	25.732 ± 6.141^{a}	0.77 ± 0.33^{a}	6.6

Means with the same superscript in the same column are significantly different ($p < 0.05$, Tukey's HSD) per group (genotype or blending ratio).

[1] Breaking index (BI) is defined as the relative compression depth before breaking of the extrudate occurs.

[2] Mathematical mean for all waxy cultivars (left of 'waxy flours'-column). Standard deviation is based on all values for waxy cultivars.

Extrudate hardness (HARD); compression distance before first peak (distance); breaking index (BI).

Analogously to the findings from the GT, a clear effect of the AM content on both hardness and compression distance is found ($p < 0.001$). Both factors steadily increased respectively from 18.688 to 25.732 N and 0.39 to 0.77 mm upon increasing AM concentrations (lower percentage WWF). On the basis of the BT it was observed that extrudates produced with pure WWF needed 7.044 N less compression force before breaking occurred than extrudates made from regular wheat flour (Figure 7.4).

> Extrudates produced using regular wheat flour (containing amylose) were less brittle and required a higher breaking strength although genotypic variation for the latter factor was observed.

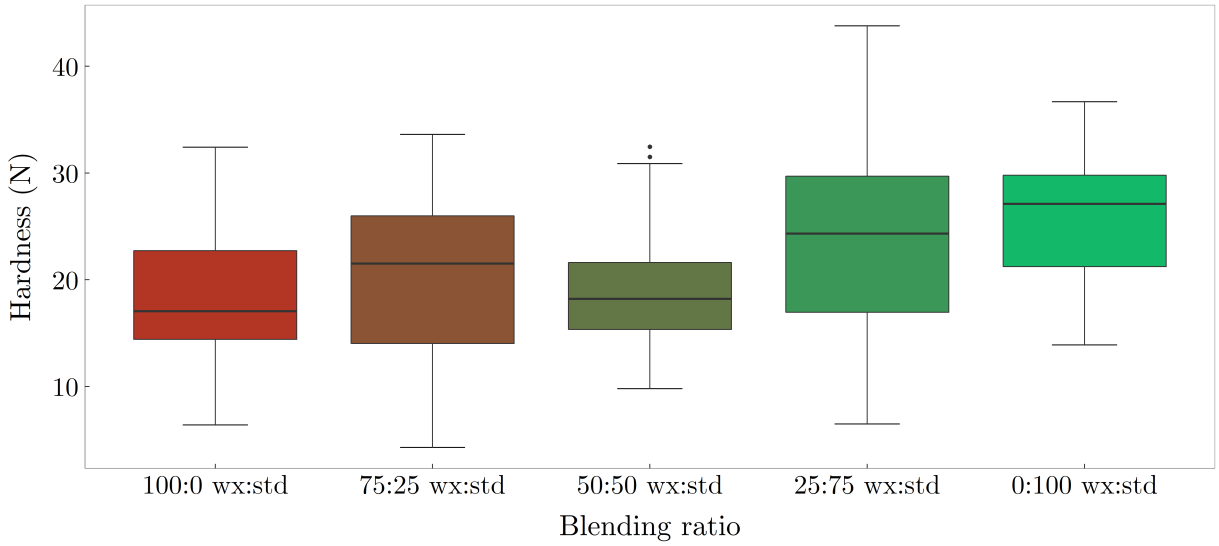

Figure 7.4: Effect of an increasing amylose concentration, from 0.34 % (●) to 21.96 % (●) by blending of waxy and regular wheat flour, on extrudate hardness.

7.3.2.1 Pasting behavior

In order to obtain an insight in the water binding capacity and the presence of intact (*i.e.* unpasted) starch granules after extrusion cooking, the pasting behavior of the ground extrudates was determined. The resulting curves for both the GT and BT can be found in Figure 7.7.

As the ground extrudate was added to the distilled water during the pre-shear phase, a first peak occurs at the beginning of the diagram (Figure 7.7**A**) as a result of the hydration of the starch components (AM, AMP and fragmented polysaccharides) in the extrudate. For the PV, genotypes P+A (73.2 ± 3.7 mPa s) and WxM (75.5 ± 3.5 mPa s) have lower values than D11 and WxD (93.9 ± 2.4 and 98.0 ± 6.1 mPa s respectively). Overall, viscosities remain low. During the heating phase, at 93.8 and 94.2 °C respectively, WxD and P+A show a small viscosity increase (first arrow around minute 11) whereas all other genotypes evolve towards a stable viscosity during the holding phase at 95 °C. These two genotypes also show a drop in their viscosity during cooling (second arrow at 23 minutes) making them return towards the expected viscosity range. FVs range from 79.0 ± 2.0 to 89.0 ± 5.2 mPa s, following the same order as the viscosity values of the primary peak.

Samples from the BT only show an initial peak at the hydration of the powder PV as is illustrated in Figure 7.7**B**). The effect of the AM content in the extrudates clearly comes to expression in these pasting curves. PV values increase in an exponential way (R^2 = 0.998) from 74 ± 3 to 556 ± 42 mPa s.

Ground extrudate powder was also subjected to DSC analysis, performed using the same methodology as used for the flour but did not show any significant endotherm over the entire scanning range (30–130 °C) (Figure 7.5). This may indicate that no ungelatinized starch (*i.e.* intact granules) remains after extrusion cooking as was also reported by Ozcan and Jackson (2005).

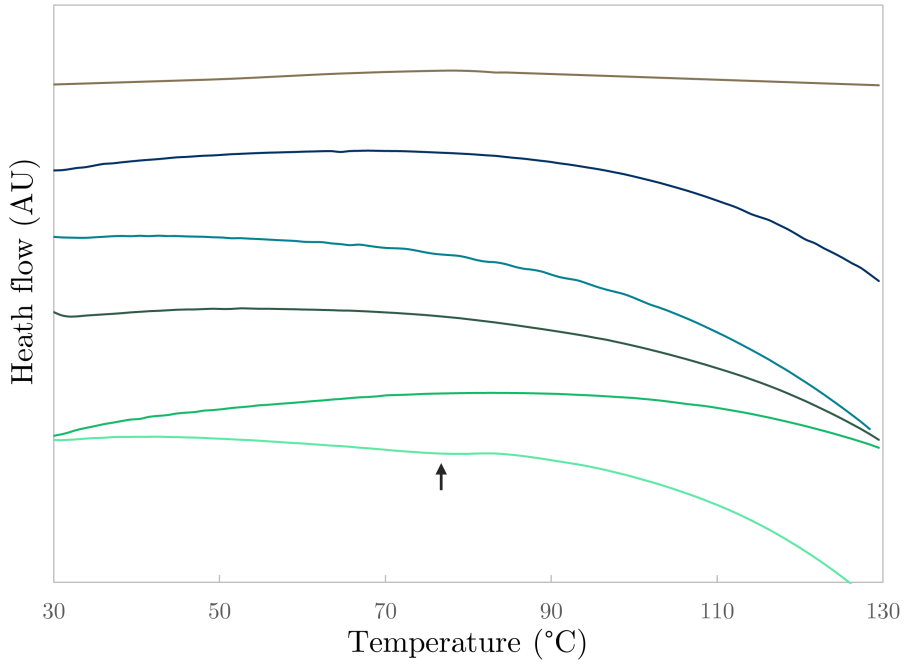

Figure 7.5: DSC curves of the ground extrudates based on pure waxy wheat flours and standard export flour (WxD —, D11 —, WxM —, NXY —, P+A —, EXP —).

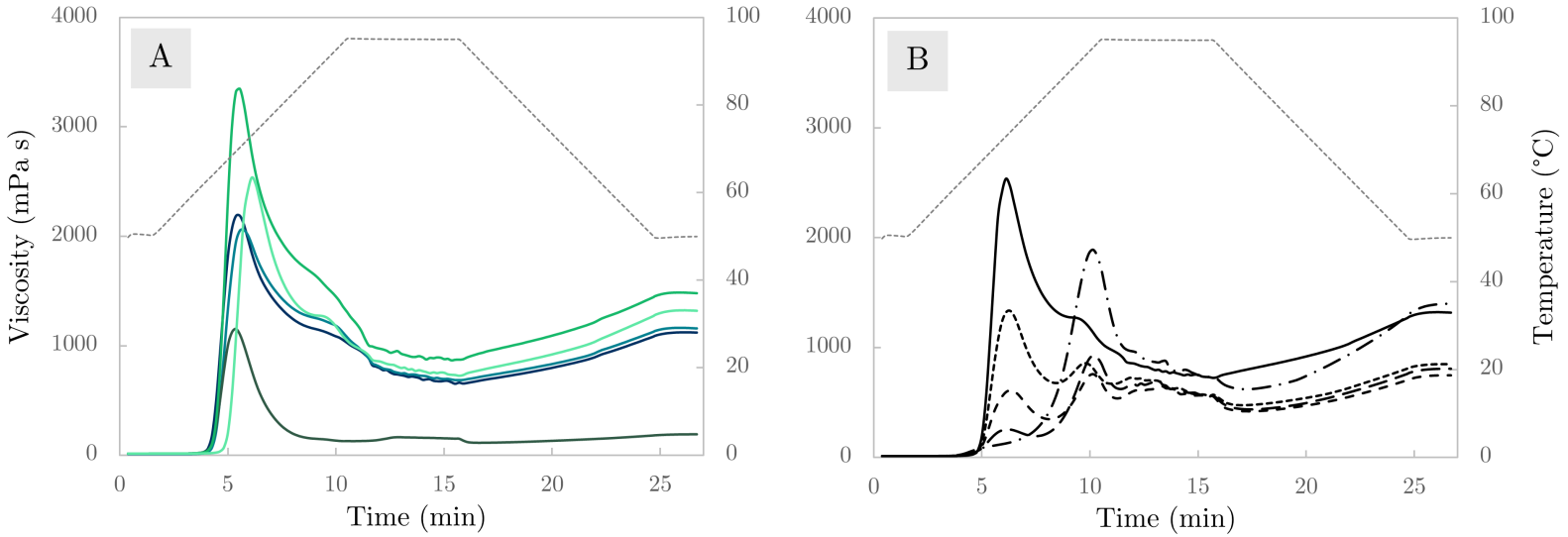

Figure 7.6: Pasting profiles of **A** pure waxy wheat flours (WxD —, D11 —, WxM —, NXY —, P+A —) and **B** blends of waxy and regular wheat flour (100:0 wx:std —, 75:25 - -, 50:50 - -, 25:75 - , 0:100 - ·, temperature - -).

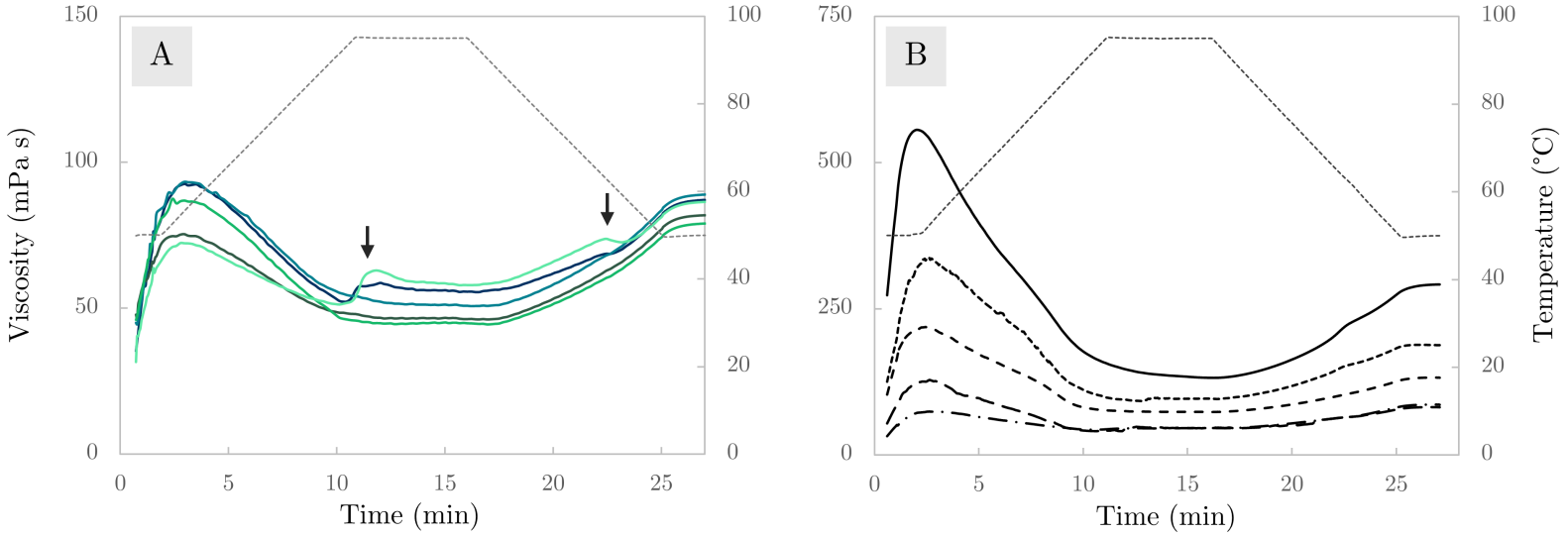

Figure 7.7: Pasting profiles of ground extrudates produced from **A** flour from five pure waxy wheat cultivars (WxD —, D11 —, WxM —, NXY —, P+A —) and **B** blends of waxy and regular wheat flour (100:0 wx:std —, 75:25 - -, 50:50 - -, 25:75 - , 0:100 - ·, temperature - -).

7.4 Discussion

The variation in the PROC of the wheat flour from the different genotypes may have influenced the end-product quality of the extrudates, mainly the EI. Ghorpade et al. (1997) stated that frequently, inconsistent results are reported for this relation. In general, expansion will decrease at increasing PROCs with fewer but larger gas cells at high (>16 %) wheat gluten enrichment. In addition, this was found to be dependent on the composition of the (gluten) proteins as a result of their botanical origin (*e.g.* soy or wheat proteins), the genotype and the growth conditions. On the contrary, compared to pure wheat starch, addition of wheat gluten showed to have a stabilizing effect on gas cell formation (in terms of obtaining a more homogeneous internal structure in the extrudates) implying that an optimum for EI in function of protein content exists (Moraru and Kokini, 2003; Draganovic et al., 2013). This was also shown in Chapter 6 in which gluten composition, protein content and their interaction showed to largely influence the expansion besides other extrudate quality attributes such as the hardness.

A positive correlation ($p = 0.03$, $R^2 = 0.715$) between dynamic rheological parameter complex shear modulus ($|G^*|$) and water absorption at 500 BU (WA_{500}) (Farinograph) could be observed. However, this result was in contrast with Van Bockstaele et al. (2008), who has reported a *negative* correlation ($R^2 = 0.548$). This can be explained by the use of a fixed water addition while preparing the dough instead of 95 % of the optimal WA. Water is an important factor in determining the viscoelastic properties of dough as it has a dual role as inert filler and lubricant (Masi et al., 1998). In this study, an increase in the $|G^*|$ resembles a higher resistance to deformation as a result of different water absorptions of the flour constituents (mainly proteins and starch). Both their concentration and composition contributes to these differences. Therefore, $|G^*|$ and phase shift angle ($\tan(\delta)$) may be appropriate variables for predicting end-product quality attributes of extrudates such as expansion and hardness. On the contrary, it can be argued that correlations between $|G^*|$ or $\tan(\delta)$ and end-product characteristics are lacking as during measurements, forces within the linear viscoelastic region were applied. Van Bockstaele et al. (2008), however, found a strong correlation ($R^2 = 0.74$) between $|G^*|$ measured on dough and the bread volume. Kristiawan et al. (2016) hypothesized that these parameters could provide insight in the viscoelastic behavior of the melt (mainly the storage modulus) during extrusion which, on its turn, has been related to macroscopic expansion behavior. Relations are however not straight-forward as the latter is obtained through a complex combination of nucleation, bubble growth, coalescence, shrinkage and fixation.

Granule size distributions are strongly differing for the different genotypes which may affect starch gelatinization both in excess of water as well as during extrusion processing. As investigated by Singh et al. (2009b), starch rheology is mainly influenced by granule size whereby suspensions of large size particles tend to be more viscous compared to those of the counterpart smaller size (*i.e.* A and B-type granules respectively). In this study, the relative proportion of B-type granules (≤10 µm) showed a significant negative correlation with the pasting temperature (T_g) ($p \leq 0.050$, $R^2 = 0.776$) whereas, for A-type granules (10–35 µm), positive correlations ($p \leq 0.050$) were found for the HS ($R^2 = 0.815$) and FV ($R^2 = 0.831$). The latter finding is in accordance with results from Kumar and Khatkar (2017) and can be attributed to the occupation of a relatively larger volume in the solution compared to B-granules at a similar quantity. Increased interaction between surfaces of unpasted (remnants of) starch granules after reaching the peak viscosity will thus contribute to a higher HS and FV (Hellemans et al., 2017). Also, the

decrease in PV for EXP compared to WWFs was related to an increase in AM content (Singh et al., 2009b; Kumar and Khatkar, 2017; Zi et al., 2019). Under no-shear conditions, Abdel-Aal et al. (2002a) found that waxy wheat starch granules show a greater swelling and more complete disintegration (gelatinization) compared to non-waxy wheat, particularly at high temperatures (90 °C). The relative higher proportion of AMP in the granules lays at the basis of this phenomenon.

> At every blending ratio, starch granules were fully degraded as no gelatinization was observed through DSC-measurements or when measuring the pasting behavior of the milled extrudate. Apart from complete gelatinization, extrusion processing will promote molecular degradation of amylopectin resulting in a decreased network formation as mainly short chains (low DP) are remaining. This can be studied using high-performance size-exclusion chromatography (HP-SEC).

By means of principal component analysis (PCA), compositional and functional attributes of the raw materials and quality characteristics of the extrudates were related. In total, 68.9 % of the variance in the dataset could be explained by the two first PCs (Figure 7.8). Colors of the arrows indicate the contribution of each variable going from red (low) to green (high). The first PC (PC1, 44.8 %) is mainly correlated with starch related attributes such as TSt content, granule size distribution parameters and thermal properties (DSC and pasting) whereas the second PC (PC2, 21.8 %) largely resembles protein properties (WA, $|G^*|$ and tan(δ)).

The WA of the flour (determined by Farinograph) is positively correlated with the $|G^*|$ ($R^2 = 0.71$) whereas a strong negative correlation with the EI ($R^2 = 0.90$) of the extrudates was also noted. Although shear applied during extrusion processing is many orders larger, this relation may imply the ability to estimate expansion on the basis of functional properties, both fundamental or empirical. However, an improved insight in protein and starch transformations (both separate and in a combined fashion) taking place during extrusion remains highly recommended. The angle between EI and AM content, pasting and gelatinization-related properties indicates the significant contribution of these elements. Flour WA and dough consistency also play a role in the development of this parameter. Moreover, the WA is negatively correlated with both PV and PROC, thereby again indicating that interactions between protein and starch take place during mixing.

Increased molecular degradation (depolymerization and debranching) of AMP during extrusion of waxy wheat (Ozcan and Jackson, 2005) may result in an elevated moisture loss through evaporation as gas cells will more easily rupture during expansion. The reduced ability of short amylopectin chains to form a strong network will result in a decreased gel strength (Blazek and Copeland, 2008), thus promoting rupturing upon fast expansion during flash evaporation. Contrastingly, debranched amylopectin—occurring mainly at the α-(1→6)-bonds—will result in an increased hydration of the obtained components (short chains and dextrins), thereby promoting water binding. However, as AMP contents are not significantly differing between the waxy genotypes, its contribution is considered to be limited. In addition, this will also be highly dependent from the protein properties. An increase in the PROC may improve gas cell formation by the development of a strong but flexible gluten-starch film around the gas cells (Sroan et al., 2009). Its strength is also determined by the protein composition, primarily the proportion of high molecular weight glutelin-subunits (HMW-GS). As reported by Purna et al. (2011), gas cell formation during breadmaking

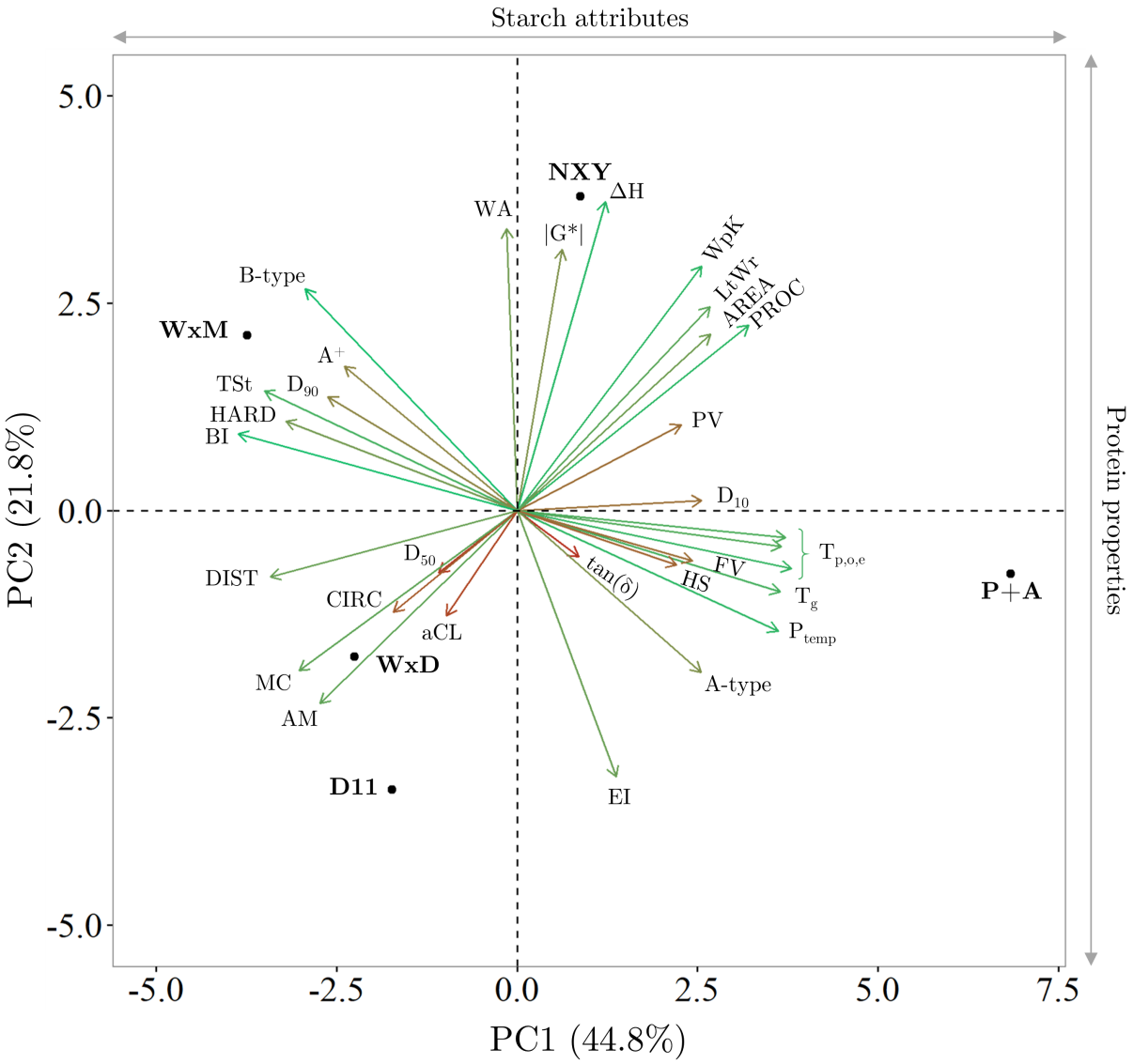

Figure 7.8: Biplot from the two first principal components (PCs), together explaining 66.6 % of the variance in the dataset, based on compositional, functional and end-product quality attributes of the samples used in the genotype trial. The color of the arrows indicate the contribution—from red (low contribution) to green (high contribution)—of each variable to the principal component analysis.
i^{th} percentile of the starch granule size distribution (D_i); extrudate hardness (HARD); breaking index (BI); compression distance before first peak (DIST); onset, peak and endset temperature ($T_{o,p,e}$); area size (AREA); enthalpy (ΔH).

also requires a good balance between elasticity and extensibility to allow the gas cells to expand and to rupture, thus enabling vapor to go out of the product.

Proposition

Variation in the amylose content is determining for extrudate quality trough altering the melt viscosity and its viscoelastic behavior which, on its turn, impacts expansion behavior and thus, texture properties. Analogously to bread production, an increased expansion at die emergence can be obtained at slightly lowered amylose concentrations although gas cell collapse can intervene with this effect implying the presence of a lower limit. The minimal required concentration can be varying according to the protein properties but requires further investigation as interaction effects between starch granules, (depolymerized or debranched) starch biopolymers, protein and water complexes the effects on melt viscosity and expansion. This impedes the feasibility to correlate compositional attributes to end-product characteristics thereby unraveling the working mechanisms behind the observed phenomena.

Both TSt and the D_{90} (or alternatively the proportion of A- and B-type granules) are positively correlated with extrudate hardness. Based on various research outcomes, it can be stated that both parameters will influence melt viscosity which was related with extrudate hardness by Zhang et al. (2016b). These authors found that the thermal properties (mainly ΔH) can affect the textural characteristics of the extrudates by influencing the melt viscosity (and specific mechanic energy) and thus, the gas cell formation. In this light, it has to be kept in mind that, on one hand, the melt viscosity should be low enough to promote bubble growth but, on the other hand, should be high enough to prevent bubble collapse and coalescence (Kristiawan et al., 2016).

7.5 Conclusion

This study has shown that, when applying waxy wheat flour in extrusion processing, an important genetic factor should be taken into account as both protein and starch related attributes influence its processability and thus, end-product quality. Nevertheless, a minimal addition of waxy wheat flour to regular wheat flour (25:75 wx:std) enhances the expansion ratio of the extrudate while resulting in more brittle products with an overall lower hardness. Effects are however not linear with the amylose-to-amylopectin ratio and the protein composition (and their mutual interaction) being crucial factors which should be taken into consideration when assessing the relation between composition and end-product quality. Results showed that also starch granule size distribution influences the pasting and gelatinization behavior, although the primary effect may remain the starch composition as this is found to be related with the size distribution pattern. Contrastingly, no clear evidence of a relation between the average amylopectin chain length and extrudate quality was found although waxy genotypes in this study had a higher proportion short branch-chains resulting in a significantly lower average chain length.

When studying different genotypes with an altered composition in terms of protein and starch, a broad screening of all molecular and macromolecular attributes should be attained to properly investigate the possible protein-starch interactions which are determining for extrudate quality. In addition, an enhanced simulation of the pasting behavior of the flour during extrusion (under high pressure, shear

and temperature and at low moisture contents) or on-line viscosity measurements should be included. An improved understanding of both the factors influencing melt viscosity as well as how this is related to end-product quality is highly recommendable. This would however require a mechanistic modeling approach.

Inherent to the use of blends to study the effect of a single attribute, is the undesirable shift obtained for other compositional properties (in this case: protein content and composition). A possible solution to overcome these secondary effects would be the use of near-isogenic lines. The added value of using lines with a similar genetic background in this type of research did already come to expression through the highly comparable results observed for WxD and D11 which are known to be closely related.

Highlights

- Starch content and composition influences raw material pasting properties whereas granule size distribution is related to extrudate texture.
- Amylose content affects the expansion index, water absorption and texture of extrudates.
- Interaction between starch composition and protein content and quality is observed for different waxy genotypes.
- Maximum expansion was obtained for blends containing 25 % waxy flour.

CHAPTER 8

The potential of wheat genotypes with an altered starch composition for breadmaking

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8.1 Introduction

The perceived quality of bread is mainly determined by the properties of the bread itself (food properties), including the sensory (color, taste, aroma) and nutritional (energy density, glycemic response, and fiber content) attributes (Gellynck et al., 2009) as well as preservation characteristics on a physiochemical (staling) (Angioloni and Collar, 2009) and microbiological (spoilage) level (Debonne et al., 2018). Continuously, researchers are attempting to improve the various quality attributes of bread by the addition of (natural) ingredients (enzymes, hydrocolloids, lipids, *etc.*), the application of alternative processing techniques (sourdough, par-baking, freezing) or the use of (wheat) flour (or starch-gluten mixtures) with an altered composition (Fadda et al., 2014). Besides minor cereals, ancient crops and pseudo-cereals, novel wheat varieties such as amylose (AM)-free (waxy, < 1 % AM), low-AM (partial waxy, 3–22 %) and AM-rich (elevated (≥ 30 %) or ultra-high (≥ 50 %) AM wheat varieties are studied for their breadmaking potential (Van Hung et al., 2006).

The application of waxy wheat flour (WWF) has been studied extensively in the last fifteen years (Fadda et al., 2014; Zhang et al., 2014) and has been related to a decreased initial firmness and a lowered staling rate. A trade-off exists between an overall prolonged freshness and the markedly diminished visual appearance and generally lowered consumer acceptability at high blending ratios of WWF (15–30 % in conventional wheat flour). Although limited research which includes breadmaking trials is available, all studies observe an instantaneous collapse of the loaf ('keyhole'-shape) and an excessive post-bake shrinkage (Peng et al., 2009; Zhang et al., 2014; Blake et al., 2015). Following Purna et al. (2011), these phenomena could be attributed to the presence of an open crumb structure resulting from the virtually complete loss of starch granule rigidity during baking, despite the limiting water availability in dough.

Furthermore, as dough kneading is equally important for the quality of the crumb structure (Rathnayake et al., 2018), the increased susceptibility for overmixing of waxy wheat dough—shorter dough development times were reported for WWF (Abdel-Aal et al., 2002b; Blake et al., 2015)—may further contribute to this effect. Contradictory findings were however obtained by Guan et al. (2009) who postulated that the higher water absorption of WWF retarded the hydration of the gluten-forming proteins, thus delaying dough development.

On the contrary, wheat cultivars with an elevated AM content have been introduced recently as an approach to increase the consumption of dietary fibers (Li et al., 2019b). However, as the complexity of AM biosynthesis (Section 1.2.3.2, page 17) allows to elevate the AM content through two entirely different synthetic events, variation of the AM content, starch structure, granule morphology and eventually, its functionality will differ largely within high amylose wheat flour (HiAm-WF). The most frequently considered approaches to elevate AM contents in wheat are (a) suppressing the glucan elongation step by starch synthase (SS) IIa (thereby also decreasing the amylopectin (AMP) synthesis) or (b) suppressing the starch branching event through either starch branching enzyme (SBE) IIa or a combination of SBEIIa and SBEIIb. For example, results from Hazard et al. (2012) showed that only the combined down-regulation of SBEIIa and SBEIIb resulted in a significant increase (115 % increase compared to wild-type) in the resistant starch content. McMaugh et al. (2014) found that starch swelling decreased as a result of an increased AM content although effects were dependent on the suppressed protein (SSI versus SSIIa). Additionally, these authors related AMP branch chain-length distribution (CLD) with differences in the

pasting behavior of the starch which was found to be genotype dependent. In contrast to the potential added value of applying HiAm-WF in bakery products (mainly nutritionally), the rapid retrogradation properties of AM may negatively influence crumb firmness thereby producing food products which have an even shorter shelf-life.

Despite the high potential of (partial) waxy and high-amylose varieties to diversify the current wheat market (for applications in both food and non-food products) (Šárka and Dvořáček, 2017), still too much uncertainty about the effects on the end-product quality remains existent. By enlarging the compositional and functional variation in the starch component, unforeseen interaction-effects (with proteins) can be expected. Hence, predicting end-product quality will be further complicated as extrapolation from current models—derived from experiments on wheat with a narrow diversity in starch compositional properties—may yield incorrect results. Moreover, usability of conventional techniques for quality screening is questionable as fundamental assumptions are invalid for these varieties with an altered starch composition. These hurdles retard commercial availability and application, despite their large potential.

8.2 Material and methods

In the current chapter, nine genotypes with distinct starch properties in terms of AM concentration are subjected to compositional and functional analysis to assess their baking quality using conventional techniques. In addition, breadmaking trials are performed to measure end-product characteristics, allowing to quantify the contribution of starch properties to these quality attributes. In this way, their potential for breadmaking applications is investigated as well as the hurdles accompanied by applying conventional techniques (for analysis and processing) on these samples.

8.2.1 Sample collection and flour extraction

'Waxydie' (WxD) and genotype '1154.06' (D11) were provided by Dieckmann seeds (Rinteln, Germany), 'Waxymum' (WxM) was obtained from Limagrain Céreal Ingredients (Sain-Beauzire, France) and 'NX12Y8222' (NXY) was collected from the University of Nebraska-Lincoln through the USDA germplasm database. These waxy genotypes, which were also applied in Chapter 7, were supplemented with KBA, a cross of Kanto107\\Bai Huo. As partial waxy genotype, its parent 'Kanto 107' (KAN) was used. Both were obtained from the USDA National Small Grains Collection. As reference, the regular wheat *flour* 'Epi B' was bought from Paniflower (Zwijnaarde, Belgium). The diversity was further extended by the introduction of two high-amylose (HiAm) wheat cultivars. ADE (Adelaide HiAm), which was kindly provided by John Carragher from the University of Adelaide (Adelaide, Australia), and ultra-high amylose *flour* which was obtained from Arista Cereals Technologies (Eric Vaschalde, Sydney, Australia).

All genotypes were initially obtained as kernels (15–30 g) and were propagated during three crop cycles to obtain enough material for milling. A primary propagation under greenhouse conditions was immediately followed by a secondary propagation step on a field. Due to the intense cultivation conditions during the first two crop cycles, only material from the final field trail were used in this research. During 2017–2018,

winter (WxD, D11, WxM, NXY) and spring (KBA, KAN, ADE) wheat genotypes were cultivated as described in Section 7.2.1.1 (page 214).

Cultivated samples were harvested at maturity and were prepared for flour extraction as described in Section 3.2.2 (page 99). Subsequently, kernels from all genotypes except ADE were tempered overnight to a moisture content of 15.5 %. A lower feed rate was set during milling on the Bühler ML-202 laboratory mill to prevent ceasing of the sieves. As preliminary experiments indicated that HiAm genotypes could not be milled using this mill, a Quadromat Junior (Brabender, Duisburg, Germany) was used to obtain flour from ADE. Moreover, no tempering was applied as this would increase stickiness of the flour. Yet, as considerable amounts of flour were noticed in the bran fraction, it was chosen to clean this fraction more intensely (6 times) compared to the samples milled on the Bühler mill (bran: single time and shorts: twice).

8.2.2 Chemical composition

From the obtained flour, moisture content, total starch content, amylose concentration and amylopectin fine structure and the protein content are determined using methods described in Section 3.2 (pages 98–115).

8.2.3 Particle size distribution

Flour particle size was measured by laser diffraction using a Beckman Coulter LS13 320 (Beckman Coulter, California, USA) equipped with the Tornado module for powder measurements. Ten to fifteen grams of flour was used to obtain a measurement duration of approximately 40 seconds at an obscuration of 5–10 %. Using the Fraunhofer theory, volume percent distributions were obtained from which the relative proportion small (2–40 µm) and large (40–400 µm) particles was calculated.

8.2.3.1 Alpha-amylase activity

In addition to these properties, α-amylase activity was measured using the Ceralpha method from Megazyme (Wicklow, Ireland) according to the procedure described in the accompanying documentation. A wheat flour standard delivered with the kit was included in each test series to evaluate the measurement performance. An absolute deviation of 0.006 Ceralpha units (CU) per gram compared to the reference value (0.060 CU g^{-1}) was allowed. All samples were measured in triplicate with a maximum RSD of 10 %.

To standardize the enzyme activity in the breadmaking trials—conventionally to a Hagberg falling number (HFN) of 250 seconds—by addition of malt flour, CU g^{-1} was correlated with the HFN using the reference flour (‘Epi B’). Flour samples containing 0.2 and 0.5 % malt flour were prepared and tested using both the Ceralpha method and the HFN (executed in quadruplicate). An exponential relation was fitted to the data, allowing the translation of Ceralpha-units to HFNs.

8.2.3.2 Cryo-scanning electron microscopy

The shape of the starch granules was visualized using a JSM-7100F TTLS LV TFEG-SEM (Jeol Europe BV, Zaventem, Belgium) under high vacuum and at an accelerated voltage of 3.0 keV. Prior to electron beam targeting, the samples were vitrified in liquid nitrogen and transferred to a PP3000T cryo-transfer system (Quorum Technologies Ltd., East Sussex, UK) at -140 °C. The samples were subjected to a sublimation step of 30 min at -90 °C. Prior to the transfer from the cryo-preparation room to the scanning electron microscope (SEM) chamber, a thin layer of a conductive metal (Pt) was deposited on the samples during 90 s.

Starch extracts were obtained using the dough-ball method. In essence, flour was mixed with water (150 % hydration) and manually kneaded until a firm dough was obtained. The dough ball was rinsed using distilled water over a gluten washer disc (mesh opening = 90 µm). The washed out starch was collected in a 50 ml tube and centrifuged for 5 min at 3,000 × g. After decanting the supernatant, the pellet was rinsed with acetone and again centrifuged. Finally, the starch was left to dry overnight.

8.2.4 Functional analysis

Functionality of the samples was tested by means of rheological measurements on the flour in water limiting conditions (*i.e.* dough) and excess of water. Besides starch pasting, the Farinograph mixing test was performed to measure dough consistency according to standard method ICC 115/1 using the 50 gram mixing chamber. In addition, dynamic oscillatory and static rheology was used to gain a broadened insight in the viscoelastic behavior of the dough within and outside the linear viscoelastic region.

8.2.4.1 Pasting behavior

Starch pasting behavior was measured using a Physica MCR 101 stress-controlled rheometer (Anton Paar, Ostfildern, Austria) equipped with a pressurized starch pasting cell using a six-blade vane as spindle. During the entire measurement, high pressure (0.6 MPa) was applied using filtered pressurized air.

Analog to the previous pasting measurements, a 14.0 % (corrected to 14 % moisture) flour-water suspension was prepared by mixing approximately 2.1 g flour with 15 ml distilled water. For measurements in which α-amylase activity had to be inhibited, 300 µl of a 0.1 M silver nitrate ($AgNO_3$) solution was added before mixing the flour with the water. A homogeneous suspension was obtained by intensively shaking the flour and water in a 50 ml centrifuge tube with a conical bottom. After transferring the suspension to the measuring cup, the top part of the pressurized starch pasting cell was screwed on tight, the pressurized air hose was connected and pressure was applied carefully. Within 2 minutes after mixing, the measuring cycle was started.

Before the actual measurement commenced, a pre-shear was performed at 960 rpm to further homogenize the sample. During this phase, temperature was gradually increased to 50 °C. After a short holding period (1 min), the suspension was heated to 140 °C at a heating rate of 5.0 °C min^{-1} at a constant rotational speed of 160 rpm. A 10 min holding period was followed by a cooling phase equal to the heating phase to 50 °C. The measurement was finished after a final holding phase at 50 °C for 2 min. Data was collected

using the RheoLab software (Anton Paar, Ostfildern, Austria) and parameters were calculated using an automated R-script (version 3.4.3, R Core Team (2018)). Measurements were performed in duplicate.

8.2.4.2 Dynamic oscillatory rheology

Posterior to a stress sweep through which the upper boundary of the linear viscoelastic region was determined, a frequency sweep was performed on dough samples using the method described in Section 7.2.2.7 (page 217). At least four replicates were performed per sample. Besides the storage modulus (G') and loss modulus (G"), dough viscoelastic properties complex shear modulus ($|G^*|$) and phase shift angle ($\tan(\delta)$) were determined. Values at 10 Hz were used for further analysis.

8.2.4.3 Static rheology

In addition to the rheological measurements in the linear viscoelastic region (thereby preserving the microstructure in the sample), creep-recovery measurements were performed. As the applied shear is one order of magnitude larger than the shear applied during frequency sweep measurements (250 *versus* 25 Pa), tests could be performed subsequently.

A 300 s creep-phase, during which a stress of 250 Pa is instantaneously applied, was followed by a 600 s recovery-phase in which the stress is reset to 0 Pa. From the recorded curves, the maximum deformation (DEF_{max}) and elastic recovery ($\%REC_{el}$) were calculated as the maximum strain (in percent) during the creep-phase and the relative return (compared to this maximum) to the initial form during recovery. In addition, the Burgers model (Equation 8.1a) (Steffe, 1996) —most frequently presented as a compliance function (Equation 8.1b)—was fitted to the creep-curve and the transformed (ascending) recovery-curves after determining the optimal number of Kelvin-Voigt elements (summation part in Equations 8.1c and 8.1d). Elements are added to better represent experimental data. After removing the part describing the long-term viscous flow (t/μ_0) which represents the permanent deformation, a function similar to Equation 8.1c can be used to describe the recovery phase (Equation 8.1d).

$$\gamma = f(t) = \frac{\sigma_0}{G_0} + \frac{\sigma_0}{G_1}\left(1 - \exp(\frac{-t}{r})\right) + \frac{\sigma_0 t}{\mu_0} \tag{8.1a}$$

$$J = f(t) = J_0 + J_1\left(1 - exp(\frac{-t}{r})\right) + \frac{t}{\mu_0} \tag{8.1b}$$

$$J_c = f(t) = J_0 + \sum_{i=1}^{m}\left[J_i\left(1 - exp(\frac{-t}{r_i})\right)\right] + \frac{t}{\mu_0} \tag{8.1c}$$

$$J_r = f(t) = J_0 + \sum_{i=1}^{m}\left[J_i\left(1 - exp(\frac{-t}{r_i})\right)\right] \tag{8.1d}$$

The optimal number of components for the two resulting models was determined using an F-test for model comparison as described in Van Bockstaele (2011). Respectively, an 8 (instantaneous response, J_0; delayed elastic deformation, J_{1-3}; retardation times, r_{1-3}, and Newtonian viscosity, μ_0) and 9 component (J_0, J_{1-4}, r_{1-4}) model was retained for the creep-phase and recovery-phase respectively. Using principal component

analysis (PCA), insight was obtained in the parameters which provide supplementary information. The first two principal components (PCs) explained 78.7 % of the variance of which 55.7 % was explained by the first PC. The loading plot (**B**) in Figure 8.7 (page 254) indicates the main contribution of the instantaneous response (J_0) and the first coefficient of the delayed elastic behavior (J_1) of the recovery phase. Moreover, C-r_1 and R-r_{1-4} are not strongly correlated thereby providing additional information and thus discriminative power. Therefore, these are retained for further data analysis.

8.2.5 Breadmaking tests

Breadmaking tests were performed as described in Section 3.2.6.1 (page 112). Malt flour addition was calculated according to the predicted HFN numbers. Dough temperature after mixing ranged between 26.2–28.1 °C. As flour of HiAm ADE was limitedly available, only a single pan loaf and two plate loaves were produced.

After baking and cooling, loaf volume (per kilogram flour) (VOLfc), height-to-width-ratio (HtWr), and crumb and crust color were measured in a similar way as described in Section 3.2.6.2 (page 112). By subtracting dough height before baking from the height of the bread after baking, the oven spring (OvnS) (absolute and relative) could be calculated. No reproducible data could be obtained for the crumb coarseness (gas cell distribution analysis) and crumb texture of breads made using WxD, D11, WxM and NXY due to a premature collapse of the loaves.

8

8.3 Results and discussion

8.3.1 Starch and protein content

Large variations in the total starch (TSt) content of the flours could be observed with KAN containing only 71.67 $\pm$ 1.13 % starch whereas WxD, D11, WxM and NXY all had a similar content of approximately 81.13 %. Initially, a low TSt content of 51.54 $\pm$ 1.10 % was obtained for ADE. This remarkably low value may be attributed to the high resistance of the starch granules to temperature degradation resulting in a low amount of starch molecules released during the cooking step in the method. However, an actual lower concentration is also expected due to the very high protein concentration (PROC) (18.9 $\pm$ 0.0 %). Also after excluding the value for ADE, a significant negative correlation between PROC and TSt (r = -0.887, $p \leq 0.05$) existed. Also KAN and KBA have relatively low TSt contents of 71.67 $\pm$ 1.13 % and 73.37 $\pm$ 1.84 %. Lower starch contents will alter the dough rheology as less starch granules will be incorporated in the gluten network. Moreover, stability of the dough during baking–mainly upon transforming from a foam to sponge structure—will decrease as less material is available to provide structure.

Also for the PROC, a broad variation was observed with values ranging from 9.7 to 18.9 %. On average, waxy genotypes showed to contain less protein with exception of KBA which had a PROC of 14.4 %. Although the generally lower protein content (and quality) of waxy genotypes was postulated in early literature, Graybosch et al. (2003) stated that this varies in a manner similar to that observed for non-waxy wheats.

8.3.1.1 Starch composition

The sample set was mainly compiled on the basis of the differences in the amylose (AM) contents as waxy (virtually free from AM), partial waxy (AM contents of 3–22 %), regular (25–28 %) and high-amylose ($\geq$ 30 %) genotypes were included (Van Hung et al., 2006). This is also reflected by the wide diversity in AM contents ranging from 0.22 % (NXY) to 48.90 % (ARI) amylose (Table 8.1). For the partial waxy genotype 'Kanto 107', a *wx-A1*/*wx-B1* double null line (Nakamura et al., 1995), an AM concentration of 7.09 % was obtained. In previous research, AM concentrations for this genotype ranged from 18.3 to 24.4 % (Sasaki et al., 2004; Yamamori and Quynh, 2000; Kristiawan et al., 2018; Kim and Qin, 2014), depending on the analytical technique and starch extraction method used. The significant difference with the value obtained in this research may be attributed to the problems encountered during the determination through high-performance size-exclusion chromatography (HP-SEC) or may come from a lower number of active wx-D-alleles. According to Yamamori and Quynh (2000), a single dosage of wx-D in combination with a double null for wx-A and wx-B-alleles would result in amylose concentrations of approximately 8 %.

By altering the biosynthesis of starch components AM and amylopectin (AMP) through modification of the isoforms of the starch branching enzyme (SBE) and the glucan subtrates (Tetlow and Emes, 2014), differences in the chain-length distribution (CLD) of AMP are expected. However, as the chain lengths (*i.e.* degree of polymerization (DP)) are expressed relatively with a classification in short (DP = 6–12, A-type), medium (13–24, B_1), medium-long (25–36, B_2) and long (37–65, B_3^+) chains, differences between samples cannot be related unambiguously to a single class. As shown in Table 8.1, a generally shorter average chain length (aCL) was found for both the regular (EPB) and high amylose wheat flour (HiAm-WF) ADE (aCL = 20.43 and 20.36 respectively) whereas ARI had the highest aCL (22.19). Only marginal differences were observed between waxy genotypes WxD, D11, WxM and NXY with aCLs ranging from 20.50 to 20.79 whereas on average, AMP-chains of waxy genotype KBA contained one extra glucose molecule ($\overline{DP} = 21.70$). This can be attributed to the generally low concentration of short, medium and medium-long chains (23.87, 46.78 and 17.22 % respectively) compared to the other waxy genotypes (25.26, 47.50 and 17.67 %). Self-evidently, this also resulted in a markedly higher concentration of long side chains for KBA (12.13 % versus 9.57 %). An apparent inverse relation existed between the AM content and the concentration of medium-long chains (B_2) with highest concentrations in waxy genotypes (17.22–18.37 %) and lowest in high-amylose (HiAm) genotypes (14.50–15.74 %). Highest variance was however observed for the long chains with concentrations ranging from 9.22 to 14.93 %.

8.3.1.2 Alpha-amylase activity

A commonly used technique for measuring the activity of glycosidases (mainly α- and β-amylase) in wheat flour is the Hagberg falling number (HFN). However, as this is a viscosity based method, a relation with the starch properties is inevitable. As discussed in Section 1.3.1.1 (page 23), pasting behavior and thus, viscosity, is strongly affected by the AM concentration and can also vary between different (waxy) genotypes. For all waxy genotypes, HFNs of 62 s, the lowest possible value, were recorded due to the low viscosity pastes obtained from waxy wheat flour (WWF). This is in accordance with findings from Graybosch et al. (2000) who stated that falling numbers are independent of α-amylase levels due to the lower peak times (peak viscosity is attained earlier) and increased susceptibility to breakdown.

By means of the Ceralpha Method (AACC International., 2000a) (Megazyme, Wicklow, Ireland), α-amylase activities of the flour samples were measured. Lowest activities were found for KAN (0.042 ± 0.004 CU g^{-1}) and WxD (0.068 ± 0.007 CU g^{-1}) whereas all other genotype had a higher value between 0.100–0.196 CU g^{-1} for EPB and ARI, respectively (Table 8.1). Three out of five waxy genotypes and both high-amylose genotypes had a significantly higher α-amylase activity (0.142 CU g^{-1} and 0.179 CU g^{-1}) compared to the reference wheat flour which is in accordance with findings from Garimella Purna et al. (2015). It is assumed that genetic differences and varying milling conditions have contributed to these results as no heavy rainfall or heat shocks—which are correlated with increased pre-harvest sprouting and late maturity alpha-amylases—were recorded during cultivation (Newberry et al., 2018). Moreover, the intensive cleaning of the bran fraction of ADE may have contributed to the higher enzyme activity as more of the enzyme-containing aleurone layer will be included in the flour (De Brier et al., 2015). It has to be noted that growth and milling conditions of both ARI and EPB are unknown as both samples were obtained from commercial mills.

8.3.2 Functional properties

8.3.2.1 Particle size distribution

Kernel morphology, hardness and composition affects the flour particle size distribution which, on its turn, influences water absorption and surface interactions (Campbell et al., 2007, 2012). Graybosch et al. (2016) also stated that differences in the fracture pattern and consequently, the flour particle size distribution, may arise from the more crystalline nature starch granules. When the flour is mixed with water and shear (and heating) is applied, the flour particles will be disrupted, depending on their initial properties and the prevailing mechanical conditions. In this way, flour particle size distribution may affect functional properties at various levels.

Figure 8.1 shows the size distribution of the flour particles as determined by laser diffraction using pressurized air. All samples had a trimodal distribution in which two main groups could be distinguished: small (2–40 µm) and large (40–400 µm) particles (Table 8.2). A similar distinction was made by Hareland (1994) and Kim and Qin (2014) who associated the volume fraction of small particles mainly with free starch granules whereas the large particles indicated gluten-starch adhesion.

With exception of ADE, a third group of extra small particles ($\leq$ 2 µm) accounting for 1 to 2 % of the total volume could be noted. The HiAm genotype, on the contrary, contained no small particles but a fraction larger than 400 µm. This is considered to be a result of the used mill (Quadromat Junior) and the intense cleaning of the bran fraction. Additionally, the high protein content of the sample may have promoted the agglomeration of starch granules. Cryo-scanning electron microscopy recordings (Figures 8.2 and 8.3) also show the higher degree of agglomeration in the ADE starch extracts. A generally lower concentration small granules was observed for both HiAm genotypes (18 and 19 volume% for ADE and ARI respectively). This can be partially attributed to the elongated or filamentous shape of the starch granules in ARI as was also observed by McCann et al. (2018) and Li et al. (2019a). According to these researchers, this is proposed to be an effect of increased interaction on the outer layer of starch granules and consequent agglomeration. Moreover, waxy genotypes WxD and D11—of which the latter is the progeny of the former—contain a similar fraction small (44 and 49 volume% respectively) and large

Table 8.1: Compositional attributes of the tested genotypes with altered starch composition.

No.	Code	Protein	Starch composition				Amylopectin fine structure					
		PROC (%)	*α-amylase activity* (CU g^{-1})	*TSt* (%)	*AM*[1] (%)	*AM:AMPr*	A-type *DP6–12* (%)	B_1 *DP13–24* (%)	B_2 *DP25–36* (%)	B_3^+ *DP36–65aCL* (%)		*SLr*
1	WxD	9.7	0.068 ± 0.007	80.49 ± 4.23	0.54	0.01	24.7	47.3	18.4	9.7	20.79	7.42
2	D11	10.0	0.104 ± 0.024	80.75 ± 0.80	0.57	0.01	25.0	47.7	18.0	9.2	20.54	7.89
3	WxM	10.2	0.130 ± 0.016	81.64 ± 2.57	0.58	0.01	25.6	47.4	17.2	9.8	20.60	7.47
4	NXY	12.0	0.160 ± 0.012	81.64 ± 1.83	0.22	0.00	25.8	47.5	17.1	9.6	20.50	7.65
5	KBA	14.4	0.137 ± 0.031	73.37 ± 1.84	1.31	0.01	23.9	46.8	17.2	12.1	21.70	5.82
6	KAN	13.2	0.042 ± 0.004	71.67 ± 1.13	7.09	0.08	25.5	46.8	16.4	11.2	21.17	6.44
R	EPB	13.1	0.100 ± 0.003	76.58 ± 2.07	25.24	0.34	26.8	47.9	15.5	9.8	20.43	7.61
8	ADE	18.9	0.162 ± 0.002	-[2]	31.68	0.46	29.1	45.1	15.7	10.1	20.36	7.38
9	ARI	13.7	0.196 ± 0.018	75.74 ± 1.90	48.90	0.96	25.3	45.3	14.5	14.9	22.19	4.73

[1] Calculated according method Ea as discussed in Section 3.2.4.3 (page 104).
[2] Removed from further data analysis as the obtained value was diverging (51.54 ± 1.10 %) due to method incompatibility.
Ceralpha units (CU); AM:AMP-ratio (AM:AMPr); amylopectin chain-length distribution classes (A/B_{1-3^+}); short:long-ratio (SLr).

Table 8.2: Flour particle size distribution and pasting behavior (measured at high pressure in excess of water) under enzyme inhibited (EI) and regular (NEI) conditions.

No.	Code	Particle size		Pasting behavior (NEI)							Pasting behavior (EI)						
		Small (2–40 µm) (%)	*Large* (40–400 µm) (%)	*Tg* (°C)	*PV* (mPa s)	*PTemp* (°C)	*HS* (mPa s)	*FV* (mPa s)	*BD* (mPa s)	*SBp* (mPa s)	*Tg* (°C)	*PV* (mPa s)	*PTemp* (°C)	*HS* (mPa s)	*FV* (mPa s)	*BD* (mPa s)	*SBp* (mPa s)
1	WxD	44	54	66.5	1874	73.3	196.8	481	1677	-1393	66.5	2787	73.3	140.3	347	2646	-2440
2	D11	49	49	67.4	1727	74.8	179.4	347	1548	-1380	65.7	2905	71.8	234.7	588	2670	-2317
3	WxM	27	72	66.5	1258	73.3	91.8	171	1166	-1086	66.5	2606	74.0	234.5	630	2371	-1976
4	NXY	24	74	68.1	2484	74.0	263.6	655	2220	-1829	66.5	3038	73.3	181.8	462	2856	-2576
5	KBA	40	58	71.0	1935	77.8	211.4	565	1723	-1369	71.0	2358	77.0	151.8	370	2206	-1988
6	KAN	37	61	68.9	1759	99.7	168.5	504	1591	-1255	69.6	2019	101.3	149.0	402	1870	-1617
R	EPB	27	71	65.7	1859	99.7	173.8	730	1685	-1128	64.9	2225	99.7	180.1	751	2044	-1474
8	ADE	18	80	61.8	88	136.2	72.6	255	16	166	50.4	198	131.7	108.1	471	90	273
9	ARI	19	79	68.3	321	128.7	99.2	626	221	305	50.4	447	126.3	83.0	611	364	164

particles. The same was observed for KAN and KBA (37 and 40 volume% respectively), implying the presence of a genetic effect. Although the reference flour (EPB) is milled in an industrial mill, a similar size distribution as for NXY and WxM was found (small: 24–27 volume%).

> Variation in the particle size distribution of flour is attributable to the packing density and size of the starch granules (surface area and non-covalent bond formation) and the composition of the protein-starch matrix. Softer kernels tend to result in lower *flour* particle sizes and higher mean *granule* sizes as a negative correlation between both distributions exists. (Gaines et al., 2000; Kim and Qin, 2014)

In addition to the height differences, a similar shift in the mean diameter of the class of small particles can be noticed. This shift was however not seen for the large granules. As a result, median particle size (D_{50}) varied in a similar fashion with values ranging from 37 µm for D11 to 99 µm for ADE. The volume percentage of particles smaller than 150 µm—the smallest sieve size used in conventional milling—was highest for WxD and D11 ($\geq$ 90 %) whereas 85 % of the particles in EPB and 88 % for ARI was smaller than this threshold. WxM, KBA and KAN were slightly coarser. NXY and ADE contained respectively 23 and 32 % particles larger than 150 µm.

> Genetic differences majorly contribute to the variation observed for the flour particle size distribution. Not solely the starch composition forms a driving factor but interaction with granular morphology and starch-protein interactions are postulated.

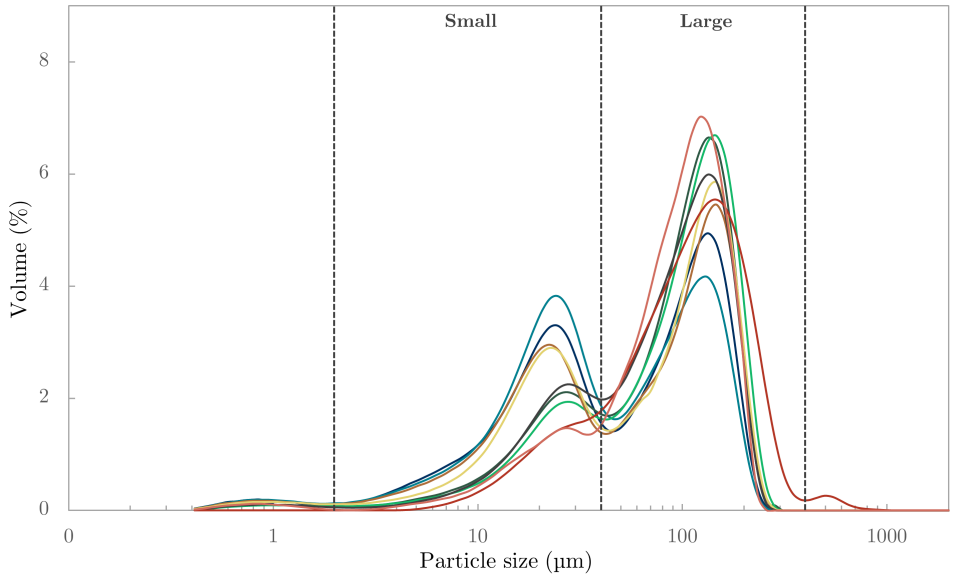

Figure 8.1: Particle size distribution of the flour obtained through milling grain samples on a Bühler ML-202 laboratory mill, a Quadromat Junior mill (ADE ●) or industrial mill (EPB ● and ARI ●). Areas between dashed lines indicate size ranges for small (2–40 µm) and large (40-400 µm) particles.
Coding for the genotypes can be found on page xii.

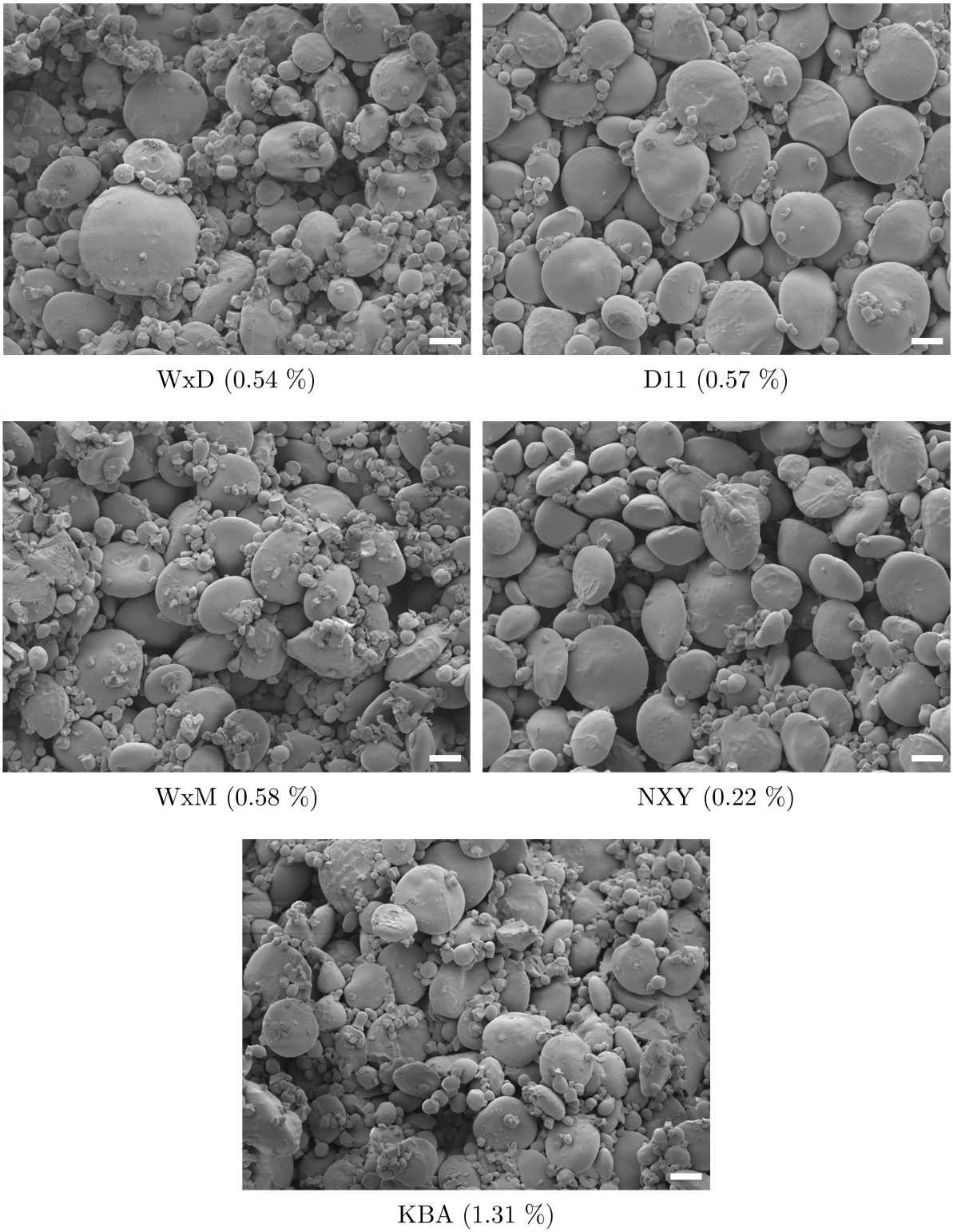

Figure 8.2: Cryo-scanning electron microscopy recordings of starch extracts (dough-ball method) at 800× magnification (scale bar 10 µm). Values between brackets are the according amylose contents.

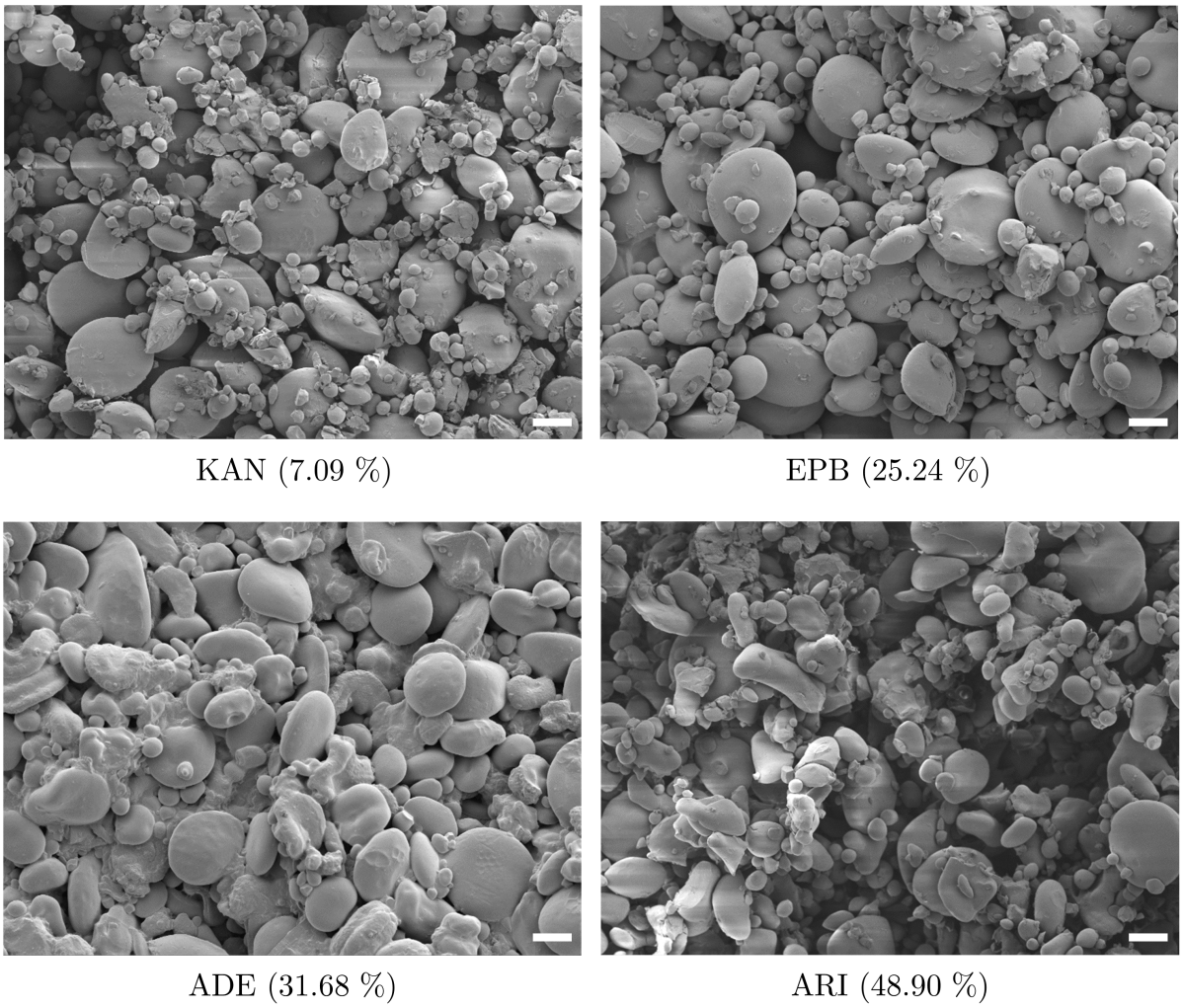

Figure 8.3: *continued* Cryo-scanning electron microscopy recordings of starch extracts (dough-ball method) at 800× magnification (scale bar 10 µm). Values between brackets are the according amylose contents.

8.3.2.2 Pasting behavior

Thermal processing in excess of water and under shear conditions results in an irreversible swelling and, eventually, disruption of the starch granules resulting in a full hydration of the starch components and, consequently, a viscosity increase. The magnitude of the loss of crystalline structure is dependent on the ratio of both starch molecules (AM:AMP-ratio), the aCL and the AMP CLD, granule morphology, granule surface properties and the flour particle size distribution (see Section 1.3.1.1 for more details). Despite the added value of this analysis (when applied on conventional wheat flours) for predicting bread quality (Barak et al., 2013), literature on the pasting behavior of HiAm-WF is scarce (Rakszegi et al., 2015). Moreover, knowledge of the origin of variation in the pasting behavior of WWF is limited extent. Graybosch et al. (2003) however found that genetic ($\mathbb{G}$) effects contributed more than environmental ($\mathbb{E}$) attributes.

Figure 8.4 shows the pasting behavior of the wheat flours using distilled water (non-enzyme inhibited ($_{NEI}$) conditions) and in an $AgNO_3$-solution ($_{EI}$ conditions). All measurements were performed under pressure (0.6 MPa). Although high-pressure conditions may further interfere with the relation between

pasting behavior and bread quality, this allows to observe pasting for the HiAm-WF. It has to be noted that the excess of water and the application of shear during pasting measurements also do not correspond with the conditions during breadmaking. Still, pasting properties has been frequently correlated with end-product quality attributes of bakery products, including bread (Blazek and Copeland, 2008).

Major differences are noticed between waxy, partial waxy, regular and high-amylose wheat flours in terms of pasting temperature (Tg), peak temperature (PTemp) and peak viscosity (PV). At first instance, an immediate and steep increase in the viscosity is observed for the WWF without showing a prior small viscosity increase as is seen for KAN and EPB as well as for the HiAm-WF, although to a lesser extent. This may be attributed to the presence of amylose-lipid complexes (in the starch granule or formed intercellular at temperatures below 60 °C) or could result from surface interactions of swollen starch granules (Gerits et al., 2015). Alternatively, it can be postulated that in WWF, the primary viscosity increase occurs together with the immediate swelling of starch granules due to their reduced granular rigidity and, thus, Tg. Malumba et al. (2013) clearly showed that granule swelling in wheat starch starts at temperatures above 50 °C, reaching an equilibrium in their mean diameter at 60 to 75 °C, depending on the applied heating rate. The mean Tg for WWF in this research (± 66.3 °C) is in line with this. PV for the waxy genotypes under $_{NEI}$-conditions is varying between 1258 and 2484 mPa s which is therefore not significantly different from the PV of KAN (1759 mPa s) and EPB (1859 mPa s).

Within the WWFs, insignificant shifts in the PTemp were found with exception of KBA for which a delayed pasting was observed. This difference might be related to the presence of a minor fraction of AM (1.31 %) thereby contributing to a higher granular rigidity and thus, more resistance against thermal disruption. In contrast, KAN and EPB have an equal Tg and PTemp despite their different AM concentration (7.09 % *versus* 25.24 %). It can be postulated that a further increase of the AM concentration in the granules has no negative impact on the granular rigidity (indicated by differences in the Tg). However, different breakdown (BD) behavior can be observed with KAN showing a steep decrease after reaching its PV whereas a slower BD of the granules is seen for EPB. This rapid and more extensive BD for WWF has already been reported by various researchers (Garimella Purna et al., 2015; Li et al., 2016a) and is related to the lack of AM to retain the granular substructures (Sasaki et al., 2000).

> Amylose concentration is strongly correlated with the pasting temperature (+) as well as with peak viscosity (-) and temperature (*i.e.* time) (+). A plateau appears to be present after reaching a threshold in the amylose content.

HiAm-WFs have a very low PV and holding strength (HS) which can be partially attributed to the lower AMP content in the starch. However, ADE, which has an elevated AM concentration of 31.68 %, had an almost three times lower PV compared to ARI, despite the higher AM content in the latter (48.90 % AM). Furthermore, the apparent coarser particle size distribution of the flour may have also contributed to the low overall viscosity, as well as the high protein content of ADE as this will further impede granular swelling. From this point of view, it can be hypothesized that under the prevailing measuring conditions, pasting did not occur to a full extent. Contrastingly, ADE had a positive setback from peak (SBp) (198 mPa s) implying that enough starch molecules (mainly amylose) were able to leach out of the granules enabling the formation of a starch network upon cooling.

In order to observe pasting in high-amylose wheat flour samples, measurements had to be conducted using high temperatures (140 °C) obtained by increasing hydrodynamic pressure. This may have impacted granular degradation as well as the network formation between released starch molecules. Relating parameters to end-product quality attributes may be further aggravated.

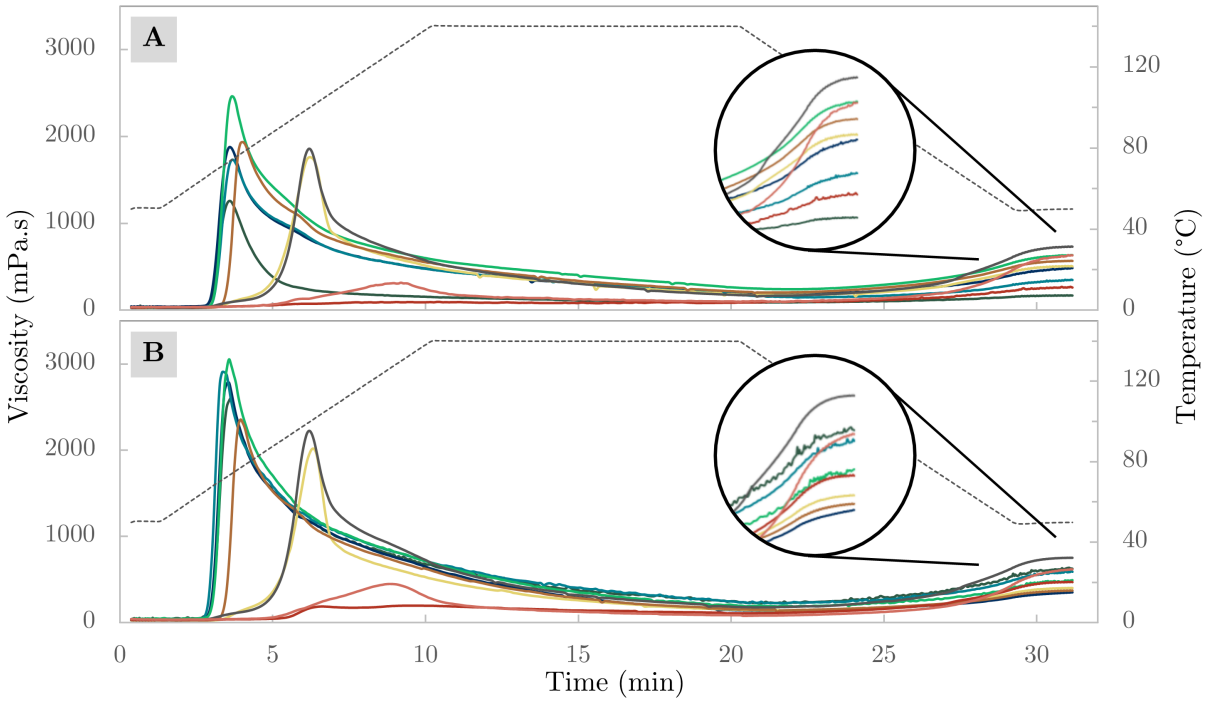

Figure 8.4: Pasting profiles of waxy, partial waxy, regular and high-amylose wheat flours (14.0 % db) in **A** distilled water and **B** 0.1 M $AgNO_3$-solution (300 µl).
Coding for the genotypes can be found on page xii.

Alpha-amylase inhibition Similar pasting profiles were observed for all genotypes compared to the measurements without enzyme inhibition. However, the variation in the PV is reduced with a narrower range (RSD of 9.7 compared to 23.7 %). Garimella Purna et al. (2015) also observed a wide diversity in the PV. According to their findings, this was not affected by the α-amylase activity whereas a protease treatment prior to the measurement resulted in a similar PV. An overall 50 % increase in the PV was noted with NXY, KBA, KAN and EPB showing the smallest increases (15–22 %) whereas the PV for WxM and ADE rose by 107 % and 124 %. This also resulted in a stronger correlation between the AM content and PV (r = -0.885) (and the related parameters BD and SBp) whereas Tg and PTemp also showed to be significantly correlated (r = -0.872 and 0.913 respectively) with AM under $_{EI}$-conditions.

After 20 min, at the end of the holding period at 140°C, viscosities evolved towards an equally low value for all samples (under both conditions, $_{NEI}$ and $_{EI}$), except for the HiAm-WFs which were consistently lower. Contrastingly, differences in the network formation upon cooling were noted as the order for the final viscosity (FV) varies for both conditions. Despite no correlation with any of the starch compositional attributes or the enzyme-activity could be noted, setback from trough (SBt) showed to be influenced by the AM concentration (r = 0.725, p = 0.027) under $_{EI}$-conditions. EPB and ARI show under both conditions

a significant viscosity increase between 80 and 50 °C resulting in a markedly higher SBt ($_{EI}$-conditions: 571 and 528 mPa s *versus* 207–395 mPa s).

> Enzyme inhibition results in an overall higher and more stable peak viscosity for all genotypes. High-amylose flours remain however far below viscosity levels of regular or waxy wheat flours showing limited or spread out granule swelling. Breakdown of starch granules occurs more rapid and to a fuller extent for (partial) waxy genotypes compared to regular wheat flour.

Correlation analysis also showed the relation between AMP CLD, mainly the presence of medium (DP 12–24) and medium-long (DP 25–36) chains, and peak properties (both PTemp and PV). An increased concentration medium-long chains resulted in higher PVs ($r = 0.797$, $p = 0.010$) and decreased PTemps ($r = -0.870$, $p = 0.002$). Although these relations were also seen for branch chains with a medium DP, correlations were less robust due to the limited variation for this parameter between samples. Moreover, the relation between Tg and AMP CLD was impacted by the enzyme activity. When using $AgNO_3$, no correlation was found ($p = 0.073$) whereas without enzyme-inhibition ($_{NEI}$-condition), a consistent decrease in Tg was visible upon higher concentrations AMP chains with DPs between 6–12. Similar findings are reported by Nivelle et al. (2019) stating that both gelatinization temperature and enthalpy are negatively related to the proportion of short AMP branch-chain as the double helices formed from these chains are unstable and, therefore, require less energy for gelatinization (Singh et al., 2009b). The role of enzymes therein remains however uncertain.

> The relative concentration of medium-long chains of amylopectin is strongly correlated with pasting properties such as peak temperature and peak viscosity and may influence granule swelling.

8.3.2.3 Dough rheology

Various researchers experienced difficulties when applying conventional techniques for determining protein quality on WWF and HiAm-WF such as the Glutomatic. According to Van Hung et al. (2006), this might be an indirect effect of limited gluten-network formation in these flours as they observed that the gluten-network in waxy wheat dough was not evenly dispersed and did not cover the starch granules entirely whereas in HiAm-WF, irregular and discontinuous gluten sheets were formed. In contrast, Guan et al. (2009) and Graybosch et al. (2016) reported that all waxy genotypes produced gluten via Glutomatic washing with gluten indexes equal to the highest ranked non-waxy line. After extensive testing, the latter authors have clearly proven that weak gluten is not a natural consequence of the waxy trait, and that waxy genotypes with acceptable gluten properties can be developed. However, upon testing the WWF in the presented sample set, non of the samples developed gluten properly. To get an indication of protein functionality, three dough rheological properties were measured using the Farinograph, dynamic oscillatory measurements (frequency sweep) and static creep-recovery tests.

> Waxy wheat genotypes may have inferior protein quality compared to regular wheat genotypes but a comparable variation can be expected. Increased granule swelling and poor gluten network formation however, may also lead to inability of conventional techniques to properly measure protein quality.

Farinograph The execution of the Farinograph mixing test showed the impact of the absence of amylose in WWFs on the water absorption. A strong correlation between the water absorption (WA) and AM content has been reported by multiple researchers (Morita et al., 2002; Niu et al., 2017; Ramachandran et al., 2016). Values for water absorption at 500 BU (WA_{500}) ranged from 65.7 to 69.7 % for WWFs with exception of D11 for which the WA_{500} was relatively low (62.5 %). The partial waxy and regular wheat flour (KAN and EPB) had even lower WA_{500}s of 56.7 and 57.3 % respectively. The relation was not linear as the lowest values for the WA_{500} were obtained for EPB and KAN whereas ARI had the highest WA_{500} (75.3 %). This finding implies that multiple components (damaged starch, protein content, fibers, *etc.*) contribute significantly to the WA. The high WA for waxy genotypes could however be attributed to the relative increase in AMP whereas the rougher surface characteristics of HiAm starch granules may promote water uptake. The latter could however not be confirmed on the basis of the SEM-recordings of the starch granules (Figures 8.2 and 8.3). Moreover, no WA_{500} could be determined for ADE as measurements were not reproducible, presumably resulting from the high dough stickiness during the measurement.

The increased WA is frequently accompanied by a decrease in the Farinograph stability (STAB) for WWF. In comparison with the regular wheat flour (18.7 min), flour stabilities were markedly lower with values ranging from 2.2 to 7.2 min. For KBA and ARI, sticky doughs were obtained around minute 10 and 15 in the analysis indicated by the large en sudden drops in the consistency (Figure 8.5). Another markable difference is the slower and more gradual dough development for WxM and NXY. Dough development time (DDt) ranges from 2.5 to 3.2 min for ARI and WxD respectively (2.9 min for EPB), which is also in accordance with results obtained by Blake et al. (2015), whereas WWF from WxM and NXY had a DDt of 6.0 and 6.2 min respectively. This phenomena may be caused by a decreased accessibility of the starch granules for water due to variations in the particle size distribution of the flour or by encapsulation in the gluten-network. As such, the WA of the intact granules may be lowered. However, no strong correlations with any of the starch or protein parameters could be established. The higher DDt for KBA (3.5 min) resulted from a poor overall WA as can be seen from the delayed consistency increase.

Dynamic oscillatory rheology By means of a frequency sweep, dough rheological properties can be studied while maintaining the micro-structure thereby providing information on the contribution of molecular and macromolecular attributes to its viscoelastic behavior. From the storage modulus (G') and the loss modulus (G"), the complex shear modulus ($|G^*|$) and phase shift angle ($\tan(\delta)$) can be determined which, in turn, are measures of the rigidity of a material and the viscoelasticity (Table 8.3). As illustrated in Figure 8.6, plotting both variables orthogonally revealed the presence of three clusters, however without a clear attribute relating to this distinction. HiAm-WFs had a lower $|G^*|$ in the same magnitude as WxD, NXY, WxM and D11. Within this cluster, samples with a slightly lower (0.291–0.313 rad) and higher (0.331–0.344 rad) $\tan(\delta)$ can be observed whereas KBA, KAN and EPB—which also showed more

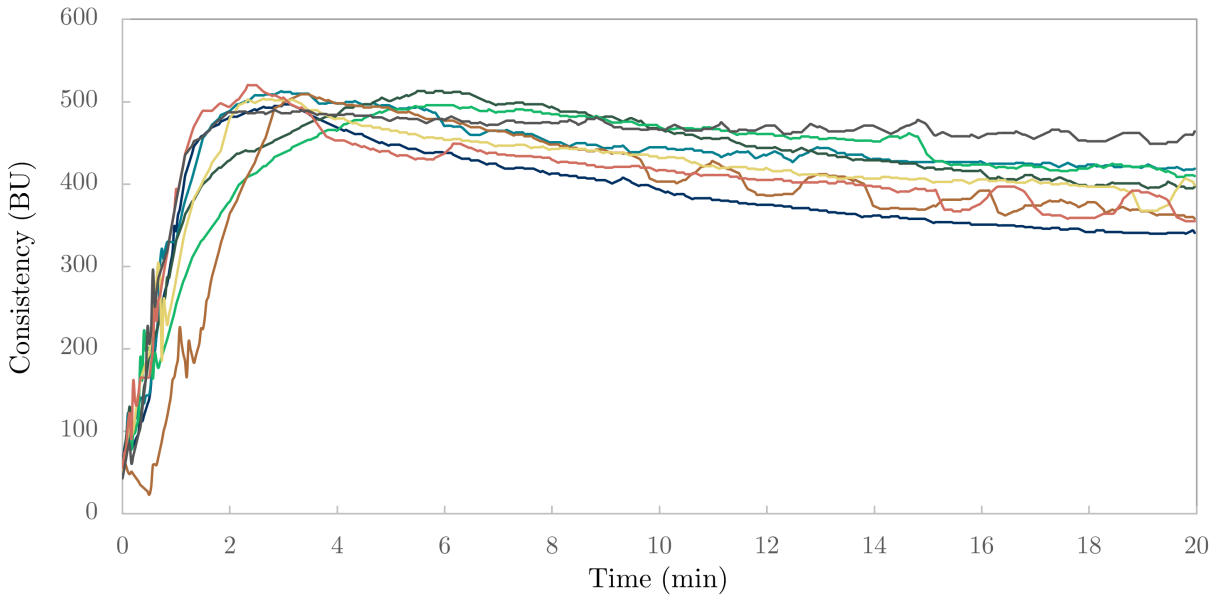

Figure 8.5: Farinograph mixing curves (according to ICC 115/1) for flours from different (partial) waxy, regular and (ultra) high-amylose genotypes.
Coding for the genotypes can be found on page xii.

resistance against deformation—had intermediate values for the tan(δ) (0.317–0.325 rad). Due to the small differences between samples and the high variance, which may be attributed to inconsistencies in the mixing time and intensity, a plain distinction between stiff and elastic doughs (clusters I and II) and rigid but more extensible doughs (cluster III) can be made.

Correlations between dough rheological parameters and end-product quality attributes of the breads may be lacking as a result of the sensitive sample preparation and the dissimilarity with the breadmaking process. As all flour samples, except KAN and EPB, had higher WAs compared to the added amount (58 % of the flour weight), gluten-forming proteins may not have been hydrated optimally. Findings from Jekle and Becker (2011) however showed that the proteins in dough are already hydrated at water additions of 10 % less than the optimal WA.

Thermal processing will furthermore drastically change the structure of all components in the matrix thereby concealing possible relations. The moisture limited conditions in dough will result in a restricted swelling of the granules during baking and less AM leaching out. According to Blake et al. (2015), this would result in a weaker and less elastic gluten–amylose network during breadmaking.

Static rheology Complementary to the small strain oscillatory measurements, dough rheology outside the linear viscoelastic range is examined by means of creep-recovery measurements. Besides maximum deformation (DEF_{max}) (maximum strain in the creep-phase) and the elastic recovery ($\%REC_{el}$) (relative return to initial form), parameters from the fitted Burgers model (for the creep (C) and recovery (R) phase) were studied separately to discriminate the dough properties of all samples on the basis of the entire curve.

The score plot (**A**) reveals an entirely different behavior in comparison to the frequency sweep results with no ability to clearly distinct clusters. The complete separation of EPB from all other genotypes results from the low instantaneous response during the recovery-phase indicating its large (delayed) deformability.

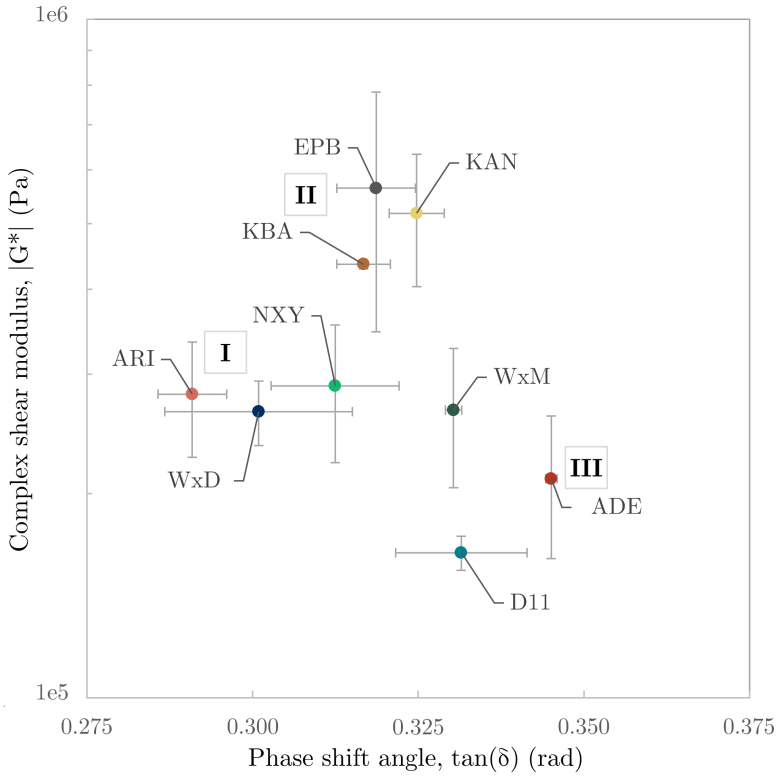

Figure 8.6: Orthogonal representation of the phase shift angle ($\tan(\delta)$) and complex shear modulus ($|G^*|$) obtained from frequency sweep measurements from 0.1 to 30 Hz at a constant pressure of 250 Pa.
Coding for the genotypes can be found on page xii.

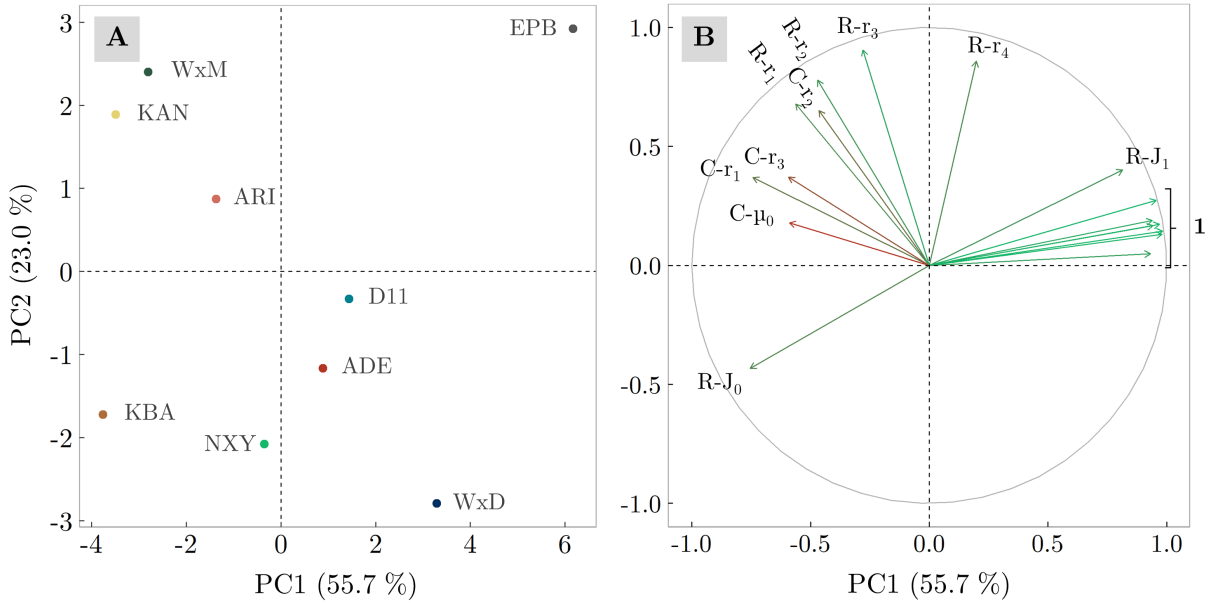

Figure 8.7: Score (**A**) and loading (**B**) plots for the parameters of the 8- and 9-component Burgers models for the creep (C) and recovery (R) phases respectively, fitted to the creep-recovery curves for dough prepared with a fixed water addition (58.0 %) at a stress of 250 Pa.
Group 1 in the loading plot contains all remaining variables.

Contrastingly, KBA has a significantly higher instantaneous response upon removal of the shear. All other genotypes are mainly separated by the second PC which is highly correlated with the retardation times from both creep and recovery phases. However, no interpretation on a functional level can be connected

to these parameters and no statistically significant correlations could be established. In theory, retardation coefficients can be good indicators of different gluten behavior when the number of Kelvin-Voigt elements are chosen correctly. The higher steady state viscosity (μ_0) for cultivars in the first quadrant (WxM, KAN and ARI) may imply an overall low deformability as the directional coefficient from the long term viscous flow decreases upon increasing μ_0-values. The opposite counts for the genotypes in the 3rd quadrant (D11, ADE and WxD).

> Markable difference between dough rheological attributes were observed for the various genotypes. However, non of these could be related to compositional attributes or other functional properties. Furthermore, sticky doughs resulting in poor reproducibility of measurements and overall large variations were noted for some samples.

Although no significant correlations with the instantaneous or delayed responses were found, the %REC$_{el}$ for EPB is also markedly lower (27.5 %) compared to the %REC$_{el}$ of other genotypes which ranged between 39.0–66.3 % (ADE and NXY respectively). Moreover, a high DEF$_{max}$ for EPB (2.36 %) implied that the dough has mainly extensible characteristics without showing high elastic behavior. In contrast, WxD and D11 have a comparable but lower DEF$_{max}$ of 1.90 and 2.01 % respectively whereas their %REC$_{el}$ is consistently higher (51.1 and 54.9 %). Compared to all other genotypes (for which DEF$_{max}$ ranged from 1.04 to 2.01 %), values for KBA and KAN were 0.52 and 0.61 % respectively. Upon removing EPB from the dataset, a weak correlation between dough rheological parameter %REC$_{el}$ and compositional attribute TSt became visible (r = -0.748, p = 0.033). Also negative correlations between DEF$_{max}$ and the storage and loss modulus (r = -0.839 and -0.841 respectively, p $\leq$ 0.05) were noted.

Proposition

The large diversity in protein and starch content and composition demands for techniques which can be uniformly applied on all samples thereby providing information on the dough properties. As pasting behavior and water absorption varies to a very high extent, the combination with heat treatment during measurements is most desirable. The sole trivial correlation between total starch and protein contents implies the inability of current techniques to fully capture interaction events.

8.3.3 Breadmaking tests

As reported in literature, waxy wheat genotypes gave rise to an extremely high loaf volume (per kilogram flour) (VOLfc) between 7000 ml kg^{-1} flour and 8500 ml kg^{-1} flour whereas bread produced using a strong, conventional wheat flour (EPB) had an almost 40 % lower VOLfc (Table 8.4). A slightly higher VOLfc was obtained for the partial waxy genotype KAN while ARI had significantly lower VOLfc. In contrast, very low and dense breads were obtained from the ADE flour. Besides the negative correlation with the AM content (r = -0.845, p = 0.004), the relative amount of short (+), medium (-) and medium-long (+) branches of AMP contributed significantly to VOLfc ($|r| \geq 0.710$, p $\leq$ 0.022).

Table 8.3: Dough viscoelastic behavior measured through static (creep-recovery) and dynamic oscillatory (frequency sweep) rheology and Farinograph measurements.

		Dough consistency				Dough viscoelastic behavior			
No.	**Code**	WA_{500} (%)	*DDt* (min)	*STAB* (min)	*SOFT* (BU[1])	$\|G^*\|$ (kPa)	$tan(\delta)$ (rad)	DEF_{max} (%)	$\%REC_{el}$ (%)
1	WxD	65.9	3.2	2.4	143	264 ± 29	0.301 ± 0.014	2.01 ± 1.53	51.1 ± 15.4
2	D11	62.5	3.0	4.3	86	164 ± 10	0.331 ± 0.010	1.90 ± 0.04	54.9 ± 5.5
3	WxM	65.7	6.0	5.6	116	266 ± 62	0.330 ± 0.001	1.04 ± 0.25	52.3 ± 14.2
4	NXY	69.7	6.2	7.2	73	288 ± 66	0.312 ± 0.010	1.56 ± 0.77	66.3 ± 10.9
5	KBA	68.8	3.5	2.8	128	436 ± 7	0.317 ± 0.004	0.52 ± 0.30	42.9 ± 32.6
6	KAN	56.7	2.7	2.6	98	518 ± 115	0.325 ± 0.004	0.61 ± 0.44	48.3 ± 10.9
R	EPB	57.3	2.9	18.7	13	564 ± 218	0.319 ± 0.006	2.36 ± 2.22	27.5 ± 22.2
8	ADE	-	-	-	-	210 ± 50	0.345 ± 0.001	1.37 ± 0.38	39.0 ± 3.6
9	ARI	75.3	2.5	2.2	129	280 ± 54	0.291 ± 0.005	1.31 ± 0.22	49.2 ± 8.4

Water absorption (500 BU[1]) (WA_{500}); dough development time (DDt); stability (STAB); softening (SOFT); complex shear modulus ($|G^*|$); phase shift angle ($\tan(\delta)$); maximum deformation (DEF_{max}); elastic recovery ($\%REC_{el}$).

[1] BU, Brabender Units, arbitrary unit for dough consistency.

Table 8.4: End-product quality attributes of the breads baked using the wheat genotypes with an altered starch composition.

		Dimensions			Crust color			Crumb color			Texture	
No.	**Code**	*VOLfc* (ml kg^{-1} flour)	*OvnS* (%)	*HtWr*	*L*	a^*	b^*	*L*	a^*	b^*	*HARD* (N)	*Staling*[1] (%)
1	WxD	8113 ± 449	37.8 ± 7.9	0.651 ± 0.031	44.0 ± 2.6	18.8 ± 0.4	36.7 ± 2.0	56.3 ± 7.7	-1.1 ± 0.1	8.4 ± 0.4	-	-
2	D11	7620 ± 143	29.1 ± 1.8	0.676 ± 0.032	48.1 ± 0.9	20.0 ± 1.1	40.7 ± 1.2	67.4 ± 2.6	-0.4 ± 0.4	15.4 ± 2.2	-	-
3	WxM	8103 ± 298	27.3 ± 8.2	0.583 ± 0.023	34.4 ± 0.5	19.6 ± 1.3	29.4 ± 2.5	59.6 ± 2.3	-1.3 ± 0.2	7.5 ± 2.1	-	-
4	NXY	8456 ± 403	44.6 ± 9.2	0.634 ± 0.146	39.2 ± 5.1	19.5 ± 0.1	33.9 ± 4.2	60.5 ± 5.2	-17.5 ± 32.3	11.3 ± 3.0	-	-
5	KBA	7378 ± 262	9.5 ± 2.9	0.530 ± 0.057	40.6 ± 5.6	18.4 ± 0.4	33.0 ± 3.5	49.2 ± 15.4	-1.0 ± 0.2	7.9 ± 6.2	254 ± 165	553
6	KAN	5075 ± 52	-2.3 ± 1.4	0.567 ± 0.044	48.4 ± 2.6	19.7 ± 0.8	40.0 ± 1.8	70.5 ± 1.7	-7.0 ± 13.3	13.7 ± 1.0	574 ± 37	124
R	EPB	4844 ± 86	9.4 ± 2.9	0.680 ± 0.020	44.3 ± 0.5	27.3 ± 9.9	37.8 ± 0.8	70.9 ± 3.8	0.3 ± 0.1	15.2 ± 1.2	866 ± 116	77
8	ADE	2417 ± 10	-0.9 ± 1.9	0.533 ± 0.000	19.1 ± 3.4	18.7 ± 1.4	9.5 ± 5.1	62.2 ± 1.9	4.4 ± 0.2	23.1 ± 0.1	4227 ± 302	18
9	ARI	4117 ± 94	-5.4 ± 5.0	0.618 ± 0.048	46.7 ± 4.5	23.2 ± 1.2	41.0 ± 2.9	75.4 ± 14.3	0.1 ± 0.1	21.7 ± 0.7	1957 ± 211	16

[1] Expressed as the relative increase in crumb hardness (HARD) over a four day period.

Lightness (L); redness (a^*); yellowness (b^*).

The higher percentage damaged starch in WWF, the increased susceptibility of AMP to enzymatic degradation, the therefrom resulting increased CO_2-production and the inability of gas to escape during baking may explain the high volumes obtained for the waxy genotypes. However, an interesting difference in the oven spring between KBA and other waxy genotypes (WxD, D11, WxM and NXY) may imply the occurrence of different mechanisms. While the latter genotypes develop their high loaf volumes in the first phase of baking (indicated by high oven spring (OvnS) (27.3–44.6 %), KBA had a slightly lower VOLfc which was mainly developed during proofing. Comparable differences can be observed for ARI and KAN for which negative OvnSs were found. The strong correlation between VOLfc and OvnS (r = 0.860, p = 0.003) implies that also correlations with AM and AMP CLD can be established for VOLfc. Interestingly, weaker correlations were present between VOLfc and OvnS on one hand and PROC on the other hand (r = -0.817 and -0.706 respectively, p $\leq$ 0.0.34).

The height-to-width-ratio (HtWr) of the plate breads provides insight in the viscoelastic behavior of the dough during baking. As most WWF breads experience a high OvnS, an unstable structure (*i.e.* low HtWr) may hint for defects in the end-product. Additionally, a correlation between HtWr and OvnS may support the influence of dough rheology on the latter factor. At first sight, no correlation could however be established (p = 0.092) as WxM and KAN have similar HtWrs (0.583 and 0.567, respectively) but very different OvnSs (27.3 % and -2.3 %). Nevertheless, within the waxy genotypes, a more linear relation is observed with KBA (lowest loaf volume) having a low HtWr supporting the hypothesis that dough rheological attributes lay at the basis of the mechanisms for loaf volume development. A strong correlation between HtWr and DEF_{max} (r = 0.853, p = 0.003) indicates that weaker doughs—those who showed less resistance against deformation (higher DEF_{max})—can still maintain their shape (higher HtWr) during baking. Contrastingly, ADE and KBA have an equal (p $\geq$ 0.05) HtWr.

> Development of loaf volume is a result of complex interactions between pasting behavior, gluten-network development and dough rheology prior to and during baking. Besides these processes, degradation (mainly starch hydrolysis) by endogenous or yeast-related enzymes majorly contributes to the final effect in the end-product. Findings imply that a *combination* of different mechanisms related to CO_2 production, retention and loss lies at the basis of the eventual loaf volume.

As can also be observed from Figure 8.8, crust color is largely varying in both its lightness as well as in its yellowness. The former is represented by the L-component in the CIE-LAB color space whereas a^* gives an indication of the yellowness of the crust. A strong correlation between both variables indicated that lighter bread crusts tend to be more yellow whereas darker crust colors have a more neutral color. However, with values for a^* ranging between 9.5 and 41.0 and b^* being consequently positive (18.4–27.3), all colors will be interpreted as variations of yellow or red.

8.3.3.1 Crumb properties

Crumb structure No quantification of the bread crumb coarseness could be obtained as no reproducible slices could be made from the loaves. This was a direct result of the collapse the loaves made with WWF (with exception of KBA). However, crumb images clearly indicate the effects of amylose in development of the crumb during baking. Breads from WxD, D11, WxM and NXY collapsed within 24 hours after

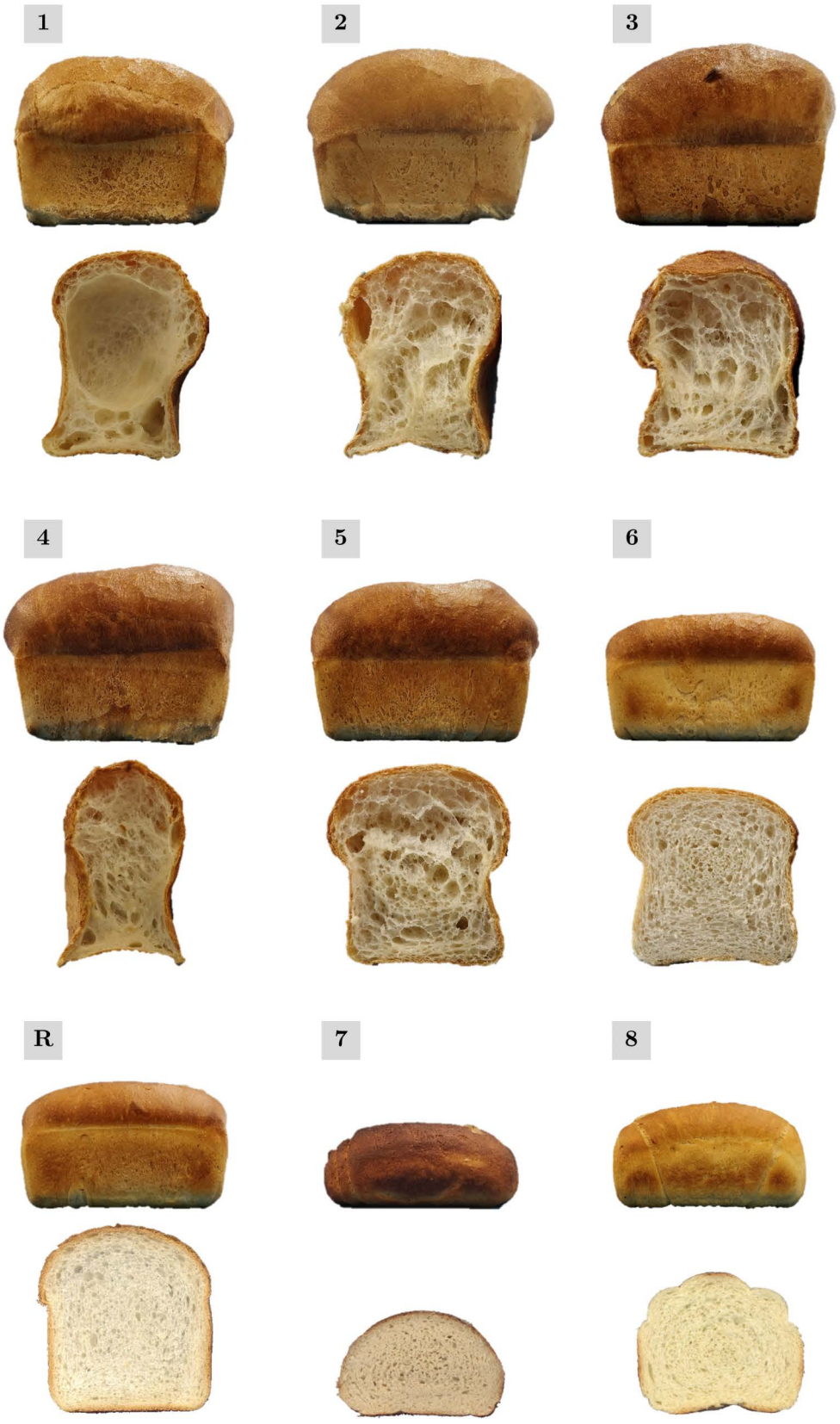

Figure 8.8: Illustration of the breads (side view and cross-section) produced using flour from 1, WxD; 2, D11; 3, WxM; 4, NXY; 5, KBA; 6, KAN; R, EPB (reference flour); 7, ADE; 8, ARI following the Standard Belgian Baking Trial.

cooling due to the lack of a uniform and elastic crumb. The crumb was sticky and gelatinous as a result of the high concentration of dextrins (obtained through enzymatic degradation during baking) which also bound more water (Every and Ross, 1996). Contrastingly, the typical 'keyhole'-effect and collapse of the loaves was only observed to a limited extent for waxy wheat genotype KBA which showed a more evenly distributed crumb structure. During baking, the stiff dough (low DEF_{max}) may promote rupture of the gas cells, thereby lowering the OvnS, and increasing the ability of the crumb to evenly fill the internal structure of the loaf. This might also result in an elevated moisture loss, thereby preventing the crumb from becoming sticky after baking. Moreover, a further improvement of the crumb structure was obtained upon a minimal increase in the amylose content as is observed for KAN (Figure 8.8 **6**).

As was stated by Ramachandran et al. (2016), a minimal AM concentration is required to form a rigid crumb structure. On the basis of the current findings, an AM content as low as 7 % (equal to a blending ratio of 28:72, assuming an AM content of 25 %) may be sufficient to obtain this effect. However, as shown earlier, dough rheological attributes—which result in turn from both protein and starch properties—interact with this effect. Moreover, it can be argued if the AM in the partial waxy genotype is correctly determined as unexpectedly low contents were obtained (as discussed in Section 3.2.4.3).

> A minimal amylose-content ($\simeq 7\,\%$) is required for the formation of a rigid crumb with uniformly distributed gas cells. However, dough rheological attributes and, thus, protein properties might also contribute to this threshold.

Crumb texture Despite the health benefits related to HiAm-WF, it is postulated that the high AM concentration in HiAm-WF will promote staling thereby lowering consumer acceptation. Initial crumb hardness (HARD) from bread produced using ARI was twice as high as EPB-based bread on the day after baking (EPB $= 866 \pm 116$ N, ARI $= 1957 \pm 211$ N). The observed phenomena can be attributed to the AM content as this starch component will retrograde within 2–24 hours after cooling. Nevertheless, it has to be noted that the VOLfc was significantly different for both samples (ARI $= 4117 \pm 94$ ml kg^{-1} flour *vs.* EPB $= 4844 \pm 86$ ml kg^{-1} flour). Moreover, the crumb hardness remains highest for the HiAm-WF based bread after a four day period although a relative increase of only 16 % (313 N) was noted. For EPB, crumb hardness increased by 77 % resulting in a final crumb hardness of 1533 N. Interestingly, the relative hardness increase over a four day period is equally low (18 and 16 %) for ADE and ARI based breads.

Although the crumb texture of only a single (partial) waxy genotype could be measured, results indicate the severe impact of an increased AMP concentrations in the flour on the staling rate in function of time. As AMP starts retrograding after one day, hardness increases over a 4-day period are significantly higher for waxy (553 %) and partial waxy (124 %) genotypes KBA and KAN. However, the open crumb results in low initial hardnesses using the current measuring method.

Proposition

The introduction of a wide diversity in starch compositional attributes, while maintaining a conventional variation in protein properties, provides a preliminary insight in the magnitude of its contribution to bread quality. The finding that both correlations between end-product quality and protein properties remain while strong relations between starch and protein properties are lacking, implies the presence of complex interactions on a functional level which cannot be easily measured using conventional techniques. Moreover, an in-depth study on the interaction between (gluten-forming) proteins and amylose during crumb formation is required in order to decipher which elements contribute to the minimal amount of AM required for breadmaking.

8.4 Conclusion

Commercial availability of wheat genotypes with an altered starch composition will significantly contribute to a diversification in approaches for improving end-product quality of bakery products from a sensory and nutritional perspective as well as from a technical point of view. However, a clear insight in the genetic variation present in these genotypes for the compositional attributes of interest—which will also have to be defined more clearly—is lacking. This also contributes to the limited understanding of the mechanisms taking place. The use of regular wheat genotypes which do not vary considerably in their starch composition, frequently allowed to relate effects observed on a functional or end-product level to one characteristic. Moreover, in the majority of the earlier research on altered starch composition of wheat, solely starch *or* protein properties were elaborately investigated. Thereby, the importance and weight of the mutual interaction between protein and starch during breadmaking is overlooked.

Firstly, the current research has shown some of the hurdles which have to be overcome when screening flour quality using conventional techniques. Hagberg falling number could not be used for measuring the α-amylase activity and no gluten were developed during Glutomatic analysis impeding a proper execution of the test. Moreover, Farinograph measurements showed poor reproducibility and sticky doughs further increased the variation. Although a (detailed) screening of the protein composition can be performed (as described in Chapter 2), the interactions become ever more difficult to capture in case variation in multiple components increases. Comprehensive strategies for standardized quality screening have to be outlined in order to accelerate the implementation of these wheat varieties in industry.

By studying the pasting behavior of all flours under high pressure, improved understanding of differences between (partial) waxy, regular and high-amylose wheat flours was obtained. Significant correlations of both amylose concentration and amylopectin fine structure with pasting temperature and peak viscosity and temperature were observed. Waxy and partial waxy genotypes also showed a larger breakdown compared to the regular wheat flour. Only the high-amylose wheat flours had a positive setback from peak confirming their gel-forming properties upon cooling. However, extremely low peak viscosities were obtained as, even under high-temperature conditions, no full granular disintegration took place. High pressure measurements also impacted network formation during cooling implying the need to further optimize the analytical method.

Loaf volume and oven spring were greatly affected by the amylose concentration although it is hypothesized that different mechanisms contribute to volume development. A collapse of all waxy breads within 24 hours after cooling also confirmed the detrimental effects on crumb rigidity using 100 % waxy wheat flour. However, both aspects are assumed to be affected complex starch-protein interactions in which water and heating play a crucial role. Therefore, it is recommendable to focus on these interactions in future research and to apply a top-down approach for obtaining an improved and broadened insight in the potential of wheat varieties with an altered starch composition.

Highlights

- Hageberg falling number, automated damaged starch determination, Glutomatic and Farinograph analysis are not (directly) applicable on flour derived from wheat genotypes with an altered starch composition.
- Broadening starch diversity (*e.g.* amylose content and amylopectin fine structure) in structure-functionality research will drastically promote the contribution of starch in defining end-product quality attributes of bakery products.
- Profound understanding of dough rheology (rigidity, elasticity and extensibility) in crumb structure and loaf volume development is crucial when using waxy wheat flour for breadmaking in order to prevent occurrence of the 'keyhole'-effect.
- High-amylose wheat flour in bread drastically lowered the staling rate although initial crumb hardness was significantly higher. Despite the high functional potential of these genotypes, a trade-off will remain existent.

General conclusion

The overall goal of this research was to investigate four approaches for wheat (*Triticum aestivum* L.) quality improvement, thereby gaining insight in some of the main lacunae currently experienced in industrial wheat processing. This has lead to the establishment of the '*four degrees of diversification*' as outlined in the introduction of this dissertation. In short, diversification can be obtained by:

- selecting cultivars with specific quality attributes for a specific, well-known application.
- using crop husbandry practices for targeted steering of the composition of wheat and its derivatives.
- broadening the knowledge on parameters and accompanying analytical techniques to screen wheat quality.
- introducing new genotypes to increase the diversity in compositional attributes thereby enabling the employment of new functionalities and their nutritional benefits.

Outcomes of the current research will encourage the translation of these pillars to concrete strategies to overcome some of the main challenges in the wheat processing chain with a more specific aim towards the bakery industry.

The current approach for wheat quality assessment and how this is embedded in the chain, hinders an optimal diversification. Besides economic motives, the build-up of the chain and the mismatch between needs and available diversity further promotes the persistence of an all-encompassing hurdle: there is a lack of fundamental knowledge on the relationship between compositional attributes and the quality of end-products. Ultimately, enhanced knowledge on this topic would give rise to universal laws for quality prediction of any type of wheat-based product. Although this is not the goal, an improved insight in the direction in which quality parameters should be searched, would greatly promote all future framing research. For example, wheat breeders will only be able to develop a genotype which is *ideal* for breadmaking if it is known which compositional attributes it has to contain, and how these can be ascertained under various growth conditions. Such holistic research can only be pursued when universally applicable and standardized methods (both analytical and statistical) are developed and adopted throughout the entire processing chain.

In the following parts, a brief overview of the major findings related to the four main topics will be presented together with conceptual thoughts on future developments on both an academic and industrial level.

Summary of major findings

The impact of Nitrogen (N) and Sulfur (S) fertilization on wheat quality and bread quality was investigated in field trials comprising the effect of both dosage and fractionation for N or dosage and timing in case of S. The generally held view that legislative restrictions for the N dosage rate are limiting for the production of high quality wheat in Flanders was refuted. A low impact on yielding parameters or determinative quality attributes (protein content, dough rheology) was observed with unclear trends between most treatments. Compared to the blanc treatment (two early N fractions), an increase in the N dosage rate was accompanied by a lowering gluten strength and decreased loaf volume. Although findings cannot be considered conclusive, the application of a fourth fraction is preferred over increasing the N dosage as, in general, no significant difference between +30 % in three fractions and -30 % at four fractions was seen. High interaction effects with environment and genotype were however noted as the quality-promoting effect was only observed for a selection of the tested cultivars. Even more, the high temperatures at the time that the fourth fraction was administered may have impacted current findings but will also impede the applicability of the proposed strategy in the future. To overcome this problem, research on the type of N-fertilizer will become more important.

Sulfur-fertilization, more specifically the timing at which S was applied, showed to impact protein content to a larger extent compared to N-fertilization. Although an overall increase was observed when S was administered, a doubling of this effect could be noted upon simultaneous application with the third N fraction. However, this also might have resulted from an earlier application. Apart from protein content, protein *composition* was also significantly impacted although differences related to timing were less pronounced. In general, a higher (wet) gluten content and increased gluten strength was noted as an effect of S-application, while doughs were also more extensible. Assumedly, this was related to increases in both the proportion of high molecular weight glutenin-subunits as well as in the prolamin concentration.

From these fertilization experiments, it was preliminary shown that restrictions are not necessarily limiting for wheat quality. It may however be valuable to investigate fertilization strategies focusing on S-application as an increase in gluten strength is not necessarily desirable. Moreover, a high dependency on the environment was noted as well as a clear interaction with genotype demanding for the incorporation of these factors in future research.

Apart from controllable environmental conditions, the detrimental impact of heat- and drought-stress on wheat yield was shown using a greenhouse trial. Although conditions were more extreme, results supported the outcomes of other researchers in terms of high protein contents ($\geq$ 100 % increase) and diminished yields. Clay soil was found to mitigate the effect of drought-stress, presumably because of its higher water retention capacity. This mainly came to expression in the number of kernels per ear and thus, the total yield as well as in the protein composition. From the reversed-phase HPLC-based results, it was seen that prolamin and glutelin composition was solely altered when wheat was cultivated on a sandy soil under drought conditions. The difference between the tested cultivars remained however many times larger.

From both the multi-annual, multi-location field trials and the Nitrogen-fertilization trial, it was concluded that all main bread quality attributes are significantly influenced by the genotype with a varying behavior

over the environments. The loaf volume per unit flour—which was found to be highly suitable for describing bread quality—was unstable for all screened pure genotypes across both locations and years. Moreover, quality attributes which are currently used in industry for predicting baking quality were approximately for one-third determined by the genotype. Exceptions were the protein content, which was only influenced by environment, and the Zeleny sedimentation value (as indicator for gluten quality) which was highly genotype-dependent. Correlations with the loaf volume were, however, for both parameters insignificant. The same observation applies for all conventionally used parameters—water binding to the wet gluten, wet and dry gluten contents and Farinograph and Alveograph parameters—when considered separately. Even by applying complex, cross-validated modeling techniques on these parameters, the loaf volume could not be predicted with a high accuracy as, on average, an absolute error of approximately 10 % was obtained.

The search for parameters which could be used to improve bread quality prediction asserted two general findings. Firstly, measurements on wholemeal instead of refined flour provided supplementary information. This was assumed to be related to the water binding capacity of components present in the matrix or which were incorporated in the gluten network during mixing. Wholemeal parameters obtained from both pasting measurements and Glutomatic analysis contributed to a larger extent when modeling bread quality of breads produced using refined flour. Secondly, starch properties clearly contributed to the development of the loaf volume as some molecular (amylopectin branch chain-length distribution), macromolecular (proportion B-type granules) and functional (holding strength and pasting temperature) attributes were consistently ranked high. Noteworthy, the amylose content in the *current* sample set—for which concentrations varied between 10.7 and 28.2 %—was negligible in defining bread quality.

Conventional techniques for wheat protein characterization gave varying results in terms of their ability to discriminate between samples or their correlation with quality attributes. By means of the Zeleny sedimentation value, it was for example possible to discriminate all cultivars, however, no correlation with their breadmaking quality could be established. The same was valid for the Alveograph W-value (area under pressure-length curve) which is also one of the main parameters used in grading systems. Moreover, it was shown that results from Glutomatic analysis were prone to error or could not be obtained for samples with an undesirable protein composition. When limited amounts of sample are available, it is also difficult to perform dough rheological measurements using the Farinograph or Alveograph. Although not included in this research, similar problems are encountered for Mixolab and Extensograph analysis whereas rotational rheometry requires a thorough method standardization and highly experienced staff for both execution and interpretation of the outcomes. With these shortcomings in mind, an alternative approach which combines reversed-phase HPLC with machine learning techniques was developed thereby starting from the composition of the raw material.

The main alleles determined by SDS-PAGE which have been related to breadmaking quality—in particular *Glu-D1* alleles 5+10 and 2+12—could be predicted with high accuracy for both a set of highly diverse as well as a set of very closely related wheat cultivars. Although this provided proof that the approach was able to extract information on protein composition on a molecular level from the input data, results for the 'back-translation' to the chromatograms were less credible as different areas were selected when the same trait was predicted using a different sample set. Furthermore, the data could also be used to predict origin and genotype when information on the composition of the water-extractable proteins (albumin and

globulin) was included. However, in contrast to the genotype which could be predicted with high accuracy, no complete distinction between the five enclosed environments was obtained. Mainly an overlap between samples grown in the same harvest year was observed. These findings indicated that protein *composition* differed more between genotypes than between environments.

When data was used to predict functional attributes of the samples, promising results were obtained. Farinograph water absorption and the Alveograph configuration ratio (P/L-ratio) could be predicted with a mean absolute error of 1.34 % and 0.65 respectively. As the used data is theoretically only protein-related, it was concluded that protein was the main contributer to these properties. Nevertheless, supported by the remaining high error rate for industrial implementation, a combination with starch properties is recommendable to construct more accurate models. This effect was also observed to a larger extent for the loaf volume implying the need to include parameters which cover the non-protein related effects during bread making. In an attempt to obtain an improved model thereby starting from the HPLC-data, it was shown that chromatographic data was preferred above compositional properties from protein-related techniques. However, functional attributes (dough rheological attributes, holding strength and pasting temperature) had to be included to enhance the accurateness. Compared to the best model which includes both wholemeal and flour properties, an improvement in the mean absolute error by 0.3 % from 6.1 % to 5.8 % was achieved by using the HPLC-based approach. Although this improvement is limited, the number of techniques is reduced and only flour-based parameters are included.

Besides mutual interactions between protein and starch, the contribution of water to effects taking place during breadmaking were found to be highly determinative. Also in extrusion, its role was found to be crucial for the mechanisms taking place during this high-temperature, short-time processing technique. The presence of a lower limit in the water feed rate (and thus, feed moisture content) was observed impacting the external and internal extrudate morphology. Equally important was the protein content of the flour with visible differences in the expansion index, moisture loss and water absorption index at concentrations currently present in commercially available wheat flour (9.3–16 %). To a lesser extent, protein composition contributed to the conditions (*i.e.* feed moisture content) at which the critical point is observed. Despite no clear link with compositional attributes could be established, differences in melt viscosity and viscoelasticity were assumed to lie at the basis of the observed phenomena. Similar conclusions were also drawn from the study on the impact of differences in the amylose content in wheat flour on its extrudability. Effects were not linear in the tested range as the highest expansion at die emergence was noted at slightly lowered amylose contents. A further increase of the relative proportion of amylopectin resulted in the collapse of gas cells whereas higher amylose concentration in the flour increased the melt viscosity thereby reducing the ability to expand. In contrast, hardness consistently decreased upon increasing the content waxy wheat flour in the blends and also the water binding capacity systematically decreased.

Both studies supported the applicability of wheat flour for extrusion processing. Waxy wheat cultivars are thereby preferred for their improved crispness whereas an increased protein content may contribute to both the stability of the gas cells as well as the texture. Depending on the feed moisture content, thinner gas cell walls can be obtained at high protein concentrations. The challenge however lies in finding the optimal melt viscosity through balancing the protein content, the feed moisture content and the amylose concentration. This may be attained by blending various wheat flours or by exploring the currently present

genetic diversity. Within waxy wheat cultivars, it has been shown that both protein properties as well as starch compositional and functional attributes (pasting behavior) are largely differing resulting in a wide variety of combinations.

Similar observations were made when wheat cultivars with an altered starch composition were used for breadmaking. Loaves from four waxy cultivars collapsed whereas a fifth cultivar produced breads which remained stable at a similar amylose content. It was shown that most potential lies in the use of flour from *partial* waxy wheat cultivars as this resulted in increased loaf volumes while maintaining a an acceptable crumb structure. Moreover, a significantly lower initial and final crumb firmness, up to 20 % lower than bread from regular wheat flour after four days, was obtained despite the higher staling rate for the partial waxy wheat flour breads. The opposite was noted for high-amylose wheat flour for which—besides a consistently lower loaf volume—only a 17 % increase in crumb firmness was recorded. On a functional level, the use of a high pressure cell for measuring the pasting behavior provided new insights in the differences between waxy, partial waxy, regular and high-amylose wheat flours. A primary increase in viscosity was only observed when amylose concentrations ranged between 7–25 % implying a varying granular swelling behavior. Furthermore, pasting and peak temperatures were not linearly correlated with the amylose content as well as the peak viscosity which was highest for amylose-free (waxy) and lowest for amylose-rich wheat flours. No breakdown was noted for one of the latter genotypes implying an incomplete pasting, even under these high pressure conditions.

The overall importance of interaction effects is thought to be even greater when screening a set of cultivars which differs in both its protein and starch composition. However, applied techniques were unable to capture these effects or were not uniformly applicable. Also, more established methods (Hagberg falling number, Glutomatic, Farinograph, pasting behavior, breadmaking trials) require adaptation or have to be replaced by alternative approaches before these can be applied on such cultivars in a reproducible manner. From the current sample set, it could also be concluded that a more holistic approach in terms of included variables (protein- or starch-related) should be pursued when studying its contribution to bread quality. By enlarging diversity, different insights can be obtained with a generally lowered importance of protein properties, an increase for starch-related attributes and a significantly higher weight for measures related to their mutual interaction.

Recommendations

This dissertation has contributed to the ongoing paradigm shift in the research domain on wheat quality and how this relates to the end-product quality of bakery products. The importance of the starch fraction therein has been emphasized as well as the current need to include both functional and compositional features for quality prediction. Crucial considerations for both aspects are the resolution of the acquired data and whether the parameters provide supplementary or complementary information. Besides the numerous technical recommendations which can be proposed on the basis of this research, the current section will focus on the general lessons derived from the applied research approaches and their perceived hurdles.

When studying wheat quality, and more specifically: when estimating the contribution of compositional attributes in the breadmaking performance, a more nuanced insight in the historically grown importance of proteins has to be obtained. This can be achieved by including relevant starch-related properties which will naturally lead to an improvement of the prediction of end-product quality and will stimulate diversification. It has been shown that granular properties, mainly size and rigidity, are of importance as well as the amylopectin branch chain-length distribution. Together with crystallinity, this may form a bridge between granule morphology and its functional behavior during heath treatments.

Furthermore, the introduction of a broader diversity in the starch fraction (by the use of waxy, partial waxy and high-amylose wheat cultivars) and the employment of their nutritional and functional benefits, will also require a better understanding of the underlying mechanisms defining bread quality. This has to be accompanied by the development of empirical models encompassing a similar order of variance. Extrapolation from current models and theories will lead to unexpected and undesirable behavior, production line downtimes, economic losses and food waste. Besides limited availability, these can be considered the current main concerns for their industrial application. In this light, generally accepted theories also have to be reviewed and adapted taking into account the behavior of both starch and proteins. Hence, the moderate interest in these wheat genotypes can further increase, thereby promoting their commercial availability and application.

Frequently, functional (dough-related) properties are measured as these incorporate the interactions taking place between starch and protein. In this research, it was also shown that properties related to water binding and network formation (Farinograph water absorption, dough development time, softening and the Alveograph configuration ratio) enhance the accuracy of the model. However, proof has been provided that these properties can also be accurately predicted by using compositional attributes. This implies the ability to eventually also predict end-product quality without requiring functional properties. However, when circumventing these often flour-based and time consuming properties, it is demanded for a more elaborate understanding of the effects taking place *during* breadmaking. This includes both interactions occurring during mixing and the effect of resting and heating. One approach is the use of more complex supervised learning techniques in which non-linearity is handled better (*e.g.* artificial neural networks) and by selecting only meaningful variables in a robust manner.

When performing a similar study on an industrial level, it is highly recommendable *not* to start from the conventionally used empirical techniques. A defined set of compositional parameters of the wheat, the wholemeal, and/or the refined flour has to be measured in-house or externally and has to be combined with information on the end-product characteristics of interest. The first steps in this process are defining these properties, developing standardized measuring protocols and constructing a framework (legislative and technical) in which data is collected. The latter is of equal importance as data has to be captivated in function of time and as multiple actors are involved. Ultimately, various stakeholders (vertical) and companies (horizontal) can contribute to broaden the diversity in both incoming and outgoing products and to more efficiently provide the relevant parameters.

Only when an efficient, robust and accurate model can be constructed, concrete recommendations towards earlier actors in the chain (mainly breeders and farmers) can be made. The observed high genotypic dependency of numerous determinative parameters implies the potentially added value of breeding towards a desired quality. However, the current mindset of increasing the protein *content* together with maintaining

a high yielding potential has to be reconsidered as this does not guarantee a high baking quality. In general, exploration of relevant traits, their assured availability and stability and an application-oriented diversification have to become the common goals within the wheat-processing chain.

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Curriculum vitae

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Education

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2013–2015	Master in Applied Biosciences (2013–2014: linking program) Major: Food Industry Thesis: Determination of the starch composition and functionality of the Ethiopian potato (Plectranthus edulis) and wheat Ghent University, Ghent, Belgium

Additional training

GPC/SEC Training course
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High-resolution X-ray Computed Tomography
18–19 January 2017, Ghent University Center for X-ray Tomography (UGCT), Ghent, Belgium

Improving wheat quality for food processing and health, by Prof. P.R. Shewry, Francqui Learning Chair 2016/2017, 14–23 March 2017, KU Leuven, Leuven, Belgium

Publications

Composition, Granular Structure, and Pasting Properties of Native Starch Extracted from Plectranthus edulis (Oromo dinich) Tubers
Hellemans, T.*, Abera, G.*, De Leyn, I., Van der Meeren, P., Dewettinck, K., Eeckhout, M., De Meulenaer, B., & Van Bockstaele, F. (2017). Journal of Food Science, 82(12) 2794-2804.
DOI: 1750-3841.13971
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Impact of crop husbandry practices and environmental conditions on wheat composition and quality: a review.
Hellemans, T.*, Landschoot, S.*, Dewitte, K., Van Bockstaele, F., Vermeir, P., Eeckhout, M., & Haesaert, G. (2018). Journal of Agricultural and Food Chemistry, 66(11) 2491-2509.
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Applicability of waxy wheat in extrusion processing
Hellemans, T., Nekhudzhiga, H., Van Bockstaele, F., Wang, Y.J., Emmambux, M.N., & Eeckhout, M. (2019). Journal of Cereal Science, *submitted*

RP-HPLC and machine learning as alternative for conventional wheat quality screening techniques
Hellemans, T., Landschoot, S., Maene, P., Van Bockstaele, F., Fleitas, M.C., Chibbar, R.N., Eeckhout, M., & Vermeir, P. (2019). Journal of Chromatography A, *submitted*

Conferences contributions

Plant development, yielding parameters and kernel composition of winter wheat in relation to soil type under organically elevated temperatures.
Oral presentation, EFFoST 31st International conference, 13–16 November 2017, Sitges, Spain.

Plant development, yielding parameters and kernel composition of winter wheat in relation to soil type and irrigation treatment under an organically elevated temperature.
Poster presentation, 3rd Agriculture and climate change conference, 24–26 March 2019, Budapest, Hungary.

Baking quality of winter wheat (*Triticum aestivum* L.) is influenced by fractionation of Nitrogen fertilization.
Poster presentation, Soils and Crops, 5 and 6 March 2019, Saskatoon, Canada.

International collaborations

Technical assistance, QA/QC training on Flour Fortification
Makerere University, Kampala, Uganda
in collaboration with 'Smarter Futures', 19–28 May 2016

Research visit, Department of Consumer and Food Sciences
University of Pretoria, Pretoria, South Africa
Prof. Naushad M. Emmambux, 12–18 May and 3–22 May 2018

Research visit, Department of Plant Sciences, College of Agriculture and Bioresources
University of Saskatchewan, Saskatchewan, Saskatoon, Canada
Prof. Ravindra N. Chibbar, 7th January to 22nd March 2019

Informal talks and publications

De glycemische index van brood: een complex gegeven
Van Bockstaele, F. & Hellemans, T. (2016). NutriNews 2016/4
URL: https://www.nice-info.be/nutrinews/de-glycemische-index-van-brood-een-complex-gegeven

Influence of genotype and cultivation conditions on wheat
Clarys Claryfar Seminar, 4th October 2017
Koopmans Meel, Leeuwarden, Netherlands.

Science figured out: Van tarwekorrel tot eindproduct
Wetenschap uitgedokterd, 2018
URL: https://www.sciencefiguredout.be/node/62

Hobbybakker bakt brood met 4.500 jaar oude gist
De Standaard, 12th August 2019
URL: https://www.standaard.be/cnt/dmf20190811_04554751

Onze sector: Van tarwekorrel tot eindproduct
Onze Passie, vaktijdschrift Bakkers Vlaanderen, July 2019

Tutorship

Master dissertation students

2016–2017	Ilona Vandenberghe	Optimalisatie van een RP-HPLC techniek voor de bepaling van tarwe-eiwitten en hun functionaliteit bij toepassing in brood
	Jozefien Timmerman	De reometer als alternatief voor de amylograaf van Brabender: een vergelijkend onderzoek
	Nele Serruys	Functionaliteit en toepasbaarheid van triticale in brood
	Phara De Bock	Intra- en intersitevariabiliteit bij zachte tarwe en karakterisatie van experimentele variëteiten
2017–2018	Bert Sinaeve	Eiwitsamenstelling en zetmeelfunctionaliteit in wintertarwe (*Triticum aestivm* L.) in functie van stikstofbemesting
	Hannes De Rycke	Bemestingsadvies voor broodtarwe (*Triticum aestivum* L.): de impact op korrelsamenstelling en eindproductkwaliteit
	Kaatje Van Cauwenberg	Impact van gluteneigenschappen, vochtgehalte en temperatuur op de zetmeelverstijfseling bij extrusie
2018–2019	Estelle Yvergneaux	Eiwit-zetmeelinteractie in brood, van molecule tot eindproduct
	Free Vandevyvere	Stikstof- en zwavelbemesting voor het sturen van de samenstelling en bakwaardigheid van wintertarwe (*Triticum aestivum* L.)

Bachelor dissertation students

2017–2018	Esther Van Parys, Fien Van Schoors, Reindert Picavet	Impact van zwavelbemesting op de eiwitkwaliteit van tarwe
	Janneke Vereeken, Lotte Selis, Lotte Verhaeghe	Kwaliteitsbeoordeling van industriële glutenstalen
	Eline Decleer, Inès Depotter, Maura Lodewyckx	Definiëring van kwaliteitsparameters in bakkerijproducten

Internship students

2016–2017	Michiel De Moor	-
2017–2018	Matthijs Peperstraete	RP-UHPLC als fingerprintingtechniek voor tarwe-eiwitten
2018–2019	Matthijs van der Heyden	Optimalisatie en validatie van Englyst-methode voor in-vitro zetmeelverteerbaarheid