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Proteins of einkorn, emmer and spelt: Influence on baking quality and role in wheat-related hypersensitivities

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Table of contents

Abbreviations

AACCI	American Association of Cereal Chemists International
AGT	Aggregation time
ALGL	Albumins and globulins
AI	α-Amylase inhibitor
APEX	Absolute protein abundance index
ATI	α-Amylase/trypsin inhibitor
ATIs	α-Amylase/trypsin inhibitors
BU	Brabender Units
CAA	Chloroacetamide
CCL2	chemokine (C-C motif) ligand 2
CD	Coeliac disease
DDA	Data-dependent acquisition
DIA	Data-independent acquisition
DTT	Dithiothreitol
emPAI	Modified protein abundance index
ESI-QTOF	Electrospray ionisation quadrupole time-of-flight
FODMAP	Fermentable oligo-, di-, and monosaccharides and polyols
GLIA	Gliadins
GLUT	Glutenins
GMP	Glutenin macropolymer
GPT	GlutoPeak Test
GS	Glutenin subunit
GP	Gel permeation
HMW	High-molecular-weight
HPLC	High-performance liquid chromatography
IAA	Iodoacetamide
IBS	Irritable bowel syndrome
ICC	International Association for Cereal Science and Technology
iBAQ	Intensity-based absolute quantitation
IL	Interleukin

LMW	Low-molecular-weight
LOD	Limit of detection
LOT	Lift off time
LOQ	Limit of quantitation
MALDI	Matrix-assisted laser desorption/ionisation
MMW	Medium-molecular-weight
MBT	Microbaking test
MOMT	Micro-Opti-Mix-Test
MRMT	Micro-Rapid-Mix-Test
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MT	Maximum torque
m/z	Mass-to-charge ratio
NCGS	Nonceliac gluten sensitivity
NIRS	Near-infrared spectroscopy
PAGE	Polyacrylamide gel electrophoresis
PCA	Principle component analysis
PMT	Peak maximum time
PWG	Prolamin Working Group
r	Correlation coefficient
RMT	Rapid-Mix-Test
RP	Reversed-phase
S	Sulphur
SDS	Sodium dodecyl sulphate
SDSS	Sodium dodecyl sulphate soluble
SIDA	Stable isotope dilution assay
SI_N	Normalized spectral index
SRM	Selected reaction monitoring
TCEP	Tris(2-carboxyethyl)phosphine
TI	Trypsin inhibitor
TNF	Tumor necrosis factor

Tris	Tris(hydroxymethyl)aminomethane
TOF	Time-of-flight
UPP	Unextractable polymeric protein
WASI	Wheat amylase subtilisin inhibitor
WCI	Wheat chymotrypsin inhibitor
WDAI	Wheat dimeric amylase inhibitor
WMAI	Wheat monomeric amylase inhibitor
WTAI	Wheat tetrameric amylase inhibitor
WTI	Bowman-Birk type trypsin inhibitor

One letter code for amino acids

Alanine	A
Arginine	R
Asparagine	N
Aspartic acid	D
Cysteine	C
Glutamic acid	E
Glutamine	Q
Glycine	G
Histidine	H
Isoleucine	I
Leucine	L
Lysine	K
Methionine	M
Phenylalanine	F
Proline	P
Serine	S
Threonine	T
Tryptophan	W
Tyrosine	Y
Valine	V

1 Introduction

Maize, wheat and rice are the three most cultivated cereals worldwide. The production of wheat (2016: 750 million tons) is lower than of maize (2016: 1,000 million tons) but higher than of rice (2016: 500 million tons). Since 2013 the worldwide production of wheat has exceeded 700 million tons (http://www.fao.org/faostat/en/#data). Wheat contributes to up to 50 % of the total calory intake in wheat-producing countries and is an important staple food [1]. The term 'wheat' summarizes many species of wheat belonging to the genus *Triticum* in the family of *Poaceae*. The most widely cultivated wheat species is common wheat (*T. aestivum*) accounting for about 95 % of wheat production [2]. Alternatively, common wheat is called bread wheat demonstrating its main use. About 40 million tons of durum wheat (*T. durum*) are produced per year and mostly used for making pasta. Durum wheat is adapted to hot and dry conditions around the Mediterranean Sea and areas with similar climate [2]. Besides the modern wheat species common and durum wheat, 'ancient' wheat species are cultivated in the 21st century in very low amounts, while they were the prevailing wheat species several thousand years ago. The best known ancient wheat species in Europe are einkorn (*T. monococcum*), emmer (*T. dicoccum*) and spelt (*T. spelta*) (Figure 1) although the latter one has extensively been modified by breeding since the beginning of the 20th century.

Figure 1: From left to right: Ears of einkorn ("Tifi"), emmer ("Osiris") and spelt ("Franckenkorn"). Einkorn has smaller kernels than emmer and spelt. Contrary to spelt, einkorn and emmer mostly have awns. Pictures: S. Geisslitz

1.1 Characteristics of ancient wheats

1.1.1 Taxonomy of ancient wheats

Einkorn, emmer and barley (*Hordeum vulgare*) were the first cultivated crops about 10,000 years ago during the 'Neolithic Revolution' when the population developed from hunters and gatherers to settlers [3]. Archaeological findings report the first occurrence of domesticated einkorn, emmer and barley in the Fertile Crescent between the rivers Tigris and Euphrates [3, 4]. The history and taxonomy of *Triticum* species is not clarified in total, but the following theory is most widely accepted (Figure 2) [5].

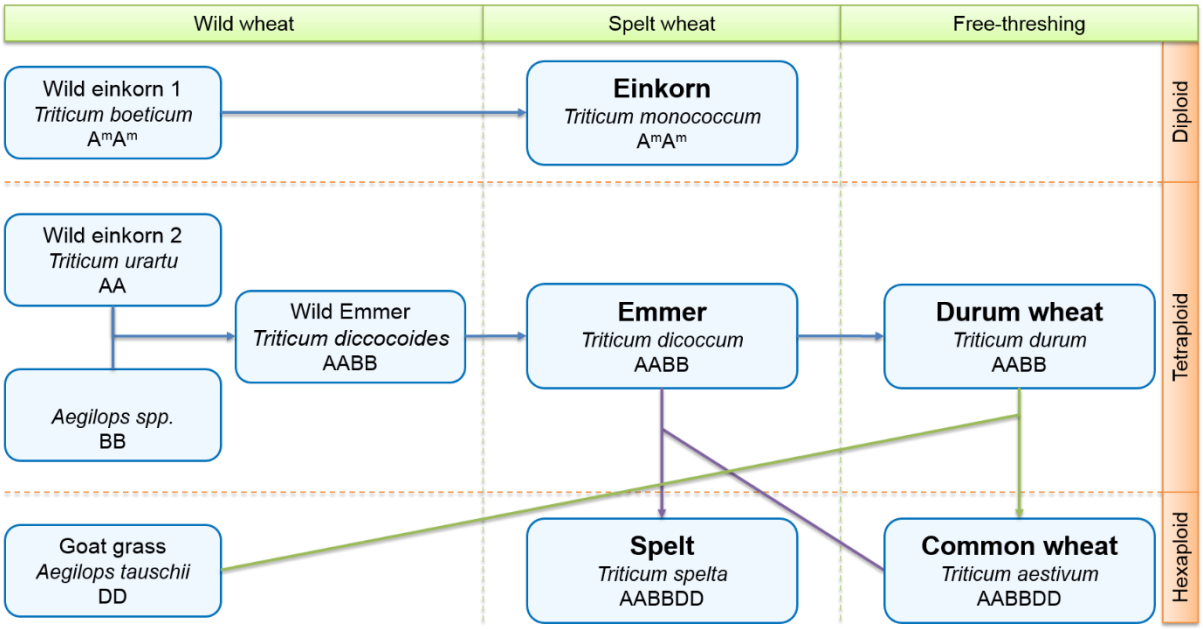

Figure 2: Taxonomy of the wheat species einkorn, emmer, durum, spelt and common wheat (modified from [5-7]); einkorn, emmer and spelt are hulled wheats and durum and common wheat are free-threshing wheats. Einkorn is a diploid wheat (genome: A^mA^m), emmer and durum are tetraploid wheats (AABB), and spelt and common wheat are hexaploid wheats (AABBDD). Their relationship is shown by arrows. The complex relationship of spelt and common wheat is shown by the violet arrow (ancestors of spelt: emmer and common wheat) and the green arrow (ancestors for spelt: goat grass and durum wheat).

T. boeticum (A^mA^m) and *T. urartu* (AA) are two well-known wild diploid *Triticum* species, which are separated by crossing barriers and have different plant morphology [8]. *T. boeticum* (alternative name wild einkorn) was domesticated by cultivation and repeated harvesting to diploid, domesticated einkorn (*T. monococcum*) in the Karacadağ mountains [4, 9]. The name einkorn originates in the German language and literally means 'single grain', because just one slender kernel per spikelet is formed. In contrast to the wild forms the domesticated crops have larger kernels and the seeds remain at the ear during harvest because of a tough rachis. The wild forms have a fragile rachis, which leads to easy shattering and dispersing of the seeds in nature. *T. urartu* has never been domesticated, but played an important role in the development of ancient and modern wheats.

Tetraploid wild emmer (*T. dicoccoides*) developed by spontaneous hybridisation of *T. urartu* and an unidentified *Aegilops* species. It was shown that the genome of *A. speltoides* has an S genome and might not be the ancestor of wild emmer [10]. On the other hand it was suggested that the B genome evolved an introgression of several parental *Aegilops* species including *A. bicornis*, *A. searsi*, *A. longissimi*, *A. sharonensis* and *A. speltoides* [11]. Therefore, the source of the B genome is still unknown and under discussion. Wild emmer still grows naturally in the Fertile Crescent. Similarly to einkorn, the domestication of emmer (*T. dicoccum*) probably took place close to the Karacadağ mountains [12]. It is not completely clarified if emmer and einkorn were domesticated at the same time or if one wheat species is younger than the other one [12]. Emmer is alternatively called '*Zweikorn*' in German, literally 'double grain', showing the presence of two slender kernels per spikelet. Einkorn and emmer are both hulled wheats with a tough glume. After the domestication of emmer, free-threshing tetraploid genotypes occurred spontaneously, which were selected by humans due to the absence of the husks. About 7,500 years ago the tetraploid, free-threshing durum wheat (*T. durum*) was cultivated in the Fertile Crescent [13].

The tetraploid wheat species wild emmer, emmer and durum wheat are interfertile with themselves and also with other tetraploid wheat species. Taxonomists suggest that these wheat species belong to same species and are subspecies of *T. turgidum* [1, 4]. Because of this, emmer and durum wheat have *T. turgidum* sups. *dicoccum* and *T. turgidum* sups. *durum* as alternative botanical names. Both botanical names are accepted equally, but the latter ones show the close relationship of emmer and durum wheat.

Some theories have been published about the evolution of the hexaploid wheat species common wheat and spelt. It is a fact that no wild hexaploid wheat species is known and therefore the hexaploid wheats resulted from crossbreeding of a tetraploid and a diploid wheat species. In the most widely accepted theory, common wheat (genome AABBDD) originated from the diploid goat grass (*A. tauschii*) with the D genome and the tetraploid, domesticated durum wheat or emmer (AABB) [6, 7, 14]. Even if emmer has been thought to be the ancestor for common wheat for a long time, more recent studies propose durum wheat as ancestor [6, 7]. The order of appearance of spelt and common wheat is not yet clarified. It is unknown whether common wheat evolved earlier than spelt or whether spelt is an ancestor of common wheat or vice versa. Another option is that both developed separately. A long accepted theory is, that spelt originated from emmer and goat grass and developed into common wheat [14]. Newer theories propose that common wheat developed first and then spelt by hybridisation of common wheat and emmer [7, 15]. This theory is displayed in Figure 2 using the green and violet arrows. The questions about when and where the development of spelt and common wheat happened are still unanswered. It is supposed that this happened about 10,000 years

ago in Transcaucasia and in regions near the Caspian Sea [7, 14, 16]. One of the most recent theories, proven by genetic investigations, suggests that first an Asian spelt was developed by crossbreeding of durum wheat and *A. tauschii* and this Asian spelt was bred to common wheat. In the review of Faris (2014) it is confirmed that European spelt was a crossbreed between emmer and common wheat [17]. Due to clarity, the evidence for Asian spelt is not displayed in Figure 2.

An alternative theory on the development of spelt and common wheat suggests that the D genome in hexaploid wheat species is a complex hybridisation of the three A, B and D progenitors [11]. This complex D genome is still present in *A. tauschii*, showing that the D genome does not have a pure genome, but is a hybrid. Secondly, it is postulated that spelt developed earlier than common wheat [11].

There are indeed some contrary theories about the development of hexaploid wheat species. It might be possible that common wheat is older than spelt and was therefore cultivated earlier. Nevertheless, einkorn, emmer and spelt belong to ancient wheats due to their dominance several thousand years ago and their almost complete absence today.

1.1.2 Cultivation of ancient wheats in the 21st century

Einkorn and emmer have disappeared almost completely from the fields since the 13th century. Today, einkorn is cultivated in very low amounts and emmer on a few thousand hectares in Italy, Hungary, France, Austria and Germany. On the other hand, emmer is more common in some regions. For example in Ethiopia, 7 % of wheat growing areas are used for emmer. In India, 250,000 tons of emmer are harvested per year [18]. Einkorn and emmer are mostly cultivated in regions with harsh agricultural (e.g. loamy or stony) and climatic (e.g. dry and cold) conditions, because both wheats are more undemanding than common wheat.

The absence of einkorn and emmer or rather the dominance of common wheat has several reasons. Emmer and einkorn are hulled wheats and the kernels are tightly enclosed by tough glumes (husks). These husks have to be removed by a special dehulling treatment in the mill to eliminate the chaff from the grain. This step is not required for common and durum wheat, because they are free-threshing wheat species. Another disadvantage of einkorn and emmer is that both have longer stalks (80 – 150 cm) than modern cultivars of common and durum wheat (up to 100 cm) and have a tendency to lodging. This complicates the harvest and may lead to reduced yields [19]. In general, the yield of einkorn and emmer is considerably lower compared to common wheat. Independent of climate and agricultural conditions, the yield of einkorn and emmer (2 – 4 t/ha) is 55 % to 67 % lower than of common wheat (6 – 9 t/ha) [19]. Furthermore, the ancient wheats einkorn, emmer and spelt show inferior baking performance and are more difficult to process than common wheat (see 1.1.4).

Until the end of the 19th century spelt was the predominant wheat species in the Southwest of Germany, but until the middle of the 20th century the cultivation area of spelt decreased continuously to almost zero. One reason for the disappearance of spelt was that the European population preferred white and fluffy breads instead of wholegrain and porridge in the post-war period. Further, breeding made common wheat more resistant to low temperatures and increased the yield compared to spelt. Last, the elaborate dehulling process was no longer economically profitable since the middle of the 20th century in the modern and industrialised mills. These reasons led to the dominance of common wheat. Especially in the 21st century and with the increase of organic farming, spelt has been attracting attention again. In most statistics common wheat and spelt are summarised and data for the cultivation of spelt are not available. Surveys estimated that Germany is the most important producer with about 100,000 ha followed by Austria, Belgium and France (20,000 ha each) [18].

Simultaneously to the increase of the cultivation of ancient wheats the spectrum of products made of einkorn, emmer and spelt rose. The offer of bread, pasta, muesli and beer made of 100 % ancient wheat increased considerably. Another example shows the growing attention on spelt: Bread made of wholegrain spelt flour was awarded as the 'bread of the year 2018 in Germany' [20].

1.1.3 Constituents and bioactive components of ancient wheats

The wheat kernel consists of the endosperm and the germ and is enclosed by the bran and in the case of spelt wheat (einkorn, emmer and spelt), additionally with non-edible husks. The relative kernel yield of einkorn (about 60 %), emmer and spelt (both about 70 %) is considerably reduced due to the high amount of husks [19]. This means that over 30 % of the einkorn, emmer and spelt kernels are husks. After removing the husks, the endosperm corresponds to over 80 %, the germ to about 2 % and the bran to about 15 % of the wheat kernel [21]. The endosperm is rich in starch (about 80 %) and proteins (about 10 %) and contains low amounts of minerals (0.5 %) and lipids (1.6 %) [21]. In contrast, the germ is rich in proteins (34 %), minerals (6 %) and lipids (28 %) [21] and the bran mostly contains crude fibre (up to 63 %), proteins (up to 18 %) and minerals (up to 8 %) [22].

These differences in the distribution of ingredients in the different parts of the kernel are important, because flours made of smaller kernels may have higher contents of crude fibre, minerals and other components concentrated in the bran (see 1.1.4). The size of kernels is reflected in the thousand kernel weight, that is slightly higher for emmer (55 g) and spelt (50 g) than for common wheat (47 g), but considerably lower for einkorn (30 g) compared to common wheat [19]. However, the differences in the major components starch, protein, fat and minerals between ancient and modern wheats are very low (Table 1) [23]. The mineral and protein

contents of the ancient wheats are generally higher than those of modern wheats, which have more crude fibre than the ancient wheats.

Table 1: Composition (minerals, starch, protein, fat and crude fibre) of wholegrain flours of common wheat, spelt, durum wheat, emmer and einkorn (%, dry basis, [23])

Wheat species	Minerals	Starch	Protein	Fat	Crude fibre
Common wheat	1.9	59.7	15.7	1.8	3.1
Spelt	2.0	52.4	19.1	2.2	2.6
Durum wheat	1.9	61.1	16.8	2.3	3.0
Emmer	2.1	60.0	19.1	2.0	1.7
Einkorn	2.2	50.5	20.0	2.4	1.3

Besides the energy intake further aspects such as health benefits and risk reduction to diseases are gaining importance in the 21st century. Thus, the question if “ancient types of wheat have health benefits compared to modern bread wheat” has been raised [1]. An example for bioactive components is dietary fibre, which can positively influence the blood glucose level, the cholesterol level and the whole digestion process. The major components of dietary fibre are cell wall components such as the polysaccharides arabinoxylan, cellulose, β-glucan and Klason lignin. Other dietary fibres are fructans (fructooligosaccharides) and resistant starch. The latter is concentrated in the endosperm, whereas the other dietary fibres are enriched in the bran and the outer layers of the kernel. The contents of total dietary fibre and β-glucan seem to be higher in common wheat compared to the ancient wheats einkorn, emmer and spelt [24], even if einkorn has the smallest kernel. The levels of fructans are higher in einkorn and common wheat than in durum wheat and emmer with spelt in between all of them (see 1.3.2) [25].

Further examples for bioactive components are phytochemicals such as phenolic acids, alkylresorcinols, tocols, carotenoids and folates, which can have positive effects on health [2]. However, the differences in the contents of these components are very small or even not present in the various species. There are no differences in the contents of phenolic acids including ferulic acid [2, 26] and tocols including α-tocopherol (vitamin E) [27, 28]. In contrast, the content of alkylresorcinols is higher in durum wheat, emmer and einkorn [29, 30]. This difference is incidentally a suitable biomarker for the distinction of different ploidy degrees (hexaploid vs. tetraploid vs. diploid) [30]. Vitamin B9 (folates) was the only vitamin determined in modern and ancient wheats. First studies indicate that ancient wheats have slightly higher contents of folates [31]. The high contents of carotenoids and especially the major carotenoid lutein leads to a deeper yellow colour of durum wheat, emmer and einkorn compared to common wheat and spelt. Carotenoids are more abundant in durum wheat, emmer and einkorn

(reviewed by [2]). Lutein may have health benefits in improving eye health and visual function. This could be true in particular for einkorn, which has high contents of lutein [32, 33].

1.1.4 Baking quality of ancient wheats

The term ‘baking quality’ includes various properties of flour according to the application and demands specific to the end product. Important properties are on the one hand high bread volumes and high water absorptions but on the other hand also an easy handling through the whole process such as high dough stability and low dough stickiness. In the following, the main focus is on good baking properties with respect to yeast fermented breads.

The interaction of gluten proteins (see 1.2) and starch is the main factor for good baking quality on the molecular level. During mixing and kneading, the gluten proteins bind water. The proteins are denatured during the baking process by heat and the starch absorbs the released water, which leads to gelatinisation of the starch. The result is bread with a crispy crust and a fluffy crumb. Intense thermal or mechanical treatment during grinding influences the interaction of gluten proteins with starch, thus water absorption and bread volume are decreased [34]. Therefore, this negative treatment should be avoided in the mill or during storage. The baking quality is not only influenced by the gluten proteins and the starch. Artisanal baking skills can have an enormous effect on the resulting bread, which is especially true for the ancient wheats. Examples for various parameters are the usage of baking aids, the optimal kneading time and intensity, the time for dough rest and fermentation, and the baking temperature.

For the most important product made of wheat, the bread, ungerminated wheat kernels are milled to flour. According to the HEALTHGRAIN definition the ‘wholegrain’ (*AE*: whole-wheat flour; *BE*: wholemeal flour; German: *Vollkornmehl*) is defined as “the intact, ground, cracked or flaked kernel after the removal of inedible parts such as the hull and husk. The principal anatomical components – the starchy endosperm, germ and bran – are present in the same relative proportions as they exist in the intact kernel” [35]. In contrast, refined (white) flours are produced by separating the bran and the germ from the endosperm by sieving or sifting. Compared to wholemeal flour, white flour has lower contents of protein (80 % of the amount in wholemeal flour), vitamins (e.g. 30 – 70 % vitamin B_1 and B_2), fibres (35 %) and ash (25 %) and thus has a lower nutritional value [21, 36]. There are different types of flour commercially available, which are specific for each individual country. In Germany, the DIN standard 10355 [37] is used to specify the types of flour according to their ash content and therefore the amount of bran, which is present in the flour. Wholemeal flour is not considered in this classification and is sold as ‘wholemeal flour’ without statement of the type. For common wheat, five types of flour are available: Type 405 (for e.g. fine bakery), 550 (for e.g. white breads or toasts), 812, 1050 (both for breads) and 1600 (for dark breads). For spelt, the three types 630, 812 and

1050 are common. Flours made of ancient wheats are mostly sold as wholemeal flour, but there are white flours made of emmer kernels available with types of 630, 812 and 1300, as well.

Wholemeal flour has a higher water absorption than white flour [38]. One reason for this fact is the high water absorption capacity of bran that may be several times its own weight [39]. Further, wholemeal flour has a higher protein content (6 – 11 % more) than white flour. The higher water absorption and protein content of wholemeal flour might lead to higher bread volumes but this is not the case. Bread made of wholemeal flour has a lower specific bread volume (3.0 mL/g) than white flour (4.5 mL/g) [38]. Gluten proteins, which have a positive effect on the baking performance (see 1.2) are enriched in the endosperm and metabolic enzymes, which play a minor role for the baking quality in the bran. Therefore, white flour contains more gluten proteins, which positively influence the baking quality and lead to higher bread volumes. Second, the starch and proteins form a strong matrix with high gas-holding capacity during mixing and kneading. The bran particles cause discontinuities and inhomogeneities in this matrix that decrease the gas-holding capacity and consequently the loaf volume [38].

The baking quality of flours can be analysed with baking tests (see 1.4.1). The baking quality of common wheat flours has been already studied extensively by baking tests and measurement of the resulting bread volume. Average bread volumes related to the flour weight are 5.0 mL/g of flour with a range of 3.4 – 7.8 mL/g for common wheat [40]. Einkorn wholemeal flours yield much lower bread volumes with a maximum of 3.0 mL/g [41]. Little is known about the exact bread volume of spelt and emmer flours. One study investigated one cultivar of spelt, which resulted in a bread volume of 3.6 mL/g that lies in between einkorn cultivars and weak common wheat cultivars [41]. It is stated that very good emmer cultivars can reach the bread volume of spelt cultivars [18]. Due to the lack of extensive studies on the baking quality of ancient wheats, more data is required.

1.2 Wheat proteins

The total or crude protein content of wheat is affected by the genotype (wheat species and cultivar) and by the growing conditions, including soil properties, climate and nitrogen fertilisation [42]. The average crude protein content of common wheat is typically 11 – 15 %, but can also vary in a larger range between 6 % and 20 %. In general, the protein content of spelt (9 – 15 %) durum wheat (12 – 16 %), emmer (12 – 15 %) and einkorn (11 – 15 %) is higher compared to common wheat [19]. The high protein content is the most important reason why especially durum wheat is mainly used for making pasta. Depending on the cultivar, spelt has the highest protein content with up to 18 % compared to the other wheat species [43].

1.2.1 Osborne fractions

Cereal proteins are classified into four fractions (albumins, globulins, prolamins and glutelins) according to their solubility as first described by T.B. Osborne in 1907 [44]. Albumins are soluble in water and globulins in diluted salt solution. In case of wheat including spelt, emmer and einkorn the prolamins and glutelins are called gliadins (GLIA) and glutenins (GLUT), respectively. The gliadins are soluble in aqueous alcohols (e.g. 60 % ethanol) and the glutenins are insoluble in the above named solutions. In the modified Osborne fractionation [45] (see 1.4.3) the albumins and globulins (ALGL) are extracted in the first step with phosphate buffered saline (Figure 3). In the second step, the residue is extracted with aqueous ethanol (60 %) to obtain the gliadins and finally, the glutenins are extracted with a mixture containing 1-propanol (50 %), reducing agents (1 % dithiothreitol, DTT), and disaggregating compounds (urea). Insoluble structural proteins remain in the residue. In the modified Osborne fractionation buffered salt solution is used in the first extraction step to avoid partial dissolution of some gliadin types. In particular, ω-gliadins are partially soluble in water.

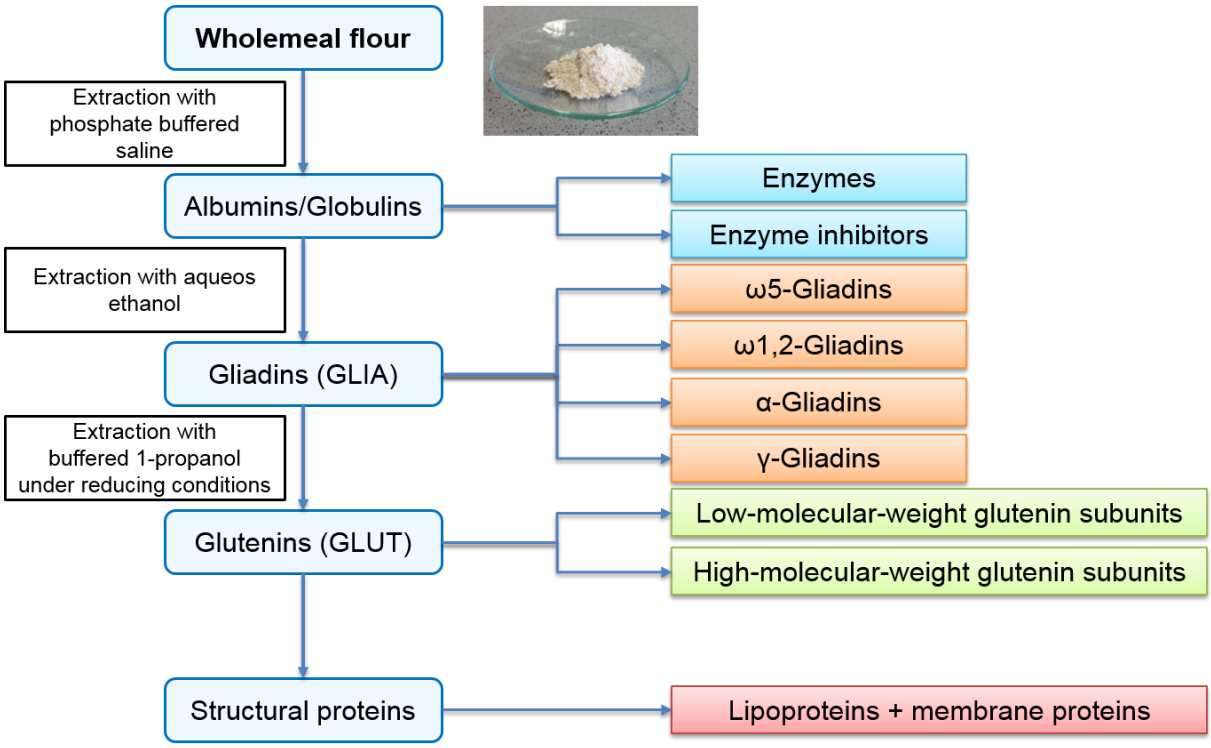

Figure 3: Procedure of the modified Osborne fractionation and overview of proteins belonging to the respective fraction [45]. Wholemeal flour is extracted stepwise with phosphate buffered saline, then with aqueos ethanol and last with buffered 1-propanol under reducing conditions. Structural proteins remain in the residue. In the albumin/globulin fraction enzymes and enzyme inhibitors are predominant. The gliadin fraction (GLIA) is divided in ω5-, ω1,2-, α- and γ-gliadins and the glutenin fraction (GLUT) in low- and high-molecular-weight glutenin subunits.

The predominant proteins in the ALGL fraction are metabolic proteins, mainly enzymes and enzyme inhibitors. These proteins are either located in the germ and the outer layer or are present throughout the endosperm. The proteins belonging to ALGL have a nutritionally better amino acid composition than the other cereal proteins such as GLIA and GLUT [46, 47]. For example, the content of the essential amino acid lysine is higher in ALGL compared to that in the other fractions [47]. The most important enzymes are those, which hydrolyse carbohydrates and proteins and provide the germ with nutrients and energy during germination. The carbohydrate-degrading enzymes α- and β-amylases hydrolyse 1,4-glucosidic bonds of amylose and amylopectin of the starch granules. The α-amylases produce a mixture of dextrins and smaller amounts of oligosaccharides and maltose, whereas β-amylase is more specific and produces maltose and the so-called ß-limit dextrins. Debranching enzyme, and α-glucosidases hydrolyse the 1,6-glucosidic bonds of amylopectin and support the amylases during starch degradation. The amounts of other carbohydrate-degrading enzymes such as β-glucosidases, cellulases, and arabinoxylanases are very low compared to the amount of amylases. Proteins in the wheat kernel are degraded by proteolytic enzymes called proteinases, proteases or peptidases. These enzymes hydrolyse the peptide bond

between amino acid residues and produce peptides and free amino acids. Their activity is at their maximum during the germination process when they provide free amino acids for the germ. Other enzymes are lipases, which hydrolyse ester bonds of triacylglycerols to yield mono- and diacylglycerols and free fatty acids. Phytase hydrolyses phytic acid to inositol and free phosphoric acid and thereby reduces the strong complexation of cations such as zinc, calcium and magnesium. The last group of enzymes includes the oxidoreductases lipoxygenase, polyphenoloxidase, ascorbic acid oxidase, and glutathione dehydrogenase. The wheat enzyme inhibitors mainly inhibit hydrolases and reduce the degradation of polysaccharides and proteins. The group of α-amylase/trypsin-inhibitors (ATIs) is the most important member of enzyme inhibitors (see 1.2.3).

The GLIA and GLUT fractions are divided into protein types and subunits. Because the characterisation of wheat proteins lasted many years, the nomenclature of the proteins belonging to GLIA and GLUT is confusing and inconsistent. According to their electrophoretic mobility the gliadins are divided into ω5-, ω1,2-, α- and γ-gliadins. On the other hand, for the GLUT fractions the terms of high-molecular-weight (HMW-GS) and low-molecular-weight glutenin subunits (LMW-GS) are based on the molecular weight of the proteins. In contrast to this classification, the nomenclature based on molecular weights is accepted. In this nomenclature the storage proteins (GLIA and GLUT) are divided in three groups: 1) HMW group; 2) medium-molecular-weight (MMW) group and 3) LMW group [48]. In the HMW group, the HMW-GS are included with molecular weights of 67,000 Da up to 88,000 Da (Table 2). The MMW group represents proteins belonging to the ω1,2- and ω5-gliadins with molecular weights between 40,000 and 55,000 Da. In the LMW group, the LMW-GS, α- and γ-gliadins are summarised with molecular masses between 30,000 and 42,000 Da. A third alternative classification system based on primary structure (amino acid sequence) uses the term ‘prolamins’ for all gluten proteins even the glutenins. The prolamins are then subdivided into sulphur- (S) rich (α-, γ-gliadins and LMW-GS), S-poor (ω5- and ω1,2-gliadins) and HMW-prolamins (HMW-GS) [49].

Table 2: Classification and molecular weights (based on mass spectrometry analysis [50, 51]) of wheat storage proteins according to their molecular weight (group); in the groups (HMW, high-molecular-weight, MMW, medium-molecular-weight, and LMW, low-molecular-weight) different protein types and subunits of the gliadin and glutenin fraction are present.

Group	Fraction	Types/subunits	S-rich or S-poor	Molecular weight [Da]
HMW	Glutenins	HMW-GS	-	67,000 – 88,000
MMW	Gliadins	ω1,2-Gliadins	S-poor	39,000 – 42,000
		ω5-Gliadins	S-poor	47,000 – 55,000
LMW	Glutenins	LMW-GS	S-rich	30,000 – 42,000
	Gliadins	γ-Gliadins	S-rich	30,000 – 40,000
		α-Gliadins	S-rich	30,000 – 34,000

The gluten proteins contain all 20 proteinogenic amino acids, but their composition is unbalanced and in general, the biological value of wheat protein is rather poor. The contents of all essential amino acids are rather low compared to those of the amino acids glutamic acid, glutamine and proline (46 %) [52]. The name of the cereal prolamins reflects this dominance of *prol*ine and glut*amin*e. The high amounts of proline and glutamine have important effects on the proteins. The amino acid glutamine contains two nitrogen atoms, which is important for the nitrogen supply during the germination process. The secondary amino group of proline causes turns in the secondary structure of proteins (interruption of α-helix and ß-sheet) and allows a close protein packing in the endosperm. Further, this complex secondary structure leads to prevention of degradation of the storage proteins by external enzymatic attacks. Therefore, the high amounts of proline and glutamine are reasons for the immunoactive properties of gluten proteins (see 1.3). In the human digestive tract, proteins are usually hydrolysed to amino acids and short peptides by gastric, pancreatic, and intestinal enzymes. The efficiency of the proteolytic enzymes trypsin, chymotrypsin and pepsin is noticeably decreased, when proline is present close to the cleavage sites. Thus, long peptides with a length of more than 30 amino acids reach the small intestine, enter into the intestinal tissue and are detected by T-cells [53]. The analysis of one of the most immunodominant peptide (33-mer) revealed the occurrence of this peptide in all investigated common wheat and spelt samples, but not in durum wheat, emmer and einkorn [54].

As mentioned earlier, the gluten proteins are divided in S-rich and S-poor units. Sulphur is present in the amino acids methionine and cysteine. The latter one can form disulphide bonds, which are the most important determinants of gluten structure. These disulphide bonds are either inside one protein (intrachain) or between proteins (interchain) [55]. With some exceptions, the ω5- and ω1,2-gliadins contain no cysteines and occur as monomers. Most of the α- and γ-gliadins have six and eight cysteine residues, respectively, which form three or four homologous intrachain disulphide bonds [55]. The proteins belonging to LMW-GS have

eight cysteines, of which six cysteines form three intrachain disulphide bonds. The remaining two cysteines are involved in interchain bonds mainly with cysteines of LMW-GS, but also with cysteines of HMW-GS and form large polymers called glutenins [56]. The HMW-GS are divided in the x-type with four cysteine residues and in y-type with seven cysteine residues [55]. Similarly to the LMW-GS, the HMW-GS form intra- and interchain disulphide bonds. So-called terminators such as glutathione and cysteine stop the polymerisation [55].

1.2.2 Glutenin macropolymer (GMP)

The LMW-GS and HMW-GS form linked polymers with molecular weights between 600,000 Da and up to 20 million Da by disulphide bonds. This glutenin fraction with the highest molecular weight is called glutenin macropolymer (GMP) or unextractable polymeric protein (UPP) and might be one of the largest protein complexes in nature [57]. The diameter of GMP particles can be beyond 80 µm, but the majority of GMP particles is below 12 µm or even below 5 µm [58]. It has been assumed that there is a positive correlation between the amount of larger GMP particles and higher GMP contents. The amount of GMP has a remarkable effect on the baking performance of common wheat flour and, therefore, the amount of GMP is an indicator for good baking performance [59, 60]. The GMP refers to the protein fraction that is insoluble in diluted sodium dodecyl sulphate (SDS) solution (e.g. 1.5 % SDS) [61]. By centrifugation of the flour suspension in SDS solution, a gel layer is formed on the surface of the starch pellet. After scraping the GMP gel, the gel is washed with ethanol and water and lyophilised. It has been shown that the GMP gel contains a maximum of about 35 % proteins and up to 70 % starch [62]. The composition of the GMP gel is influenced by the genome. The GMP gel of the cultivar “Akteur” had a protein content of 35.0 % and that of the cultivar “Winnetou” only 7.9 %. Other components of the isolated gel are, among others, SDS (4.5 – 15.5 %) and water (1.8 – 7.7 %). For this reason, the gravimetrical analysis of the GMP gel is not at all suitable and as alternative, the protein part of GMP is quantitated by high-performance liquid chromatography (HPLC). For this, the GMP gel has to be dissolved either by sonication [63] or by extraction with 50 % 1-propanol in phosphate buffer under reducing conditions (e.g. DTT) [59].

1.2.3 α-Amylase/trypsin-inhibitors (ATIs)

ATIs are proteins of the ALGL fraction and are soluble in aqueous salt solutions in the Osborne fractionation. ATIs are a group of low molecular weight proteins with about 12,000 to 16,000 Da and inhibit amylases and/or proteases from different sources. The first report of ATIs in wheat was published by Silano et al. in 1973 [64]. The terms α-amylase/trypsin-inhibitor (ATI), α-amylase-inhibitor (AI) and trypsin-inhibitor (TI) are not clearly defined in the literature and are often used in the same context [5, 65, 66].

ATIs are inhibitors of α-amylase and/or trypsin from insects, mites and mammals [65, 67, 68], whereas the reactive site of the bifunctional inhibitors was identified by Cuccioloni et al. [67]. In contrast, the wheat ATIs do not inhibit the cereal enzymes [69]. There are three groups of ATIs: The first two ones show mainly inhibitory activity against α-amylase and the third one includes bifunctional inhibitors of α-amylase and trypsin [66, 70]. The first group, called wheat monomeric amylase inhibitors (WMAI), includes the monomeric ATI 0.28, which is encoded on the short arm of the group 6 chromosomes on the B and D genome (6B+6D) [66]. The second group referred to as wheat dimeric amylase inhibitors (WDAI) includes the homodimeric ATIs 0.19 and 0.53. These proteins are encoded on the short arms of the group 3 chromosomes on the B and D genome (3B+3D) [66, 71]. The third and last group is called wheat tetrameric amylase inhibitors (WTAI) and includes the heterotetrameric ATIs CM1, CM2, CM3, CM16 and CM17. Their short form of CM indicates the proteins' solubility in a mixture (7:1, v/v) of chloroform and methanol [70]. Genome mapping of immunoresponsive proteins elucidated the genomes of CM1 (7B), CM2 (7A+7D), CM3 (4B+4D), CM16 (2A+4B+4D) and CMX (4A+4B+4D) [71]. ATI CM17 was not detected in this study, but this is encoded similarly as CM16 by the genes 4B and 4D [66]. Two other protease inhibiting proteins included in the group of ATIs are called wheat chymotrypsin inhibitor (WCI) and wheat amylase subtilisin inhibitor (WASI). WCI is encoded by 1B+1D+4A and WASI by 2A+2D [71]. One last protease inhibiting protein is either called Bowman-Birk type inhibitor or wheat trypsin inhibitor with inhibitory activity against trypsin but not against amylase [72]. In total, there are 13 ATIs known in wheat, which have evidence at protein level and are not only predicted based on genome sequencing (Table 3). Their amino acid sequences exist in the UniProtKB database [73]. With the exception of CM17, all amino acid sequences are reviewed and have evidence on protein level.

Table 3: Overview of different α-amylase/trypsin inhibitors (ATIs) in wheat. The abbreviations are used as short forms due to the length of the UniProtKB names. The UniProtKB accession number is unique for every type and is used as reliable identification in the UniProtKB database [73]. Except CM17 all proteins are reviewed proteins and have evidence at protein level. The number of amino acids and the molecular weights (calculated from the amino acid sequence) refer to the proteins without signal peptide. All cysteine residues are present in the chain and not in the signal peptide.

Abbr.	UniProtKB name	UniProtKB accession	Amino acids	Molecular weight [Da]	Cysteine residues
ATI 0.28	Alpha-amylase inhibitor 0.28	P01083	123	13,326	10
ATI 0.19	Alpha-amylase inhibitor 0.19	P01085	124	13,337	10
ATI 0.53	Alpha-amylase inhibitor 0.53	P01084	124	13,185	9
CM1	Alpha-amylase/trypsin inhibitor CM1	P16850	120	13,086	10
CM2	Alpha-amylase/trypsin inhibitor CM2	P16851	120	13,035	10
CM3	Alpha-amylase/trypsin inhibitor CM3	P17314	143	15,832	10
CM16	Alpha-amylase/trypsin inhibitor CM16	P16159	119	13,437	10
CM17	CM 17 protein	Q41540	119	13,431	10
CMX1/3	Trypsin/alpha-amylase inhibitor CMX1/CMX3	Q43723	97	11,408	10
CMX2	Trypsin/alpha-amylase inhibitor CMX2	Q43691	97	11,466	10
WCI	Chymotrypsin inhibitor WCI	P83207	119	12,944	10
WASI	Endogenous alpha-amylase/subtilisin inhibitor	P16347	180	19,633	4
WTI	Bowman-Birk type trypsin inhibitor	P81713	71	7,962	10

ATIs share ten cysteine residues, which form five intramolecular disulphide bonds except WASI (four cysteine residues) and 0.53 (nine cysteine residues) [74-76]. This leads to a compact three-dimensional structure that is supposed to be the reason for a high heat resistance and the inhibitory activity against enzymes of insects and mammals [66, 70, 77]. In contrast to gluten proteins, which contain high amounts of glutamine and proline, ATIs have a more balanced amino acid composition. Therefore, the ATIs are supposed to not only play a protective role against insects as inhibitors, but to function as storage proteins. As a result,

ATIs compensate in part the absence of essential amino acids in the gluten proteins and serve as important storage proteins for seedling growth [66, 78].

In general, the amino acid sequences of most of the 13 ATIs have a very high homology in their sequence (Figure 4). The ATIs WASI and WTI have a very low similarity in their amino acid sequence to the other ATIs (0.4 %) and are very dissimilar between each other (8.7 %). The ATIs 0.19 and 0.53 (94.4 %), CMX1/3 and CMX2 (99.2 %, one amino acid exchanged), CM1 and CM2 (90.3 %), and CM16 and CM17 (87.4 %) have large areas with homologous amino acid sequences. The ATIs 0.19, 0.28 and 0.53 share almost half of the amino acid sequence (43.6 %) and the CM-types CM1, CM2, CM3, CM16 and CM17 more than one quarter (27.6 %). The ATI type WCI has a low similarity of 16.5 % to the CM-types.

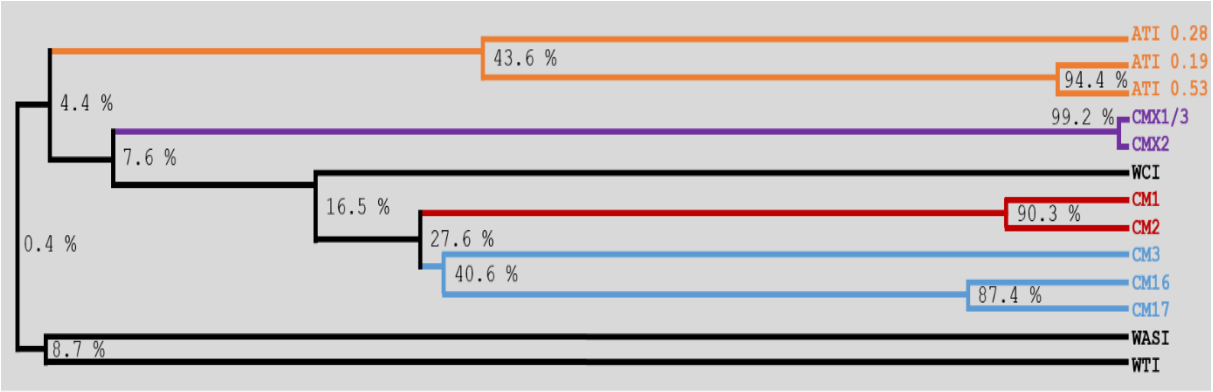

Figure 4: Alignment of 13 α-amylase/trypsin-inhibitors (ATIs) using Clustal Omega visualised as a tree [79]. Same colours indicate high homology (> 40 %) between different ATIs and the percentage numbers show the grade of homology between them. The homology between the ATIs 0.19 and 0.53 (94.4 %), CMX1/3 and CMX2 (99.2 %, one amino acid changed), CM1 and CM2 (90.3 %) and CM16 and CM17 (87.4 %) is very high, as visualized by the length of the connecting lines. The ATIs wheat amylase subtilisin inhibitor (WASI) and wheat trypsin inhibitor (WTI) have almost no homology to the other ATIs.

The high homology is caused on the one hand by the location of the cysteine residues and on the other hand by the similarity of the amino acid sequences (Figure 5). One cysteine residue (position 73 in ATI 0.28) is present in all ATIs and can be aligned. Two other cysteine residues (position 37 and 114 in ATI 0.28) can be aligned for twelve of the 13 ATIs. Because of the very high amino acid sequence homology, it is a challenge to develop analytical methods to differentiate between the ATIs. Further, the ATIs have similar molecular weights, polarities and isoelectric points, making it difficult to separate individual ATIs. For the analysis of individual activities of each ATI type it is necessary to evaluate the inhibitory activity separately and for this, very highly purified and enriched ATIs are needed. For inhibitory tests or clinical cell assays, samples enriched in one ATI type are used but there are still other ATIs present [80]. One alternative are recombinant proteins, which have to be produced by heterologous expression in microorganisms.

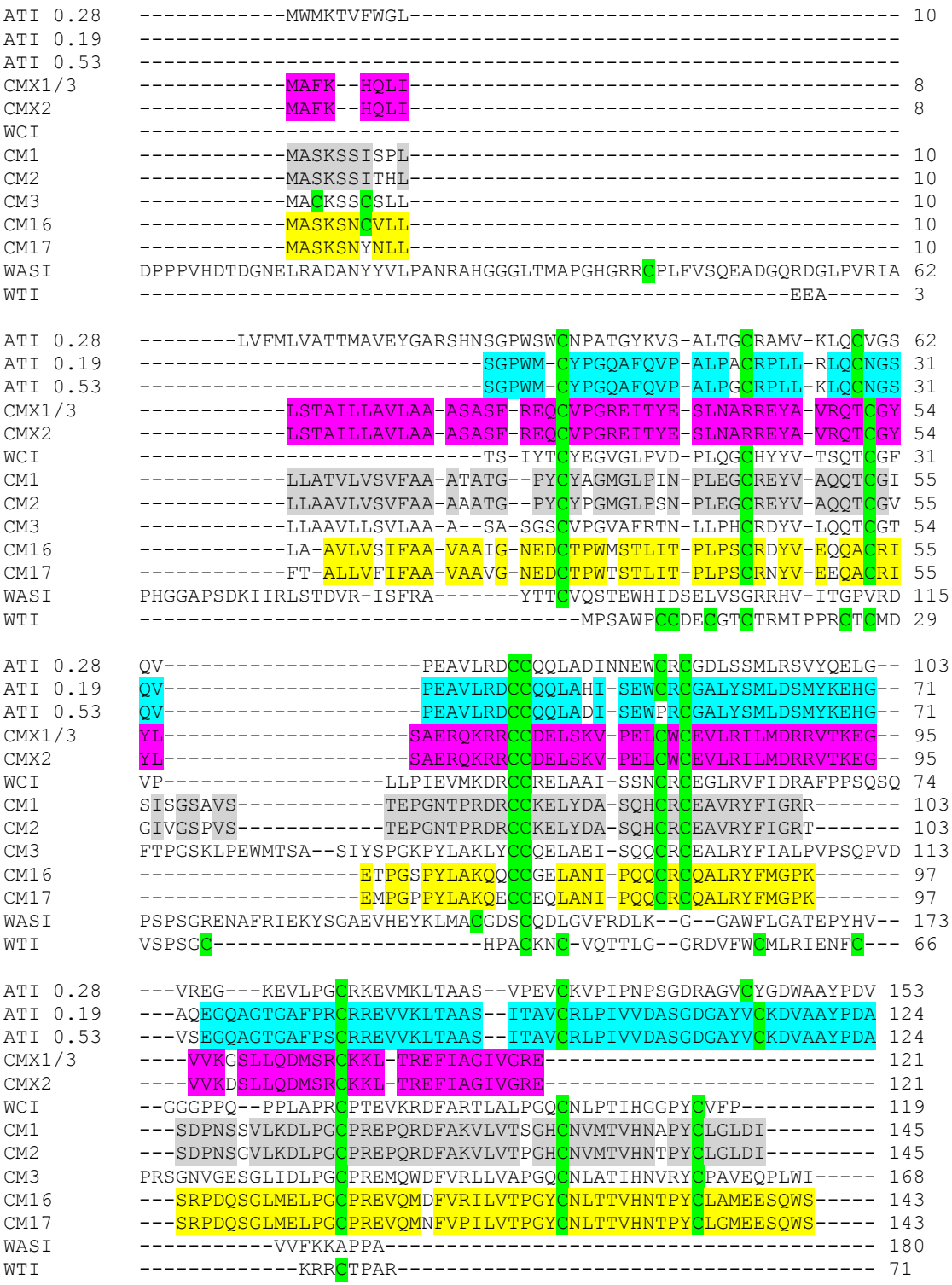

Figure 5: Alignment of the amino acid sequences of 13 α-amylase/trypsin inhibitors (ATIs) (abbreviations see Table 3) using Clustal Omega [79]. The cysteine residues are coloured in green. With the exception of WASI (four) and ATI 0.53 (nine) all ATI types have ten cysteine residues, which form five intramolecular disulphide bonds. Homologous parts of the amino acid sequences are coloured in turquoise for ATI 0.19 and ATI 0.53, in violet for CMX1/3 and CMX2, in gray for CM1 and CM2 and in yellow for CM16 and CM17.

ATIs inhibit amylases and trypsin of various sources. It was shown that water extracts of common wheat flour decrease the amylase activity of adult insect pests e.g. yellow mealworm or German cockroach, but not that of longhorned borer or Mediterranean flour moth [81]. This study was based on *in vitro* experiments in adult insect populations. An *in vivo* experiment with larvae of red flour beetle (wheat storage pests) and cowpea needle (legume storage pests) showed that ATIs have a greater effect on the growth of the cowpea needle larvae than on the red flour beetle ones [82]. For this reason, it was suggested that ATIs have indeed a protective function, but some insect pests developed mechanisms to detoxify ATIs. The inhibitory activity of ATIs on mammalian enzymes is controversial. Water extracts of flour did not decrease the amylase activity of monkey or guinea pig amylases, but that of human (saliva and pancreas) and chicken [81]. Further, one not clarified type of ATI (or even a mixture of different ATIs) decreased the amylase activity of human saliva stronger than that of human pancreas. For this, only one hundredth of the isolated inhibitor was needed to decrease the amylase activity of saliva compared to pancreatin [83].

Little is known about the amylase inhibitor activity of ancient wheats compared to that of common wheat. One study compared the inhibitory activity of a small data set with flours of emmer (1), durum wheat (6), spelt (8) and common wheat (16) [84]. Due to the small size of the data set, relationships between modern and ancient wheats are hard to establish, but no significant increase or decrease of the inhibitory activity against amylase between ancient and modern wheat species was observed. Therefore, more data about the inhibitory activity of ancient wheats against amylase is still required.

Flours of soy and pea seeds have a much higher trypsin inhibitory activity than flours of wheat [85, 86]. One study compared the trypsin inhibitory activity of soy flour with wheat gluten and this revealed a ten times higher inhibitory activity for soy flour [85]. Even if wheat gluten does not have the same composition as wheat flour, it is known that wheat gluten contains comparable amounts (about 3 %) of ATIs [87]. In comparison to wheat, the soy trypsin inhibitors are not heat stable and can be deactivated by e.g. toasting [85]. Further the two soy trypsin inhibitors ‘Bowman-Birk’ and ‘Kunitz’ have an anticancer effect [88]. In comparison to the well-studied amylase inhibitory activity, little is known about inhibitory activity of wheat extracts, especially about that of ancient wheats. A study with two common wheat cultivars cultivated in four years revealed that the inhibitory activity is influenced by genetic background (between the cultivars) and environmental conditions (between the growing years) [89].

The absolute quantitation of proteins poses several difficulties. One complex and comprehensive study investigated the amount of all wheat proteins by 2D-gel electrophoresis and comparison of spot volume [90]. The proteins of each spot were identified by untargeted

mass spectrometry (MS) analysis. This procedure requires a large and an ideally complete protein database, which does not exist for many plants. Further, the comparison of spot volumes on electrophoretic gels is not as accurate as other analyses based for example on MS, because the intensity of the spot volume also depends on the ability of the colour reagent to bind to the proteins. Compared to all wheat proteins the volume of the spots containing ATIs was 4.1 % [90]. Considering an approximate protein content of 10 % in common wheat, the ATIs account for about 4 mg/g. In the investigated cultivar (Butte 86), the ATI type 0.19 was most abundant compared to the other types with more than 15 % (Figure 6). The least abundant ATI was CMX2 with about 1 %. It has to be stated that this described distribution was evaluated for one cultivar and protein quantities are known to be affected by both genetics and environmental conditions. One study investigated these effects on the amount of one ATI type (CM3) in durum wheat cultivars using MS and showed that cultivation conditions have a higher influence on CM3 contents than the genetic background [91].

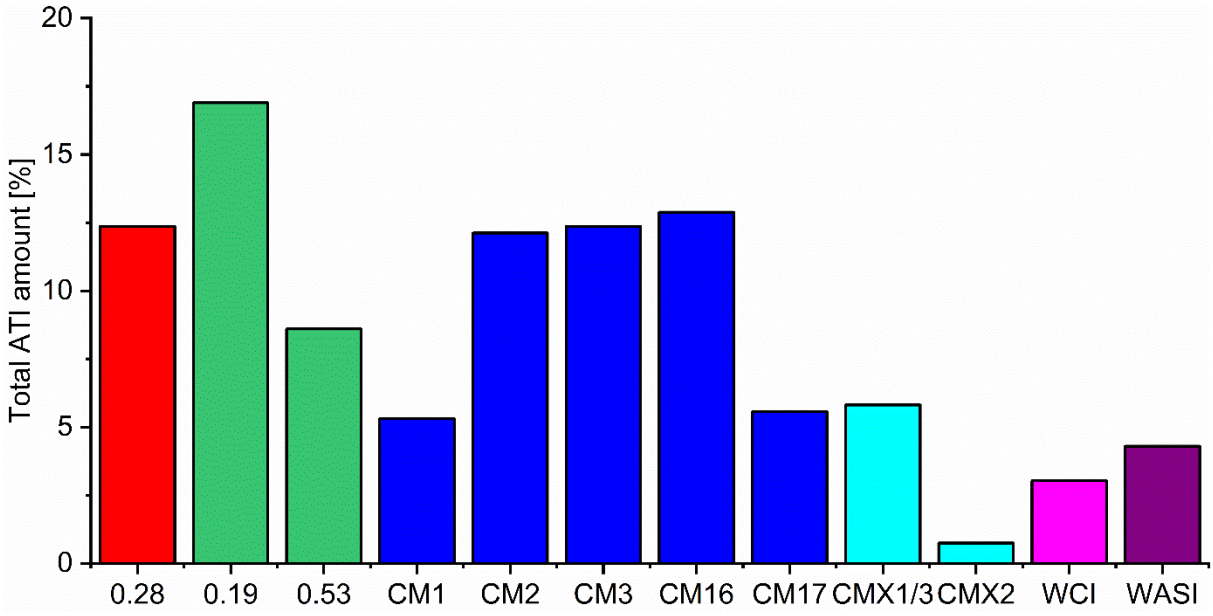

Figure 6: Distribution [%] of α-amylase/trypsin inhibitors (ATIs) in the common wheat cultivar Butte 86 (sum of all shown ATIs = 100 %). The figure is modified from [66]. These results are based on 2D-gel electrophoresis and comparison of spot volumes [66, 90]. The proteins in the spots were identified by untargeted mass spectrometry analysis. The ATI 0.19 was the most abundant in Butte 86 with more than 15 %. The ATIs 0.28, CM2, CM3 and CM16 were equally distributed with more than 10 %. CMX2 was the least abundant with about 1 %. The remaining ATIs 0.53, CM1, CM17, CMX1/3, WCI and WASI amounted to 3 – 7 %.

The presence or absence of ATIs in ancient wheats is partially confirmed. In emmer, ATIs were detected, which have similar amino acid sequences as the ATIs CM2, CM3 and CM16 of common wheat [92]. In einkorn, the genes encoding for ATIs were found, but they are not expressed. It was postulated that the genes might be silenced or expressed at very low levels

so that no proteins were detectable. One reason for this could be gene mutations hindering the translation into proteins [5].

ATIs are potential triggers of intestinal and extraintestinal immune activation. For example, rice ATIs are the major allergens in rice allergy [93, 94]. Incidentally, hypoallergenic rice was already developed by downregulating of ATIs, which might be better tolerable for rice allergy patients [95, 96]. ATIs of wheat, barley and rye are involved in various wheat-related hypersensitivities (see 1.3.1) [97]. One study showed that the wheat CM3 is the major allergen for atopic dermatitis [98]. Recombinant proteins of the wheat ATIs 0.19, 0.28, CM1, CM2, and CM3 triggered IgE reactions in a part of baker's asthma patients (37.5 %) [99]. No control patients reacted to any of these proteins. Further, the ATI CM16 had the highest allergenic activity in skin tests of patients, in whom wheat flour allergic sensitisation was detected by prick tests [100]. The most recent studies showed that wheat ATIs are adjuvants of various existing inflammations and are potential triggers of noncoeliac gluten sensitivity (NCGS) [80, 101].

Due to their resistance to intestinal proteolysis (especially tryptic hydrolysis), ATIs are able to pass the upper part of the intestine as intact proteins. Thus, they are sensed by mucosal innate immune cells and engage the toll-like receptor 4 (TLR4)-MD2-CD14 complex on monocytes, macrophages and dendritic cells (Figure 7). This leads to the release of proinflammatory chemokines and cytokines like interleukin (IL-) 8, tumor necrosis factor (TNF-) α and chemokine (C-C motif) ligand 2 (CCL2) and activation of innate immunity [80, 101, 102]. This trigger leads to movement of the activated antigen-presenting cells to extraintestinal sites, such as mesenteric lymph nodes. In the lymph nodes or lymphatic organs, T-cell mediated diseases and preexisting inflammations, such as inflammatory bowel disease, systemic lupus erythematodes, autoimmune encephalitis, or inhalative allergy, are intensified [102].

Further, *ex vivo* studies on intestinal biopsies demonstrated that added ATIs are adjuvants of the gluten specific T-cell response [101]. Therefore, it was suggested that ATIs are potential triggers of innate immunity and of symptoms, which are present in NCGS (see 1.3.2). For the TLR4-activating activity the secondary structure of ATIs is the predominant factor. After reduction of disulphide bonds, the activity is lost [101]. The ATIs 0.19 and CM3 were identified as the most active ATIs. The ATIs 0.19 and CM3 were both isolated from common wheat and expressed as recombinant proteins in eukaryotic cells and tested in cell assays for their bioactivity [101, 102].

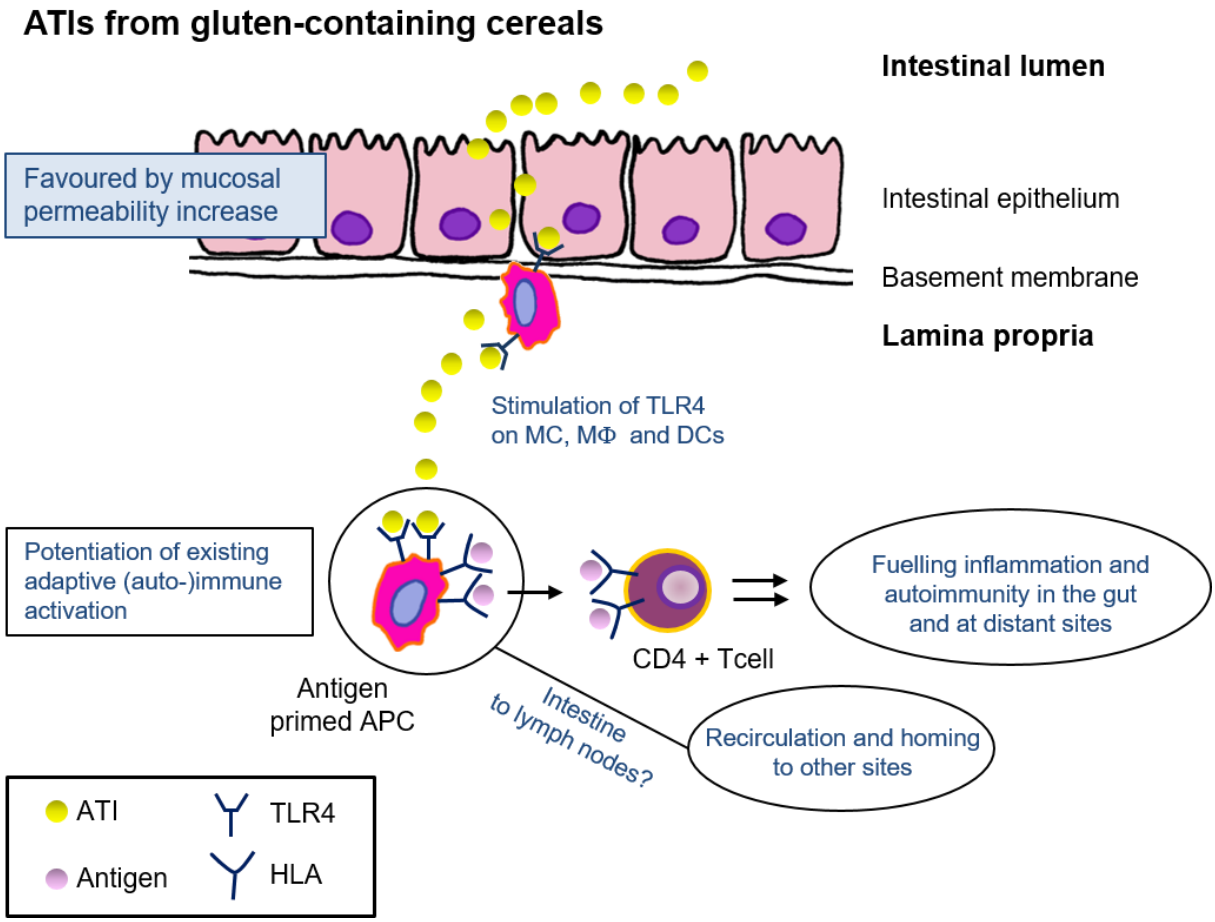

Figure 7: α-Amylase/trypsin inhibitors (ATIs) of gluten-containing cereals (e.g common wheat, spelt, rye and barley) as innate triggers of intestinal and extraintestinal immune activation. ATIs pass the intestinal epithelium as intact proteins and stimulate the toll-like receptor 4 (TLR4) on monocytes (MC), macrophages (MΦ) and dendritic cells (DC). This leads to potentiation of existing adaptive immune activation and an increase of antigen-presenting cells (APCs). These serve as adjuvants for an ongoing adaptive T-cell response and intensify chronic and especially autoimmune diseases. It is supposed that the APCs occur at extraintestinal sites, e.g. mesenteric lymph nodes. HLA, human lymphocyte antigen. Modified from [102].

Different wheat species were already tested for their immune activating potential in cell assays. It was shown that different cultivars of common wheat differ in the bioactivity (50 – 150 %) compared to a commercially available common wheat flour (set as reference of 100 %). ATI extracts obtained from flours of the ancient wheats spelt (70 %), emmer and einkorn (30 – 40 %) had a lower bioactivity in the cell assays than common wheat. The investigated sample set was very small in this study and further data with well-defined samples grown on different cultivation sites are required. Heat processing to bread, pizza and cookies reduced the bioactivity to 45 – 75 % compared to the reference flour. Gluten-free materials had a lower bioactivity of 20 % (soy, buckwheat, millet and teff) or 10 % (lentil, quinoa and oat) relative to the common wheat reference.

1.3 Wheat-related hypersensitivities

1.3.1 Overview

The ingestion of or contact with wheat can trigger diseases, allergies and immunological reactions in the human body. According to the 'Oslo definitions for coeliac disease (CD) and related terms', the term 'gluten-related disorders' includes conditions, which are related to gluten [103]. These conditions are present in CD, dermatitis herpetiformis, gluten ataxia and NCGS. Depending on the pathomechanism, these hypersensitivities are divided into autoimmunogenic immune responses (immunoglobulin A (IgA-) and IgG-mediated), innate immune responses and IgE-mediated allergies (Figure 8) [103, 104]. In the following, the focus is on NCGS.

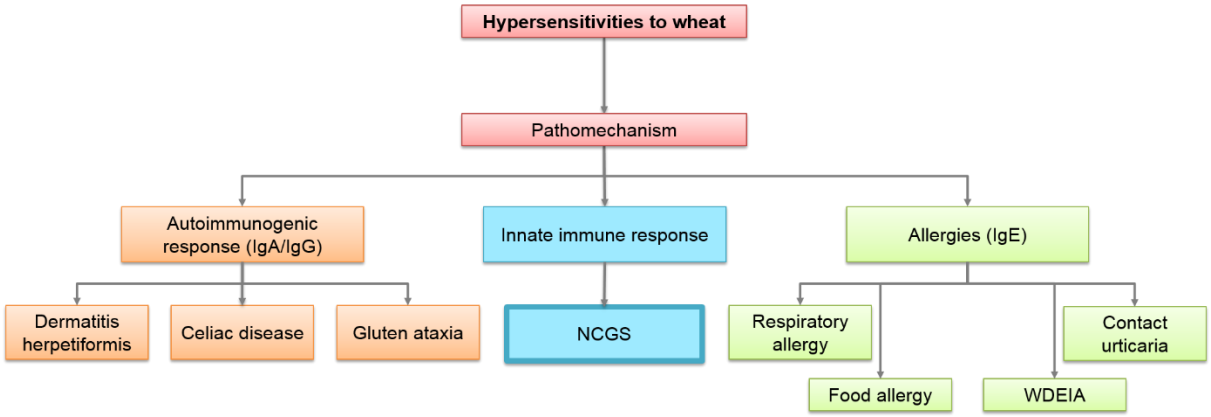

Figure 8: Classification of wheat-related hypersensitivities according to the pathomechnism. Ig, immunoglobulin; NCGS, noncoeliac gluten sensitivity; WDEIA, wheat-dependent exercise-induced anaphylaxis. Adapted and modified from [53, 104]

1.3.2 Nonceliac gluten sensitivity (NCGS)

In the 'Oslo definition', NCGS is characterised as a disorder with "a variety of immunological, morphological and symptomatic manifestations that are precipitated by the ingestion of gluten in individuals in whom CD has been excluded" [103]. For NCGS, different terms in the literature are common: noncoeliac gluten sensitivity, noncoeliac wheat sensitivity, nonallergy wheat sensitivity or wheat sensitivity [80, 105]. In the following, the umbrella term 'NCGS' is used.

NCGS was first described in the literature in 1980 [106]. Even if research was done on this issue and a large number of studies and reviews were published, many details on NCGS are still unknown. Depending on various studies, the prevalence of NCGS varies in a wide range between 0.55 % and 6 % of the population [104, 107-112]. One study in the United Kingdom reported a very high prevalence of 13 %, but the interviewed participants had made a self-diagnosis and only 1 % of the participants were medically investigated [113]. Nevertheless the prevalence of NCGS seems to be higher than that of CD (1 %) and wheat allergy (0.5 – 2%) [114, 115]. Women are affected more often than men (2.5:1 to 4:1) and young people more

often than older ones [116, 117]. Precise numbers of the prevalence are hard to obtain due to the significant overlap of NCGS and irritable bowel syndrome (IBS).

The symptoms of NCGS are similar to those of CD and IBS and occur several hours to a few days after consumption of wheat or gluten-containing products [53]. The symptoms are both intra-intestinal (e.g., bloating, abdominal pain, diarrhea and epigastric pain) and extra-intestinal (e.g., lack of wellbeing, tiredness, headache, anxiety, foggy mind and muscle pain) [118].

The pathomechanism of NCGS is still poorly understood. Potential triggers of NCGS are peptides of wheat gliadin, other gluten peptides and proteins, ATIs and fermentable oligo-, di-, and monosaccharides and polyols (FODMAPs). Peptides of wheat gliadins cause CD, but it was shown that these peptides have no inflammatory activity to the intestine of NCGS patients [119]. A double-blind randomised placebo-controlled study revealed that symptoms disappeared in patients with excluded CD during a gluten-free diet and reappeared on a gluten-containing diet [120]. The gluten-containing diet was verified to be FODMAPs-free, but commercially available gluten contains ATIs [87]. The term FODMAPs includes short chain oligosaccharides of fructose (fructans) and galactooligosaccharides (stachyose, raffinose), disaccharides (lactose), monosaccharides (fructose) and sugar alcohols (polyols). They are resistant to digestion and absorption in the small intestine and thus, are fermented in the large intestine by gut microbiota. This usually contributes to intestinal integrity and gut health, but the fermentation and formation of gas can cause intestinal symptoms typical of IBS. It was proven that FODMAPs do not cause extra-intestinal symptoms such as those frequently reported in NCGS [109]. Nevertheless, FODMAPs might be involved in symptoms typical of NCGS, but they do not trigger NCGS [121]. Small intestinal biopsies of NCGS patients showed no differences in markers of the adaptive immune response. In contrast, they had increased levels of TLR2 and TLR4, increased numbers of α- and ß-intraepithelial lymphocytes and reduced numbers of regulatory T-cells. These are all markers of the innate immune response that ATIs were found to trigger (see 1.2.3) [122].

At the moment there is a lack of specific biomarkers and tests for the diagnosis of NCGS. In most cases, patients with symptoms typical of NCGS make a self-diagnosis without clinical confirmation. The medical diagnosis relies on the exclusion of CD, wheat allergies, other food intolerances and IBS. To exclude CD and wheat allergy, NCGS patients must have a negative CD serology, normal duodenal histology (normal small intestinal mucosa) and negative results in IgE-based assays (e.g. prick test). In contrast to CD and wheat allergy, the IgA/IgG anti-tissue transglutaminase, anti-deamidated gliadin-peptides antibodies and IgE antibodies of NCGS patients are negative [117]. Further no relation appears to exist between the antibodies and the expression of HLA-DQ2 or -DQ8 genes [109, 123]. The exclusion of wheat allergy is

performed with provocation tests in most cases, which can be potentially dangerous for patients [124].

After the exclusion of the above named disorders, a diagnostic algorithm is performed, in which NCGS is confirmed in two steps using the gastrointestinal symptom rating scale [108]. In the first step, the symptoms are rated once a week during a six week gluten-free diet, after having been on a normal gluten-containing diet for at least six weeks. If the symptoms do not improve on the gluten-free diet, NCGS is excluded. If the symptoms improve, in the second step, a double-blind placebo-controlled challenge over three weeks, is applied. In the first week, the diet contains either gluten (recommended amount: 8 g) or a placebo, the second week is strictly gluten-free and in the third week, the diet contains again either a placebo or gluten. A difference of at least 30 % between gluten and placebo containing diet is required to differentiate between a positive and a negative diagnosis of NCGS. This diagnostic procedure is difficult to perform in routine clinical practice and many patients refuse to go on a gluten-containing diet due to quick re-emergence of symptoms and discomfort.

The only way to treat NCGS is similar to CD and is based on adherence to a life-long gluten-free diet. In contrast to CD, a reduction of gluten-containing food to 5 – 10 % of the amount of wheat typically consumed may be sufficient to eliminate the symptoms [80]. Therefore, NCGS patients are advised to follow at least a gluten-reduced diet or a gluten-free diet.

1.4 Methods for the determination of baking quality

For breeders, farmers, millers, (baking) industry and research, it is a necessity to monitor the baking quality and performance of wheat. Flours have different properties, which determine the application for e.g. soft dough, bread, cookies or pasta. One option for the determination of the baking quality of flour is the classical baking test. For this, dough is prepared and baked under standardised conditions and then the bread volume and texture of crumb and crust are analysed [125]. As alternative to these time consuming and work intensive baking tests, indirect quality parameters were established, which can be used to predict the baking quality. These methods are approved as International Association for Cereal Science and Technology (ICC) and American Association of Cereal Chemists International (AACCI) standards. As second alternative, analytical methods based on the characterisation of wheat proteins are prevalent in research.

1.4.1 Baking tests

Usually, baking tests are applied to determine the baking quality of flours. These tests are the most accurate and reliable. The Rapid-Mix-Test (RMT) is the standard method in Germany, but more than 1 kg flour and comprehensive equipment are required for this test [126]. If the amount of flour is limited especially in breeding trials the RMT is not the appropriate method. Thus, microscale baking tests were developed for the application in laboratories. For these tests a small flour quantity of only 10 g is required. There are different procedures to prepare the dough [59, 127, 128]: 1) Micro-Rapid-Mix-Test (MRMT), 2) microbaking test (MBT) and 3) Micro-Opti-Mix-Test (MOMT). The MRMT is the microscale version of the RMT. For the MRMT, 10 g of flour are mixed at high speed for exactly one minute with sodium chloride (2 %), sucrose (1 %), fresh baker's yeast (7 %), and ascorbic acid (20 mg/kg) based on a final water content of the dough of 46.4 %. The correlation (r) of the bread volume obtained by the RMT and by the MRMT was very high (r = 0.92) with the advantage that the MRMT leads to a remarkable decrease of material input [40]. In contrast to the MRMT, the optimal kneading time is used in the MBT and the optimal water amount is added to the flour. Therefore, the MBT is more time-consuming, but more properties of the flour are evaluated. Prior to the baking process, the optimal water absorption and the optimal dough development time are analysed in a micro-farinograph. Flour is mixed with sodium chloride (2 %) and water. A time versus torque curve is recorded during kneading. The maximum of this curve corresponds to the kneading optimum and therefore the dough development time. It has been shown that opposed to the standard method (ICC no. 115/1, 30 °C, 500 Brabender Units, BU) the conditions of 22 °C and 550 BU are more suitable to easily evaluate the kneading optimum [40]. In addition to the dough development time, the dough stability time can be taken from the farinograph curve, as the time when the dough is softened and the torque falls below 550 BU. The MBT

generally yields higher bread volumes compared to the MRMT [59]. The reason for this is that the doughs are mixed to their optimal consistency (normally between 5 and 15 min) in the MBT, leading to the optimal formation of the gluten network. In the MRMT the fixed mixing time of one minute can lead to under- or overmixing and the complete baking potential is not achieved. In summary, the MBT is the more appropriate method for a realistic description of the baking performance [59]. The MBT has later been optimised as the MOMT. A medium correlation (r = 0.68) was observed for the bread volume prepared in the micro-farinograph and in the newly developed spiral mixer of the MOMT and a high correlation (r = 0.84) between the MBT and the MOMT [56]. The newly developed MOMT has the advantages that no cooling of the mixer is needed, the cleaning process is much faster compared to the micro-farinograph and one dough preparation only takes 5 min. In comparison to the MBT, in which three doughs can be prepared per hour, with the new MOMT twelve doughs can be produced per hour thus increasing the effectiveness [56].

1.4.2 Indirect quality parameters

Besides the classical baking tests, other technological and rheological methods are applied to analyse the baking performance and quality of flour. One option is the determination of the wet gluten content with the Glutomatic® instrument and the so-called Gluten Index method (ICC no. 137/1). First, the dough is either prepared with the same procedure as described for the MBT or flour is mixed with water for 20 s [129]. Next, the water-soluble ingredients are washed out and the water-insoluble gluten remains on the sieve. After centrifugation, the wet gluten content is measured by weighing and after drying, the dry gluten content is weighed. The Gluten Index method is suitable for the determination of gluten quality of common wheat and durum wheat. An aliquot of wet gluten is centrifuged on a sieve under standardised conditions. The percentage of gluten that remains on the sieve is referred to as gluten index. Due to the very weak gluten network of the ancient wheats emmer and einkorn, this method is not eligible for those.

An alternative method is the microscale extension test combined with the use of a Texture Analyser. For this test, first, dough (e.g. according to the MBT) is prepared and divided into dough strands. After a defined resting time, the strands are extended by the Texture Analyser until rupture. During extension, the Texture Analyser monitors a force-distance diagram, the so-called Kieffer-curve [40]. The maximum of this curve characterises the maximum resistance to extension (R_{max} in mN) and the point of rupture is called ‘extensibility’. Dough characteristics such as elasticity and resistance to extension are determined by the micro-extension test. Good baking performance and high bread volume are reached when high elasticity and high resistance are balanced. Doughs made of common wheat show this good balance and thus, they have a high gas-holding capacity. In contrast, the ancient wheats form very weak and soft

doughs. Although they have a very high extensibility, their resistance is very weak. This can lead to crumbling doughs, an effect that is especially true for einkorn [18]. Conclusively, the micro-extension test is not suitable for einkorn, because either resistence is too weak to be assessed by the Texture Analyser or the extension distance is not sufficient to extend dough strands of einkorn until they disrupt.

The determination of the sedimentation volume according to Zeleny (ICC no. 116/1) is a method to approximate the baking quality. In this test, a defined amount of flour is suspended in a closed measuring cylinder with lactic acid containing bromophenol blue solution. After shaking and resting for a defined time, the sedimentation volume can be read off. The sedimentation volume describes the swelling and flocculation capacity of the gluten proteins and is called S-Value. High sedimentation volume is associated with a high volume of precipitated gluten proteins and a slow sedimentation. This leads to better baking quality and a high bread volume [130, 131]. It was shown by light microscopy investigations that the GLIA proteins are completely dissolved and only the GLUT proteins are involved in swelling [61, 132]. The SDS sedimentation test is an enhancement of the Zeleny test based on the same principle, but instead of a lactic acid containing solution, a solution containing SDS is used [133]. The ancient wheats einkorn, emmer and spelt generally have a lower S-Value than common wheat due to their weaker gluten network [134].

The GlutoPeak® test (GPT) is a fast and easy rheological method for the determination of the baking quality of flour. The method is an alternative to farinograph experiments (water absorption and dough stability time) and the determination of the wet gluten content in the Glutomatic® or the Gluten Index method. In the GPT, flour is mixed with calcium chloride solution (0.5 mol/L) at constant temperature and constant rotation of the measuring paddle [135]. The mixing process is visualised as a torque-time-curve (Figure 9).

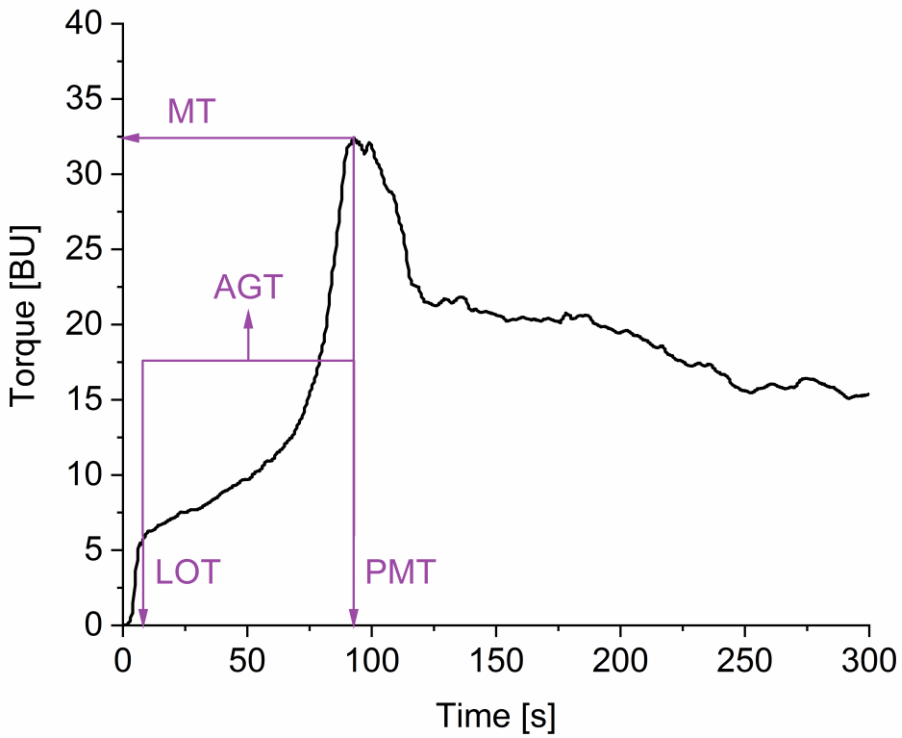

Figure 9: Typical curve obtained from the GlutoPeak test (GPT, own data). Flour and calcium chloride solution are mixed with a measuring rotating paddle. After a specific time (LOT, lift off time), the gluten proteins start to aggregate until a uniform gluten network is built up (PMT, peak maximum time; MT, maximum torque). Due to continued rotation the gluten network is degraded and the curve declines. One important evaluation parameter is the aggregation time (AGT) that is calculated as difference of PMT and LOT. BU, Brabender Units.

After a specific time, which is characteristic for each flour sample, the gluten proteins start to aggregate. This is shown by the rise of the curve. A uniform gluten network is built up, which is displayed by the shape of the peak. Due to the continued rotation of the measuring paddle, the gluten network is degraded again within a short period of time. The analysis time of only five minutes shows that high throughput screening is possible in practice. Important evaluation parameters are the lift off time (LOT), the peak maximum time (PMT) and the maximum torque (MT), which can be directly read off the curve. The aggregation time (AGT) is then calculated as the difference of PMT and LOT. Flours with good baking quality show high peaks with a fast rise of the curve. Flours with a weak gluten network have flat curves with late peaks or in the worst case even no peaks. Common wheat and durum wheat were already intensively characterised by GPT but data on ancient wheats are missing [136-140].

1.4.3 Analytical methods

Applying baking tests, the baking performance and quality is analysed directly. Besides the analysis of indirect parameters (e.g. GPT and wet gluten content), various analytical methods are used to predict the baking quality. One important prediction parameter is the crude protein content, which is analysed either by the Dumas method (ICC no. 167), the Kjeldahl method (ICC no. 105/2) or near-infrared spectroscopy (NIRS). By the Dumas and Kjeldahl methods the total nitrogen content is determined and then, the crude protein content is calculated by a conversion factor of 5.7 for wheat proteins. Thus, total nitrogen is analysed, even if the nitrogen is not part of proteins, but e.g. of free amino acids or phospholipids. Nevertheless, this amount of nitrogen is negligibly small and the conversion factor compensates this issue. The Dumas and Kjeldahl methods show generally comparable results [141]. The preferred method is the Dumas method due to high reproducibility, short analysis time and the small required sample amount [142]. The advantage of NIRS is that the protein, water and ash contents can be analysed from intact kernels in a very short time and a non-destructive way. One disadvantage is that a comprehensive calibration and a large data base are required for reliable results. However, NIRS is used as routine analysis in quality control [19, 143, 144].

Further important methods for the characterisation of flours are the determination of moisture (ICC no. 110/1) and ash contents (ICC no. 104/1). The moisture content of flours is taken into account by reducing or increasing the amount of added water during dough preparation for breadmaking. High contents of ash and, therefore, minerals enhance the nutritional value and influence the baking quality of flour (see 1.1.4).

The baking quality of wheat flour is essentially influenced by two parameters, the gliadin/glutenin ratio (GLIA/GLUT) and the quantity of GMP (see 1.2.1 and 1.2.2). The gliadins and glutenins play different roles during dough preparation. The gliadins have an effect on dough viscosity and extensibility, and the glutenins on dough elasticity and resistance [57, 145-147]. In dough, the gliadins are regarded as the ‘softeners’ or ‘solvents’ of the glutenins, as it is visualised in Figure 10. In common wheat, the GLIA/GLUT ratio is generally between 1.5 and 3.1 [59, 148]. The ancient wheat spelt has higher GLIA/GLUT ratios of about 1.7 – 9.0 and einkorn has very high ratios of 4.4 – 13.0 [41, 149]. A surplus of gliadins leads to viscous and soft doughs, which is especially true for spelt and einkorn. A surplus of glutenins makes the dough too strong so that the gas produced by the yeast is not able to produce a high dough and bread volume.

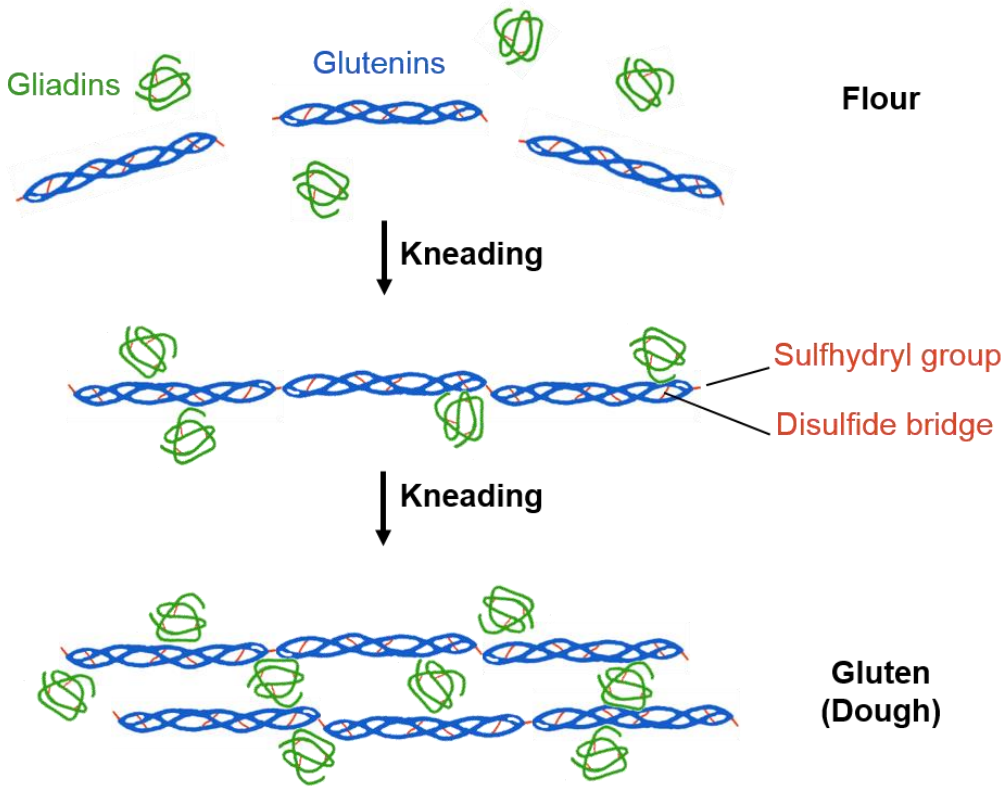

Figure 10: Schematic representation of the functional effects of gliadins (GLIA) and glutenins (GLUT) in wheat gluten. During kneading new disulphide bridges between GLUT proteins are formed. This is the reason for the elasticity and the resistance of the dough. The GLIA proteins are the softeners of GLUT and they influence the viscosity. The interaction and a balanced ratio of GLIA and GLUT are important for good baking results. Modified from [150].

The analysis of GLIA, GLUT and GMP contents can be used to predict the baking performance. One experimental option is a combined method with stepwise protein extraction followed by chromatographic separation according to the modified Osborne fractionation (Figure 11) [45]. First, the ALGL fraction is extracted with phosphate buffered saline, then, GLIA with 60 % ethanol and finally GLUT with 50 % 1-propanol under reducing conditions (DTT) (see 1.2.1). The fractions are then analysed by reversed-phase (RP-) HPLC and UV detection at 210 nm. The proteins are separated due to their polarity, with polar proteins being eluted prior to hydrophobic proteins [45, 48, 151]. For calibration, a protein mixture called reference gliadin of the Prolamin Working Group (PWG-gliadin) is used. It has been shown that the absorption at 210 nm is independent of the amino acid composition of proteins and is linearly correlated to protein concentration [45]. Thus, the protein concentration can be calculated directly from the area of the measured signals [152].

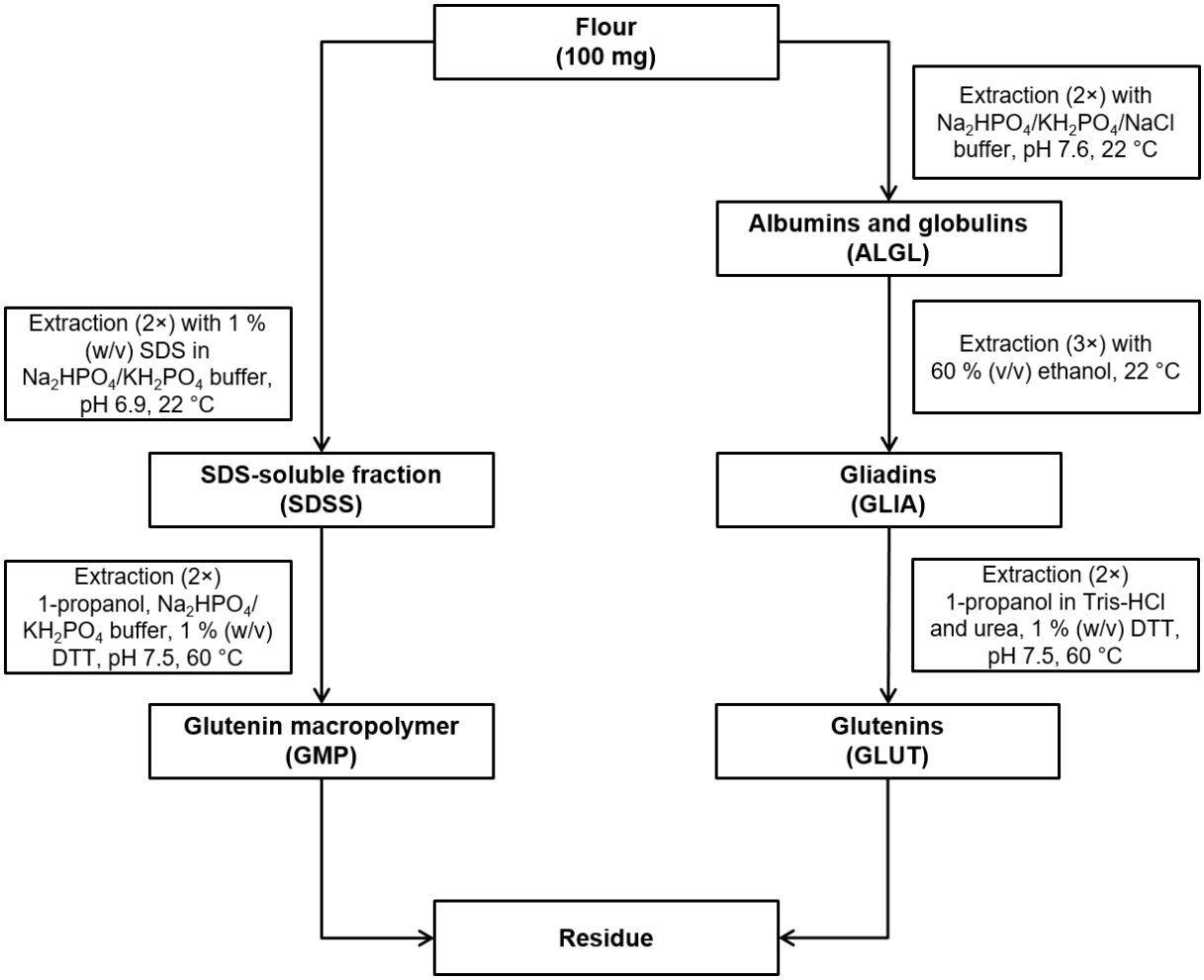

Figure 11: Scheme of glutenin macropolymer (GMP) (left) and Osborne fractionation (right). In the GMP fractionation, first, the sodium dodecyl sulphate (SDS) soluble fraction (SDSS) and then the fraction containing the GMP are extracted. Both fractions are analysed by gel permeation high-performance liquid chromatography (GP-HPLC). In the Osborne fractionation, first, the fraction containing albumins and globulins (ALGL), then the gliadins (GLIA) and finally the glutenins (GLUT) are extracted. These three fractions are analysed by reversed phase (RP-) HPLC. Modified from [45, 59]. DTT, dithiothreitol, Tris, Tris(hydroxymethyl)aminomethane.

In the GMP fractionation, the SDS-soluble proteins (SDSS) are extracted with a SDS-containing solution and afterwards the GMP layer is dissolved under reducing conditions (DTT). Contrary to the Osborne fractions, the SDSS and GMP fractions are analysed not by RP-HPLC, but by gel permeation (GP-) HPLC [60, 63, 153, 154]. Here, the proteins are separated according to their molecular weight and large proteins are eluted prior to small proteins. Analogous to the Osborne fractionation, the calibration is performed with PWG-gliadin.

Protein-specific colouring agents are alternatives to chromatographic methods. The colouring agents bind to proteins and the protein-dye-complex is measured by spectral photometric assays. A disadvantage of these assays is that the entire amount of proteins is analysed and a differentiation between subtypes (e.g. α- and γ-gliadins) or molecular weights (HMW-GS and

LMW-GS) is not possible. The Bradford assay is the best and fastest assay for the determination of GLIA and GLUT contents in comparison to assays such as fluorescamine or Naphthol Blue Black [155]. In the Bradford assay, the colouring agent Coomassie Brilliant Blue G-250 (Figure 12) is used. The negatively charged colouring agent binds to protonated amino acid side chains (e.g. arginine) at acidic pH value. This leads to a shift of the absorption maximum from 465 nm to 595 nm. The protein concentration is directly proportional to the protein-dye-complex [156].

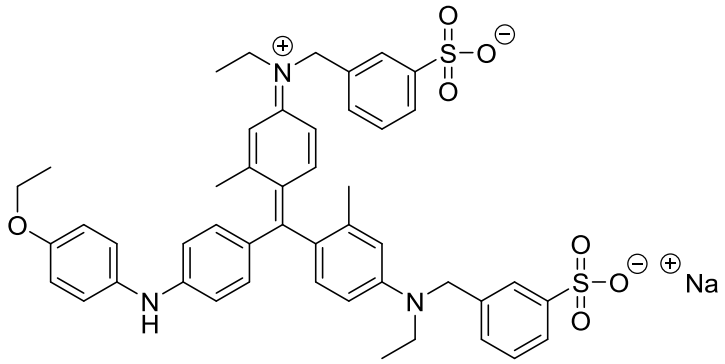

Figure 12: Structure of the colouring agent Coomassie Brilliant Blue G-250. This colouring agent is used in the Bradford assay and binds to protonated amino acid side chains (e.g. arginine) at acidic pH-value.

Bovine serum albumin is used as calibrant in most cases, because the amino acid sequence is well known and the protein is soluble in water, contrary to gluten proteins. It has been shown that the Bradford assay is compatible with the solvents used in the Osborne fractionation, but not with SDS [155]. Therefore, the analysis of SDSS and GMP is not possible with the Bradford assay. Due to the fact that the colouring agent binds to specific amino acids, it is more suitable to use calibration proteins with an amino acid composition similar to the proteins under study. For the analysis of GLIA and GLUT, isolated and purified wheat proteins are used, because there is a very high similarity in the amino acid composition.

SDS polyacrylamide gel electrophoresis (SDS-PAGE) is a well-established method for the qualitative characterisation of proteins. The proteins are extracted and denatured by an extraction buffer containing SDS and then reduced to monomers with reducing agents (e.g. DTT). The negatively charged proteins are separated by an electric field, according to their molecular weight in the respective gel. Depending on the components of the gels (tris(hydroxymethyl)aminomethane (Tris-) glycine, Bis-Tris, or Tris-acetate), on the degree of crosslinking, and on the components of the running buffer (glycine, SDS, Tris and/or tricine), different systems are suitable for the separation of proteins with low or high molecular weights. The proteins in the gels are made visible for example with Coomassie Blue, silver or copper staining. The molecular weight of the sample proteins can be estimated by comparison with marker proteins with known molecular weight.

A system consisting of a Bis-Tris gel and a running buffer based on SDS and Tris, is convenient for the separation and detection of wheat proteins, especially all types and subunits of GLIA and GLUT [157, 158]. With this system, the separation of HMW-GS was optimised compared to former approaches. Different HMW-GS are associated with good baking performance. It was shown that common wheat cultivars with HMW-GS Dx5 and Dy10 have good baking properties and cultivars with HMW-GS Dx2 and Dy12 poor properties [159, 160]. These combinations can be separated by the gel-buffer-system described above.

1.5 Mass spectrometric methods for the characterisation of proteins

RP- and GP-HPLC coupled to UV detectors and gel electrophoresis are the most commonly used methods for the characterisation and analysis of cereal and gluten proteins. The use of common analytical instruments and easy data evaluation are the most important advantages. One big disadvantage is that the information value is limited to the polarity (RP-HPLC) or the approximate molecular weight (GP-HPLC and SDS-PAGE) of the analysed proteins. Therefore, proteomics techniques based on MS are meanwhile well established to get much more information on proteins.

1.5.1 Qualitative methods

Two main questions can be answered by the qualitative characterisation of proteins: The molecular weight and the amino acid sequence. For the analysis of the molecular weight of intact proteins or peptides, either matrix-assisted laser desorption/ionisation time-of-flight MS (MALDI-TOF-MS) or LC electrospray ionisation quadrupole TOF (LC-ESI-QTOF-MS) are used. The advantage of LC-ESI-QTOF-MS compared to MALDI-TOF-MS is that the analytes are separated by LC and complex protein mixtures can be analysed. For MALDI-TOF-MS, enriched and purified proteins reveal more conclusive mass spectra than a complex protein mixture. However, MALDI-TOF-MS is much faster than LC-ESI-QTOF-MS and the data evaluation is easier. Gluten proteins including gliadins (ω1,2, ω5, α and γ) and glutenins (LMW-GS and HMW-GS) were characterised by LC-ESI-QTOF-MS and identified by comparison of the molecular weight with databases [51, 161]. The molecular weights of gliadins were in the expected range between 40,000 and 55,000 Da. The HMW-GS Ax1, Ax2*, Bx6, Bx7 By8, By9, Dx5, Dx3, Dx2, Dy12 and Dy10 were identified and characterised with molecular weights between 67,000 and 88,000 Da. The molecular weights of ATIs (13,000 – 15,500 Da) in different common wheat cultivars, one spelt cultivar and two einkorn cultivars compared by MALDI-TOF-MS showed that there are proteins with molecular masses similar to ATI in einkorn [162]. Even if the molecular weight is similar, no statement about the functionality is possible. Both MALDI-TOF-MS and LC-ESI-QTOF-MS are not suitable for quantitative analysis, because the MS signal intensities of different proteins are not comparable.

For the determination of the amino acid sequence by MS, the proteins are first hydrolysed to peptides. This is done because the analysis of peptides is more sensitive and accurate compared to the analysis of intact proteins. The typical workflow of untargeted shotgun analysis includes the extraction of proteins, reduction of disulphide bonds, alkylation of cysteine residues, enzymatic hydrolysis of proteins to peptides and peptide analysis by (nano-) LC-tandem mass spectrometry (MS/MS) [163]. The challenge in the extraction of gluten proteins is that these proteins are not soluble in water and some types have low concentrations in flour. However, the solvents used in the Osborne fractionation are suitable to dissolve gluten proteins and the extracts are supposed to be concentrated before further workup. For the reduction of disulphide bonds either DTT or Tris(2-carboxyethyl)phosphine (TCEP) can be used. In contrast to DTT, TCEP does not react with the alkylation agent. For alkylation either iodoacetamide (IAA) or chloroacetamide (CAA) is used with CAA having the higher specificity compared to IAA [164]. The choice of the enzyme for the hydrolysis depends on the amino acid sequence of the proteins. For example, trypsin is suitable for the hydrolysis of ATIs due to high arginine (R) and lysine (K) contents. Chymotrypsin is suitable for the hydrolysis of HMW-GS, due to higher contents of tyrosine (Y), tryptophane (W), phenylalanine (F) and leucine (L) compared to arginine and lysine. Besides trypsin and chymotrypsin, other enzymes, such as pepsin or thermolysin, and even combinations of several proteases (e.g. trypsin, chymotrypsin and pepsin) are used [165].

The peptides are analysed by LC-MS/MS based on different techniques such as QTOF (e.g. TripleTOF®, SCIEX, and SYNAPT G2, Waters) or quadrupole-ion trap (e.g. Orbitrap™, Thermo Scientific) either in data-dependent acquisition (DDA) or data-independent acquisition (DIA) mode [166]. The measured peptides are then identified with bioinformatic algorithms and software tools (e.g. MaxQuant [167] and Perseus [168]) and comparison with protein databases (e.g. UniProtKB [73]). These software tools are suitable for the identification of proteins with already known amino acid sequences, because all theoretical peptides are predicted with their molecular weights (precursor ion) and MS patterns (product ions) and the prediction data is compared to the measured data. This strategy is possible due to the fact that peptides fragment preferably in the same way in the MS/MS. Depending on the ionisation mode, different fragments of a, b, or c and y, x or z are generated (Figure 13). This is a huge advantage compared to other –omics approaches, e.g. metabolomics and lipidomics, where the identification of analytes is afflicted with more difficulties.

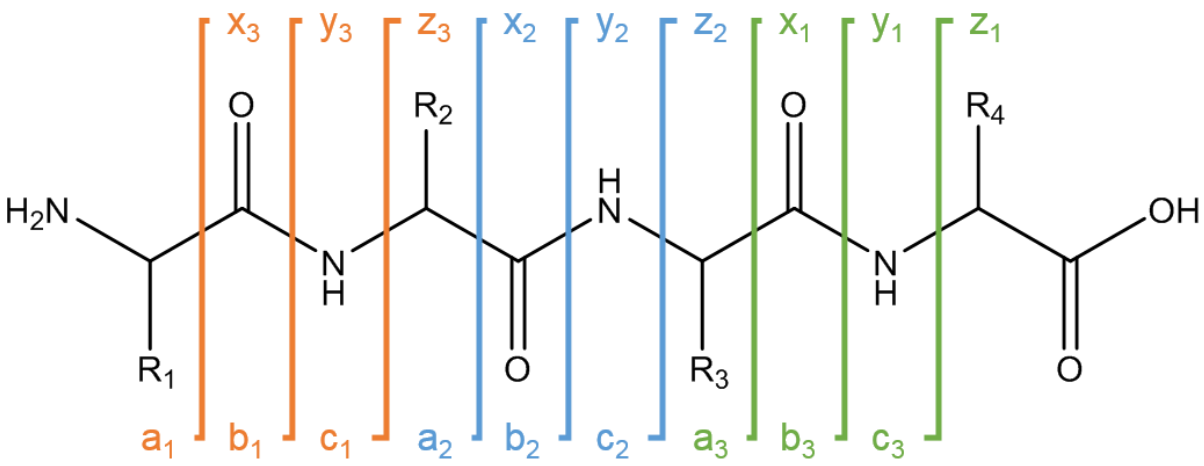

Figure 13: Peptide fragmentation notation generated by collision energy during MS/MS [169]. There are three possible cleavage points of the peptide backbone either from the N-terminus of the peptide (a_n, b_n and c_n) or from the C-terminus (x_m, y_m and z_m).

The even and steady fragmentation pattern of peptides can be used to identify the amino acid sequences of proteins, which are not present in protein databases. For this approach, software, such as PEAKS [170] or PepNovo [171], is applied for data processing. It is a benefit if the proteins are enriched and purified before digestion and LC-MS/MS. Options for this purification are SDS-PAGE or 2D gel electrophoresis, GP- or RP-(HP)LC or techniques using precipitation and size exclusion filters.

About 150,000 entries are present for the species *Triticum aestivum* (common wheat) in the UniProtKB database (status January 2019). However, the entire genome of common wheat was finally sequenced and has been published in 2018 [172]. The genome sequencing was performed with one common wheat cultivar (Chinese Spring) and this implies that not all existing sequences are contained in the data set. This is especially true for HMW-GS, for which different types exist in various cultivars (e.g. Dy10 or Dy12). Further, due to gene silencing not all genes are expressed to proteins and might be not present in the plant. Therefore, some proteins already have the status ‘evidence on protein level’ and their presence was verified. Another problem is that proteins with the name ‘uncharacterised proteins’ exist in the database. There is indeed an amino acid homology between those and proteins with an assigned name, but statements about functionality or presence in fractions (ALGL, GLIA or GLUT) are not possible. Last, some protein names, such as ‘α-amylase-inhibitor’ or ‘HMW glutenin subunit’ lead to confusion and no exakt classification (e.g. ATI 0.19 or ATI 0.28, HMW-GS Dy10 or HMW-GS Dy12) is possible.

1.5.2 Quantitative methods

After the identification of the target protein(s) by high amino acid sequence coverage rate, the next aim is their quantitation. The usage of stable isotope labelled standards has been established as the 'gold standard' for the quantitation of proteins (see below). As alternative, label-free absolute quantitation approaches based on different MS techniques, such as 'spectral counts', 'precursor ion intensities' or 'product ion intensities' are applied. The general advantages of label-free methods are high throughput for nearly any sample type with minimal costs, as well as the multiplex quantitation of large numbers of proteins. For the approach 'spectral counts', a shotgun MS technique is applied and the data is processed with different algorithms (e.g. 'exponentially modified protein abundance index', emPAI, 'absolute protein abundance index', APEX, or 'normalised spectral index'', SI_N) [172-174]. All these algorithms count the identified MS/MS spectra of the identified peptides and consider other parameters such as the number of theoretically observable peptides, differences in MS detectability and ionisation efficiency, the length of the protein and the product ion intensity. These strategies are easy to implement in data processing software and can be applied to a high number of samples even retrospectively. However, the spectral counting approach is restricted by undersampling in low concentrations and saturation effects in high concentrations [175].

Next to spectral counts, the 'precursor ion intensities' approach is used for absolute quantitation of proteins. Even if peptides are present at equimolar level, the precursor intensities vary significantly depending on their specific MS response. An option to reduce this effect is to focus on the most intense peptides per protein ('best flyer'). A linear relationship between the averaged precursor ion intensity of the three best flyer peptides per protein to the total protein abundances was reported [176]. As an alternative, the 'intensity-based absolute quantitation' (iBAQ) algorithm was developed. In this algorithm, all precursor ion intensities of all detectable peptides per protein are considered and summed up. This sum is then divided by the number of theoretically observable peptides to obtain the iBAQ intensity of a protein [177]. With the iBAQ intensity, the amounts of different proteins in the same sample are comparable and a statement about the protein composition is possible. The absolute protein abundances were already estimated in e.g. *Saccharomyces cerevisiae*, *Mycplasma pneumonia* and human tissue cell lines (reviewed by [175]).

The third technique 'product ion intensities' was recently established and uses the product ion intensities measured by selected reaction monitoring (SRM, for principle see below). The best flyers are identified with untargeted LC-MS/MS and then analysed with a targeted LC-MS/MS method. The SRM signals of the intense transitions of the best flyer peptides are summed up. A high correlation between this sum and the absolute abundance of a protein was observed and therefore an estimation of absolute protein quantities with a label-free method is possible

[178]. In summary, various comparative studies based on spectral counting or ion intensity based approaches showed that protein abundances can be estimated easily, with high throughput and with relatively low average errors (reviewed by [175]).

These techniques can be applied to issues such as the “influence of food processing on the protein composition of different wheat protein fractions” [179] or the “comparison of gluten extraction protocols assessed by LC-MS/MS analysis” [180]. The effects of processing on the solubility were high on gliadins, LMW-GS and HMW-GS due to formation of new disulphide bonds. Gliadins of bread were no longer extractable with 60 % ethanol, but together with the glutenins under reducing conditions. These findings are important for a better understanding of the influence of food processing and the detection of gluten in processed food. Enriched ATI fractions were analysed and compared by label-free quantitation [80]. The findings showed that it is a complex problem to enrich and purify ATIs of wheat flour. Nevertheless, these fractions were used in cell assays to evaluate the bioactivity of different ATIs on cells. The fractions enriched with 0.19 and CM3 showed the highest activity. However, label-free methods are not suitable for the quantitation of gluten in gluten-free products for CD patients. To legally declare foodstuff as gluten-free, a regulatory limit of 20 ppm of gluten may not be exceeded. As a label-free estimation of gluten shows low sensitivity and accuracy, it is not the method of choice. Thus, label-free quantitation cannot provide the same precision and accuracy as a stable isotope dilution assay (SIDA) using stable isotope labelled peptide standards.

SIDA and the use of stable isotopic labelled marker peptides is the ‘gold standard’ for the absolute quantitation of peptides. However, this approach is afflicted with challenges, as well. Besides complications during protein extraction (e.g. incomplete extraction due to solvent, extraction time, extraction temperature), there are two more prominent challenges: a specific and complete protein digestion and the optimal peptide selection. The digestion is affected by several factors. Therefore, the digestion of proteins to peptides is not always that complete and specific as it should be theoretically. For example trypsin does not cleave peptide bonds if proline is near to the cleavage side. Nevertheless, there have been reports that trypsin does cleave such peptide bonds [181]. Further, the digestion efficiency is influenced by the accessibility of the cleavage site due to insufficient protein denaturation or steric hindrance (e.g. bulky amino acids).

Several important considerations need to be made when selecting a peptide as internal standard [175]. The most important factor is that the peptide is unique within the target proteome. Then, the protein must be completely cleaved at the specific sites to yield the theoretical amount of peptide. For trypsin, dibasic cleavage sites (e.g. -K-K-), acidic residues

(D and E) close to the cleavage site and *N*-terminal proline (e.g. -K-P-) should be avoided. For a high MS detectability, the peptide should have a length in the range of 8 to 25 amino acids. Smaller peptides are not specific enough and larger peptides tend to have low MS intensities. Also, some amino acids are chemically modified during the analysis. Methionine and tryptophan are oxidised, combinations of -N-G- and -Q-G- can be deamidated and *N*-terminal glutamic acid and glutamine may cyclise to pyroglutamate. Further, cysteine is generally alkylated to avoid disulphide bond formation and this alkylation can be incomplete, too. In reality, it is a great challenge to fulfil all these criteria for the optimal marker peptide. The smaller the proteins, the harder it is to consider all these requirements and compromises have to be found. As alternative, labelled recombinant proteins instead of peptides can be used to compensate the above explained problems.

The workflow for the absolute protein quantitation by targeted LC-MS/MS analysis includes the extraction of the proteins, the reduction of disulphide bonds, the alkylation of cysteine residues and the enzymatic hydrolysis to peptides. In contrast to the untargeted approach (see 1.5.1) the internal standards are added as early as possible to compensate losses during the workflow. If cysteine residues are present in the internal standards, they have to be added prior to alkylation. However, the addition of the internal standard just before injection is described, as well [91, 182]. This approach at least compensates ion suppression during MS/MS [183]. The stable isotope labelled standards are usually labelled with ^{2}H or ^{13}C and ^{15}N and ideally have the same chemical and physical properties as the analytes (exception: e.g. retention time shift of ^{2}H-containing peptides). The internal standard and the analyte can be distinguished by the mass difference in the MS and MS/MS spectra. Due to the known concentration of the internal standard, the analyte content is calculated by comparison of the peak areas of analyte and internal standard. Targeted LC-MS/MS analysis using this approach were already applied to gluten proteins of common wheat, rye, barley and oats and to the ATI CM3 in durum wheat [54, 91, 182, 184, 185].

For LC-MS/MS analysis, the peptides are usually separated by RP-columns that are connected in-line to the ESI source of a triple quadrupole mass spectrometer used for SRM (Figure 14). The first quadruple (Q1) is the mass filter for the precursor ion of the target peptide and is normally set to the precursor mass using a mass window of 0.7 – 1.0 Da. The Q1 transmits precursor ions of a specific mass-to-charge ratio (m/z) value to the so-called collision cell (Q2). Here, the peptides are fragmented to at least one product ion with gas atoms (nitrogen, helium, or argon). The collision energy depends on the product ion and is between 10 and 40 electron volts. In the third quadrupole (Q3), the product ions are selected. A detector finally counts the number of product ions generated from precursor ions (transition) and thus visualises the ions as MS peaks with specific retention times and intensity values.

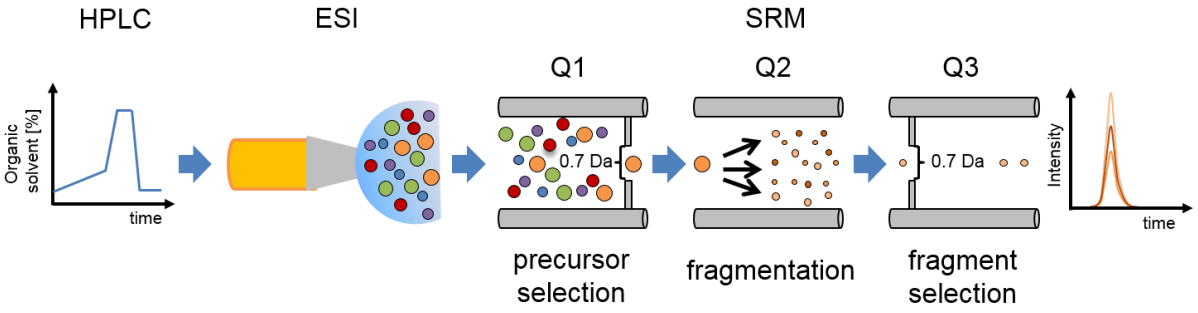

Figure 14: Principle of selected reaction monitoring (SRM) using a triple quadrupole mass spectrometer. First, the peptides of a sample are chromatographically separated on an HPLC column and directed in-line to an electrospray ionisation (ESI) source. A single transition from the precursor ion to the product ion is recorded through an *m/z* filter (0.7 Da) in the first quadrupole (Q1), fragmentation in the Q2 and a second *m/z* filter (0.7 Da) in the Q3. Figure modified from [186].

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2 Aim of the work

The ancient wheat species einkorn, emmer and spelt are nowadays cultivated in very low amounts compared to the modern wheats common wheat and durum wheat. The reasons are the low grain yield and poor baking performance of ancient wheats. The baking performance of wheat flour is mainly due to the composition of gluten proteins. In particular a balanced ratio of gliadins to glutenins is a prerequisite for the formation of viscoelastic dough with high gas holding capacity when wheat flour is mixed with water. The gluten proteins of ancient wheat species usually lack glutenins and contain excess gliadins. This leads to sticky, extensible and inelastic doughs. However, the ancient wheats have been attracting attention in the 21st century due to good sensory properties, possible health benefits and potential better tolerability for NCGS patients. In particular the content and the activity of ATIs have been under discussion during the last years. The cultivation and economic importance of ancient wheats would considerably increase, if cultivars with high baking performance would be available and if scientific data would support the hypotheses of low content and (bio-) activity of ATIs.

Therefore, the first aim of this study was to elucidate how the gluten composition of ancient wheat species affects the baking performance and to identify cultivars with good techno-functional properties. Furthermore, proteins responsible for good baking quality (high bread volume, dough stability and water absorption) should be identified to establish structure-function-relationships for all five wheat species. For this purpose, a sample set consisting of eight cultivars per wheat species common wheat, spelt, durum wheat, emmer and einkorn grown at the same location under the same climatic conditions was used. Thus, effects of geographic and climatic conditions (e.g. soil, temperature, precipitation) on the (protein) composition were ruled out, and only effects due to genetic differences (species/cultivar) were observable. The baking quality of common wheat is analysed by baking tests, but can also be predicted by indirect parameters (e.g. GPT) or by analytical methods (e.g. quantitation of GLIA, GLUT and GMP contents). It should be studied, if these methods were also suitable to predict the baking quality of the ancient wheats einkorn, emmer and spelt.

The second aim of the work was to characterise and quantitate ATIs in modern and ancient wheat species. ATIs are potential triggers of NCGS and from available literature it was supposed that the ATI content of ancient wheat species would be lower than of modern wheats, also based on suggestions that ancient wheats would be better tolerated by NCGS patients. For this purpose, a targeted LC-MS/MS method in SRM mode based on the principle of SIDA should be developed to quantitate ATIs in all five wheat species under study. This should contribute to answering the question, if ancient wheats have indeed a lower ATI content than modern wheats.

3 Results

3.1 Gluten protein composition and aggregation properties as predictors for bread volume of common wheat, spelt, durum wheat, emmer and einkorn

A sample set consisting of eight cultivars each of common wheat, spelt, durum wheat, emmer and einkorn was grown under the same environmental conditions. The kernels were milled to wholegrain flour. These flours were investigated by different methods. First, the total protein content was determined by the Dumas method. The fractions of ALGL, GLIA and GLUT were extracted according to the modified Osborne fractionation and proteins were quantitated by RP-HPLC. The fractions of SDSS and GMP were extracted according the GMP fractionation and analysed by an optimised GP-HPLC method. Sabrina Geisslitz optimised the composition of the solvents used for GP-HPLC to improve the separation of GMP proteins. Next, the aggregation properties were investigated by the use of GPT. Water absorption, dough development time and dough stability time were evaluated by micro-farinograph experiments. Bread volume and the shape of breads were investigated by baking tests.

Sabrina Geisslitz evaluated all HPLC data and calculated the amounts of ALGL, GLIA, GLUT, their types and subunits, SDSS and GMP. Further, Sabrina Geisslitz summed up all obtained data of RP-, GP-HPLC, GPT, micro-farinograph and baking tests. Sabrina Geisslitz interpreted all data by means of visualisation in figures, correlation analysis and principle component analysis (PCA). Sabrina Geisslitz wrote the manuscript, designed all figures and revised the manuscript according to the reviewer comments.

This work expanded the knowledge on the gluten protein composition and GMP content of einkorn, emmer, spelt and durum wheat. Due to the unique sample set cultivated at the same location, all differences in gluten protein composition were effects of the genotype and not of the environment. High GLUT and GMP contents were detected in common wheat and the lowest ones in emmer and einkorn. The highest bread volume was obtained for common wheat and low bread volumes for durum wheat and einkorn. Common wheat, durum wheat and einkorn formed spherical breads, but some spelt and emmer cultivars gave very flat breads. The correlation analysis showed that especially proteins of HMW-GS, GLUT and GMP were responsible for high bread volumes. In summary, the contents of GLUT, HMW-GS and GMP, and the aggregation properties of the GPT (AGT and MT) could be used to predict the baking quality of all five investigated wheat species.

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Gluten protein composition and aggregation properties as predictors for bread volume of common wheat, spelt, durum wheat, emmer and einkorn

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ABSTRACT

The technological properties of the ancient wheat species spelt, emmer and einkorn are considered inferior compared to common wheat and durum wheat, but there are only few comparative studies between ancient and modern wheat species. To help fill this gap, protein content and composition, gluten aggregation, dough and bread properties were determined in a unique set of eight cultivars each of common wheat, spelt, durum wheat, emmer and einkorn grown under standardized conditions at one location in the same year. Spearman correlations and principal component analysis (PCA) revealed that especially the contents of glutenins, high-molecular-weight glutenin subunits and glutenin macropolymer were suitable to predict the volume of breads made from wholemeal flours of the five wheat species using microbaking tests. Based on their proximity to common wheat in the PCA, one cultivar each of spelt, emmer and einkorn was identified that had similar protein analytical, functional and baking properties to common wheat. Furthermore, the characterization of gluten aggregation behavior using the GlutoPeak test (GPT) enabled an estimation of dough properties and bread volume. Therefore, the fast and easy GPT may serve as an alternative to time-consuming and labor-intensive baking tests.

1. Introduction

In the 21st century, the production area of the “ancient” (hulled) wheat species einkorn (*Triticum monococcum* L., diploid), emmer (*T. dicoccum* L., tetraploid) and spelt (*T. spelta* L., hexaploid) is negligibly small compared to that of the “modern” (naked) wheat species common wheat (*T. aestivum* L., hexaploid) and durum wheat (*T. durum* L., tetraploid). Especially in the last decades ancient wheat species were replaced by modern wheat species due to higher grain yields (spelt 37%, emmer 55% and einkorn 62% lower yield compared to common wheat). Ancient species are hulled wheats with a though glume, which has to be separated from the grain in the mill (Longin et al., 2015). Because some consumers associate the consumption of ancient wheats with health benefits, ancient wheat species have been attracting attention in the last 20 years and special products such as bread, pasta and beer have been developed (Longin et al., 2015). Studies on the contents of bioactive components (e.g. dietary fiber components, phenolic acids, folates) in ancient and modern wheats revealed only small differences between modern and ancient wheat species. For example, even though emmer and einkorn contained more of the carotenoid lutein than common wheat, durum wheat had comparable contents of lutein due to its yellow color (Shewry and Hey, 2015). More studies on a wider range of genotypes of ancient and modern wheats grown under standardized conditions are currently needed to assess possible health benefits (Shewry, 2018). Further advantages of ancient wheats include their disease tolerance, adaptation to different climatic conditions, low requirement of fertilizers and potential to increase biodiversity (Longin et al., 2015). In addition, tetraploid and diploid wheat species may contain lower amounts of immunoreactive proteins and peptides compared to hexaploid species. For example, the celiac disease-active 33-mer peptide was not detected in emmer, durum wheat and einkorn samples due to absence of the D-genome, but spelt and common wheat had comparable contents of the 33-mer (Schalk et al., 2017).

The baking quality of wheat flours is mostly determined by gluten quality and quantity. Gluten proteins are storage proteins and divided into gliadins (GLIA) soluble in aqueous alcohol and glutenins (GLUT) soluble in aqueous alcohol only after reduction of disulfide bonds. Contents and composition of GLIA (ω5-, ω1,2-, α- and γ-GLIA) and GLUT (ωb-gliadins, high- (HMW-GS) and low-molecular-weight glutenin subunits (LMW-GS)) are typically analyzed by modified Osborne fractionation followed by reversed-phase high-performance liquid chromatography (RP-HPLC) (Wieser et al., 1998). One glutenin

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subfraction with particular importance for baking quality is the polymeric glutenin macropolymer (GMP) that is insoluble in aqueous sodium dodecyl sulfate (SDS) solution. GMP is one of the largest protein-based biopolymers (Don et al., 2003; Weegels et al., 1996) and can be quantitated by gel-permeation (GP-)HPLC (Cinco-Moroyoqui and MacRitchie, 2008). There are only few studies on the content and composition of gluten proteins of the different wheat species. White flours of common wheat had GLIA/GLUT ratios between 1.7–3.1 (Wieser and Kieffer, 2001) and 1.4–2.1 (Thanhaeuser et al., 2014), whereas those of spelt (2.2–9.0) (Koenig et al., 2015), durum wheat (3.1–5.0) (Wieser, 2000; Wieser et al., 2003), emmer (3.5–7.6) (Wieser and Koehler, 2009) and einkorn (4.0–14.0) (Wieser et al., 2009) were higher compared to common wheat. The contents of GLIA ($r = 0.80$), GLUT ($r = 0.76$) and GMP ($r = 0.80$) were suitable to predict the bread volume of common wheat, because of higher correlation coefficients compared to the prediction using crude protein contents ($r = 0.71$) (Thanhaeuser et al., 2014). The GLUT content and especially that of HMW-GS was correlated to the bread volume (Wieser et al., 2009). Regarding GMP, common wheats contained 8–18 mg GMP/g of flour, but there are no studies available for spelt, durum wheat, emmer and einkorn.

Among cereals common wheat is most suitable for bread making because the flour forms a viscoelastic dough when it is mixed with water. In comparison to common wheat the flours of ancient wheat species yield softer doughs with low elasticity and high extensibility because of the poor gluten quality (Sobczyk et al., 2017; Longin et al., 2015; Wieser et al., 2009). The baking quality is usually determined by baking tests which are very time-consuming and labor-intensive. Instead, so-called quality parameters such as crude protein (CP) content (ICC method 167), wet gluten content (ICC method 137/1), micro-scale extension tests (Wieser and Kieffer, 2001) or the Zeleny sedimentation test (ICC method 116/1) are used to predict the baking quality of wheat flours. Another fast and easy method to determine quality-related parameters of wheat flour is the GlutoPeak test (GPT) that registers gluten aggregation properties (torque) during high-speed mixing for a short time (6 min). Parameters such as maximum torque (MT), peak maximum time (PMT) and aggregation time (AGT) are calculated from the respective curve. These parameters were used to predict the gluten and baking quality in white flours of common wheat (Bouachra et al., 2017; Huen et al., 2017; Marti et al., 2015a, 2015b) and durum wheat (Marti et al., 2014). However, no GPT data are available for spelt, emmer and einkorn so far.

Although gluten content and composition of a variety of common wheat, spelt and einkorn cultivars were already characterized, these studies are difficult to compare, because the samples were cultivated in different areas and harvest years, fertilized differently and the grains were milled to white or wholemeal flours. Therefore, the aim of this study was to establish suitable quality parameters to predict the baking quality of wholemeal flours of common wheat, spelt, durum wheat, emmer and einkorn. For this purpose, the results for gluten content and composition and gluten aggregation properties were correlated with the results of baking experiments using eight cultivars of each wheat species grown at the same location (Seligenstadt, Germany) and in the same year (2013).

2. Materials and methods

2.1. Chemicals

All chemicals were of analytical or higher grade and purchased from VWR Merck (Darmstadt, Germany), Serva (Heidelberg, Germany), LECO (Kirchheim, Germany) or Sigma-Aldrich (Steinheim, Germany). Water was deionized by a water purification system Arium 611VF (Sartorius, Goettingen, Germany).

2.2. Wheat samples

Eight cultivars per wheat species of common wheat, spelt, durum wheat, emmer and einkorn were cultivated by the State Plant Breeding Institute (SPBI), University of Hohenheim (Stuttgart, Germany) at Seligenstadt, Germany (Coordinates 49°52′N, 10°8′E) from October 2012 to July 2013. Seligenstadt is located 281 m above sea level and is characterized by 9.4 °C mean annual temperature and 721 mm mean annual precipitation. The fertilization was adjusted to the demands of the wheat species. Common wheat, spelt and durum wheat were fertilized with 95 kg N/ha and emmer and einkorn with 75 kg N/ha. Further information about field trials is available from Longin et al. (2015). An overview of the selected cultivars with sample codes is given in Supplementary Table 1. For common wheat, two cultivars per quality class (E, elite quality; A, high quality; B, bread quality; C, cookie quality) were chosen. For spelt, eight well known cultivars were cultivated. For durum wheat, important winter durum wheat cultivars from Germany, Austria and France were selected. Furthermore, two elite breeding lines of the winter durum wheat breeding program of SPBI were added to the list of durum wheat. As only four approved cultivars of emmer and einkorn each were available, four elite breeding lines of the emmer and einkorn breeding program of SPBI were included. Grains were dehulled in case of spelt, emmer and einkorn and milled into wholemeal flour using a cross-beater mill (Perten Instruments, Hamburg, Germany). The wholemeal flours were stored in closed bottles at room temperature for at least two weeks before they were used for all analyses and techno-functional tests.

2.3. Standard determinations

The nitrogen content of wholemeal flours was determined in triplicate by the Dumas combustion method (ICC standard 167) using a TruSpec Nitrogen Analyzer (Leco, Kirchheim, Germany). Calibration was performed with ethylenediaminetetraacetic acid and the factor 5.7 was used to calculate the CP content. Moisture and ash contents were analyzed according to ICC standards 110 and 104, respectively.

2.4. Analytical Osborne fractionation

Albumin/globulin (ALGL), GLIA and GLUT fractions were extracted stepwise from 100 mg of wholemeal flour and analyzed by RP-HPLC according to Thanhaeuser et al. (2014). Three separate extraction/RP-HPLC experiments were carried out for each flour sample. Injection volumes were optimized for each species and protein fraction. Typical injection volumes were: ALGL of common wheat, 20 µL; ALGL of the other species, 5–10 µL; GLIA of common wheat, 10 µL; GLIA of the other wheat species, 5 µL; GLUT, 20 µL.

2.5. Quantitation of SDS-soluble and GMP fractions

SDSS and GMP fractions were stepwise extracted from 100 mg of wholemeal flour and analyzed by GP-HPLC according to Thanhaeuser et al. (2014). GP-HPLC analysis was performed with slight modifications: elution system, water/acetonitrile/trifluoroacetic acid (TFA) 500/500/1 (v/v/v); isocratic flow rate, 0.3 mL/min. Injection volumes were: SDSS, 10 µL; GMP, 40 µL.

2.6. GlutoPeak test (GPT)

The GPT was performed according to Marti et al. (2015a). The parameters measured automatically by the GPT software were: Time of start of aggregation (lift off time, LOT, in s); maximum torque (MT, in Brabender equivalents, BE); time of maximum torque (peak maximum time, PMT, in s) and aggregation time (AGT, in s, difference between PMT and LOT). Triplicate determinations were made.

2.7. Microbaking test (MBT)

The MBT was carried out in triplicate according to Thanhaeuser et al. (2014). Bread volume, maximal height and maximal width were measured with a benchtop laser-based scanner (VolScan Profiler, Stable Micro Systems, Godalming, U.K.). The form factor was calculated by dividing width by height.

2.8. Statistical analysis

Mean values ± standard deviations of triplicates were calculated with Microsoft Office Excel 2013 (Microsoft Corporation, Seattle, Washington, USA). Statistical evaluation (Spearman correlation, analysis of variance, ANOVA, with Tukey's-Test as post-hoc test at a significance level of $p < 0.05$) and principal component analysis, PCA) were performed with OriginPro 2017 (OriginLab, Northampton, Massachusetts, USA).

3. Results

All determinations were performed in triplicate per sample (eight cultivars of five wheat species, n = 40). The mean values for every cultivar [mg/g flour] are displayed in Supplementary Tables 2, 3 and 4 and the distribution of Osborne and SDSS/GMP fractions in Supplementary Figs. 1 and 2. Further, the mean coefficient of variation (CV) of each analysis for each group of wheat species is shown. Table 1 shows a summary of Supplementary Tables 2, 3 and 4 with the mean value and the range (the lowest and the highest value) of the eight cultivars each. The capital letters near the mean value indicate significant differences between wheat species.

3.1. Standard determinations

Moisture, ash and CP contents of the 40 wholemeal flour samples are shown in Table 1 and Supplementary Table 3. The ash content of common wheat was significantly lower and that of einkorn significantly higher compared to the other species. The moisture contents of common wheat and spelt were significantly higher compared to durum wheat, emmer and einkorn. The CP content of common wheat was significantly lower than that of spelt, durum wheat and einkorn. Furthermore, durum wheat contained significantly higher levels of CP than common wheat and emmer.

3.2. Qualitative characterization of Osborne fractions

The differences in the RP-HPLC peak profiles were generally much higher between wheat species than those between the eight cultivars belonging to one wheat species. Therefore, the chromatograms showing the ALGL, GLIA and GLUT fractions of one exemplary cultivar of each wheat species are displayed and used for the comparison. The RP-HPLC peak profiles of ALGL from the hexaploid wheat species common wheat and spelt yielded very similar patterns and number of peaks (Supplementary Fig. 3A and B). The chromatograms of the tetraploid wheat species durum wheat and emmer did not reveal clear differences among each other except the peak numbers at a retention time of about 9 min (Supplementary Fig. 3C and D). The most noticeable differences in the chromatograms of hexaploid, tetraploid and diploid wheat species (Supplementary Fig. 3F) were identified at retention times between 8.0 and 9.0 min.

In contrast to the ALGL profiles, the differences in the GLIA peak patterns were more pronounced (Fig. 1). The RP-HPLC profile of einkorn (Fig. 1E) showed more peaks of ω5-GLIA compared to the other wheat species. In contrast, the number of peaks of ω1,2-GLIA increased from einkorn to durum wheat and emmer (Fig. 1C and D) and further to common wheat and spelt (Fig. 1A and B). The number of peaks of α-GLIA was higher for common wheat and einkorn compared to spelt, durum wheat and emmer. Chromatograms of γ-GLIA of hexaploid species showed a more complex peak profile and a higher number of peaks than the other wheat species. Thus, the variability of the peak pattern of γ-GLIA decreased noticeably from hexaploid via tetraploid to diploid species. In summary, hexaploid, tetraploid and diploid wheat species can mostly be distinguished based on the peak pattern of the GLIA fraction.

Considering the GLUT fractions, ωb-GLIA were only clearly present in common wheat (Fig. 2A) and faintly in spelt (Fig. 2B). The peak numbers of HMW-GS were comparable for common wheat and spelt, but differed compared to durum wheat, emmer and einkorn. All durum wheat cultivars had only two peaks for HMW-GS, whereas all emmer cultivars showed five major peaks. Einkorn had three major HMW-GS peaks, but the intensity was lower compared to the other species. There were also differences in LMW-GS peak patterns, again mostly between the three ploidy levels.

3.3. Quantitation of Osborne fractions

The mean contents and ranges of Osborne fractions and the corresponding gluten protein types are summarized in Table 1 (Supplementary Table 2). The mean CV of triplicate determinations were below 5.5% within the wheat species. The fractions (ALGL, GLIA, GLUT) and types are expressed as percentages of total extractable protein in the Osborne fractionation (Σ Osborne). The proportion of ALGL over all wheat species ranged between 14.5% and 32.0%, that of GLIA between 50.1% and 71.0% and that of GLUT between 5.9% and 26.0%. The percentage of GLIA from common wheat was significantly lower compared to the other wheat species and increased from common wheat to spelt, durum wheat, emmer and einkorn. GLUT showed the opposite behavior: The lowest percentages were detected for einkorn and emmer. This led to significant differences of GLIA/GLUT ratios, which were lowest for common wheat and highest for emmer and einkorn with spelt and durum wheat in between. Gluten contents were significantly higher for spelt and durum wheat compared to common wheat, emmer and einkorn, as was the sum of total extractable protein.

Within GLIA types, ω5-GLIA were more abundant and ω1,2-GLIA less abundant in einkorn compared to the other wheat species. This resulted in significantly different ratios of ω5-/ω1,2-GLIA with mean values from 0.6 to 1.6 for hexaploid and tetraploid species and 6.6 for einkorn. Einkorn contained more α-GLIA and less γ-GLIA compared to the other wheat species resulting in an α-/γ-GLIA mean ratio of 2.2, in contrast to 1.0–1.3 for hexaploid and tetraploid species.

Einkorn contained significantly less and common wheat significantly more ωb-GLIA compared to the other species. The percentages of HMW-GS and LMW-GS decreased from hexaploid to tetraploid and diploid wheat species, with higher relative differences for HMW-GS. Although the contents of both HMW-GS and LMW-GS decreased, the LMW-GS/HMW-GS ratio was significantly higher for durum wheat and einkorn than that of common wheat, spelt and emmer.

3.4. Quantitation of SDSS and GMP fractions

Compared to Osborne fractionation, GMP fractionation uses a different extraction protocol with 1% SDS solution followed by quantitation of SDSS and GMP fractions by GP-HPLC. The GP-HPLC chromatograms of SDSS showed very limited differences between the various wheat species due to the overall poorer separation performance of the GP column compared to RP-HPLC, but those of GMP revealed characteristic profiles that were divided into HMW-GMP (6.3–8.5 min) and LMW-GMP subfractions (8.5–12.5 min) based on molecular weight markers (Supplementary Fig. 4). HMW-GMP formed one and two defined peaks in common wheat and spelt, respectively, but were less clearly visible in durum wheat, emmer and einkorn. The quantitative

Table 1
Mean values and ranges obtained from the analysis of eight cultivars per wheat species (modified Osborne fractionation, SDSS/GMP fractionation, GlutoPeak test, dough properties and microbaking test). Protein fractions are given as percentages of extractable protein in the Osborne fractionation (Σ Osborne) and GMP-fractionation (Σ SDSS + GMP).

Parameter	Common wheat	Spelt	Durum wheat	Emmer	Einkorn
Standard determinations					
Moisture [g/100 g]	9.5^{B}	9.7^{B}	$8.8^{A,C}$	9.0^{A}	8.7^{A}
	9.3–9.8	9.5–9.9	8.6–9.0	8.6–9.3	8.0–9.1
Ash in dry mass [g/100 g]	1.7^{B}	2.1^{A}	2.0^{A}	2.0^{A}	2.2^{C}
	1.6–1.8	1.9–2.2	1.9–2.1	1.9–2.1	2.1–2.4
CP content [g/100 g]	11.0^{C}	$13.0^{A,B}$	14.2^{A}	$11.8^{B,C}$	$12.7^{A,B}$
	9.3–13.3	10.8–16.1	13.0–15.5	11.2–12.4	11.6–13.9
Osborne fractions[a]					
Σ Osborne [g/100 g sample]	9.3^{B}	11.6^{A}	11.3^{A}	9.2^{B}	9.7^{B}
	7.6–11.2	10.0–13.9	9.7–13.2	8.4–9.9	8.7–10.8
Gluten (GLIA + GLUT) [g/100 g sample]	7.0^{B}	9.3^{A}	9.0^{A}	7.0^{B}	7.4^{B}
	5.1–8.9	7.3–11.5	7.6–10.0	6.4–7.5	6.4–8.3
ALGL [%]	25.3^{A}	$20.8^{A,B}$	20.1^{B}	$23.9^{A,B}$	$24.1^{A,B}$
	20.3–32.0	14.5–26.7	16.7–24.8	22.4–25.3	21.5–25.9
GLIA [%]	53.3^{C}	61.2^{A}	$61.9^{A,B}$	$63.5^{A,B}$	66.7^{B}
	50.1–60.5	57.1–65.9	56.1–66.1	60.4–65.7	62.5–71.0
GLUT [%]	21.4^{A}	18.0^{A}	18.0^{A}	12.6^{B}	9.2^{B}
	16.3–25.7	14.7–21.7	12.6–26.0	9.7–17.0	5.9–14.4
GLIA/GLUT	2.6^{C}	$3.5^{B,C}$	$3.6^{B,C}$	$5.2^{A,B}$	8.0^{A}
	2.0–3.2	2.8–4.0	2.2–5.3	3.6–6.7	4.2–12.0
GLIA types[a]					
ω5-GLIA [%]	2.8^{A}	2.7^{A}	4.5^{A}	3.4^{A}	8.8^{B}
	1.9–3.7	1.8–4.0	2.8–7.2	1.4–4.5	2.4–11.9
ω1,2-GLIA [%]	3.6^{A}	4.9^{B}	3.3^{A}	5.2^{B}	1.4^{C}
	2.5–4.6	3.6–5.8	1.5–5.0	3.7–6.2	1.0–2.2
α-GLIA [%]	24.4^{B}	29.1^{A}	30.0^{A}	$27.9^{A,B}$	38.3^{C}
	21.5–27.7	25.5–31.2	25.1–38.5	25.1–31.2	36.1–40.4
γ-GLIA [%]	22.5^{A}	$24.5^{A,B}$	$24.1^{A,B}$	27.0^{B}	18.2^{C}
	20.1–25.3	21.4–26.8	19.7–29.5	23.2–30.6	13.8–22.2
ω5-/ω1,2-ratio	0.8^{A}	0.6^{A}	1.6^{A}	0.6^{A}	6.6^{B}
	0.4–1.2	0.4–0.7	0.6–3.0	0.2–0.9	2.4–10.1
α-/γ-ratio	1.1^{A}	1.2^{A}	1.3^{A}	1.0^{A}	2.2^{B}
	0.9–1.2	1.0–1.4	0.9–1.7	0.8–1.3	1.6–2.9
GLUT types[a]					
ωb-GLIA [%]	1.2^{B}	0.5^{A}	0.6^{A}	0.6^{A}	0.3^{C}
	1.1–1.4	0.4–0.6	0.4–0.9	0.5–0.7	0.2–0.5
HMW-GS[c] [%]	5.2^{C}	3.9^{A}	$3.0^{A,B}$	2.8^{B}	1.4^{D}
	3.6–6.7	2.5–5.0	2.2–4.0	2.0–4.2	0.9–2.0
LMW-GS[c] [%]	15.0^{A}	13.6^{A}	14.4^{A}	9.2^{B}	7.5^{B}
	13.2–18.9	11.5–16.1	9.6–21.2	7.2–13.1	4.4–12.2
LMW-GS/HMW-GS	3.0^{A}	3.5^{A}	4.9^{B}	3.4^{A}	5.5^{B}
	2.4–3.4	3.2–4.6	4.1–6.5	2.2–4.4	4.0–6.8
GMP fractions[b]					
Σ SDSS + GMP [g/100 g sample]	10.2^{B}	12.5^{A}	12.7^{A}	$11.4^{A,B}$	12.1^{A}
	9.0–12.8	10.1–15.6	12.1–13.8	10.6–12.2	10.8–13.7
SDSS [%]	92.2^{A}	95.4^{B}	92.4^{A}	96.6^{B}	97.3^{B}
	89.1–94.9	92.7–97.1	86.2–96.2	95.7–97.6	96.9–98.3
GMP [%]	7.8^{A}	4.6^{B}	7.6^{A}	3.4^{B}	2.7^{B}
	5.1–10.9	2.9–7.3	3.8–13.8	2.4–4.3	1.7–5.3
GMP subfractions[b]					
HMW-GMP [%]	$1.2^{A;B}$	$1.0^{A,B,C}$	1.6^{A}	$0.7^{B,C}$	0.4^{C}
	0.6–2.2	0.6–1.4	0.7–3.1	0.4–1.0	0.2–0.8
LMW-GMP [%]	6.5^{A}	3.6^{B}	6.0^{A}	2.7^{B}	2.3^{B}
	4.6–9.4	2.2–5.7	3.9–10.7	2.0–3.6	1.4–4.4
LMW-GMP/HMW-GMP	5.9^{B}	3.8^{B}	4.0^{A}	$4.4^{A;B}$	5.6^{B}
	3.8–8.2	2.9–5.8	2.5–5.2	2.8–5.5	4.8–6.8
GPT parameters[c]					
PMT [s]	69.4^{A}	$53.6^{A,B}$	67.4^{A}	$40.4^{B,C}$	26.3^{C}
	56.7–94.3	42.0–73.0	38.7–115.5	23.7–67.0	18.3–43.7
LOT [s]	6.4^{A}	7.8^{A}	8.4^{B}	7.7^{A}	8.0^{A}
	5.7–8.3	7.0–9.3	7.0–10.3	7.3–9.3	4.0–11.3
AGT [s]	63.0^{A}	$45.8^{A,B}$	58.9^{A}	$32.6^{B,C}$	17.2^{C}
	50.7–86.0	34.7–65.0	31.7–106.0	16.4–58.0	8.3–34.7
MT [BE]	38.1^{A}	36.2^{A}	33.8^{A}	30.2^{A}	15.4^{B}
	25.0–46.3	28.3–45.3	21.7–60.0	26.7–37.7	14.3–17.3

(*continued on next page*)

Table 1 (*continued*)

Parameter	Common wheat	Spelt	Durum wheat	Emmer	Einkorn
Microbaking test					
Water absorption [%]	70.1^{A}	65.2^{B}	70.4^{A}	62.5^{B,C}	60.2^{C}
	62.7–75.7	60.5–66.6	65.9–71.8	60.5–63.9	58.6–62.5
Dough development time [min]	7.8^{A}	4.2^{B}	8.6^{A}	3.9^{B}	2.4^{B}
	6.7–10.0	3.3–5.9	5.0–11.2	2.9–5.0	1.7–3.9
Dough stability [min]	7.2^{A}	4.3^{A,B}	4.7^{A,B}	0.5^{B}	3.8^{A,B}
	5.5–10.0	1.2–9.1	0.8–7.6	0.2–0.8	0.2–14.2
Bread volume [mL]	35.1^{C}	31.4^{D}	23.4^{A}	22.2^{A,B}	19.6^{B}
	29.5–37.7	27.3–36.1	18.3–27.4	20.4–26.5	17.4–21.9
Specific bread volume [mL/g]	2.2^{A}	2.0^{A}	1.4^{B}	1.4^{B}	1.5^{B}
	1.8–2.3	1.7–2.3	1.2–1.6	1.3–1.6	1.3–1.7
Form factor (width/height)	1.9^{A}	2.8^{B}	1.8^{A}	2.7^{B}	2.1^{A}
	1.8–2.1	2.4–3.2	1.3–2.4	2.4–3.1	1.8–2.3

Mean values associated with different capital letters are significantly different within each row (one-way ANOVA, Tukey's test, $p < 0.05$).

[a] CP, crude protein; Σ Osborne, sum of extractable protein in the Osborne fractionation; ALGL, albumins/globulins; GLIA, gliadins; GLUT, glutenins; GLIA/GLUT, gliadin-to-glutenin ratio, calculated as mean value of individual GLIA/GLUT ratios; ωb, glutenin-bound ω-gliadins; HMW-GS, high-molecular-weight glutenin subunits; LWM-GS, low-molecular-weight glutenin subunits; Osborne fractions, gliadin types und glutenin subunits are % of Σ Osborne.

[b] SDSS, sodium dodecyl sulfate soluble proteins; GMP, glutenin macropolymer; Σ SDSS + GMP, sum of extractable protein in the GMP fractionation; HMW-GMP, high-molecular-weight fraction of glutenin macropolymer; LMW-GMP, low-molecular-weight fraction of glutenin macropolymer; SDSS, GMP and GMP subfractions are % of Σ SDSS + GMP.

[c] GPT, GlutoPeak test; PMT, peak maximum time; LOT, lift off time; AGT, aggregation time; MT, maximum torque.

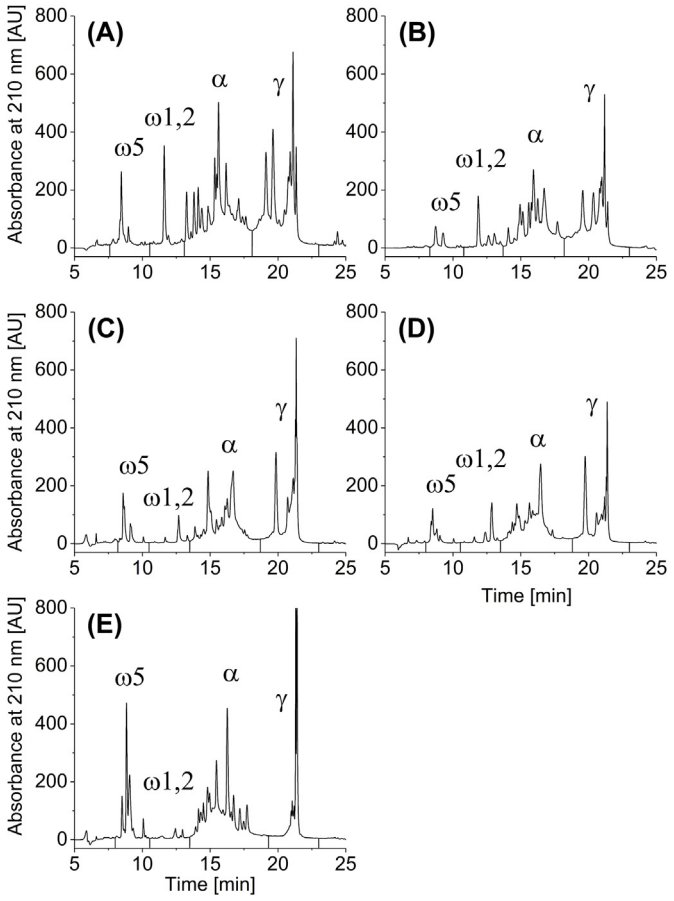

Fig. 1. RP-HPLC profiles of gliadins of **(A)** JB Asano (JBA, common wheat), **(B)** Schwabenkorn (SCH, spelt), **(C)** Lupidur (LUP, durum wheat), **(D)** 9.105/06/01 (EM4, emmer) and **(E)** MV Menket (MVM, einkorn); injection volumes, JBA: 10 µL; SCH, LUP, EM4 and MVM: 5 µL; ω5, ω5-gliadins; ω1,2, ω1,2-gliadins; α, α-gliadins; γ, γ-gliadins.

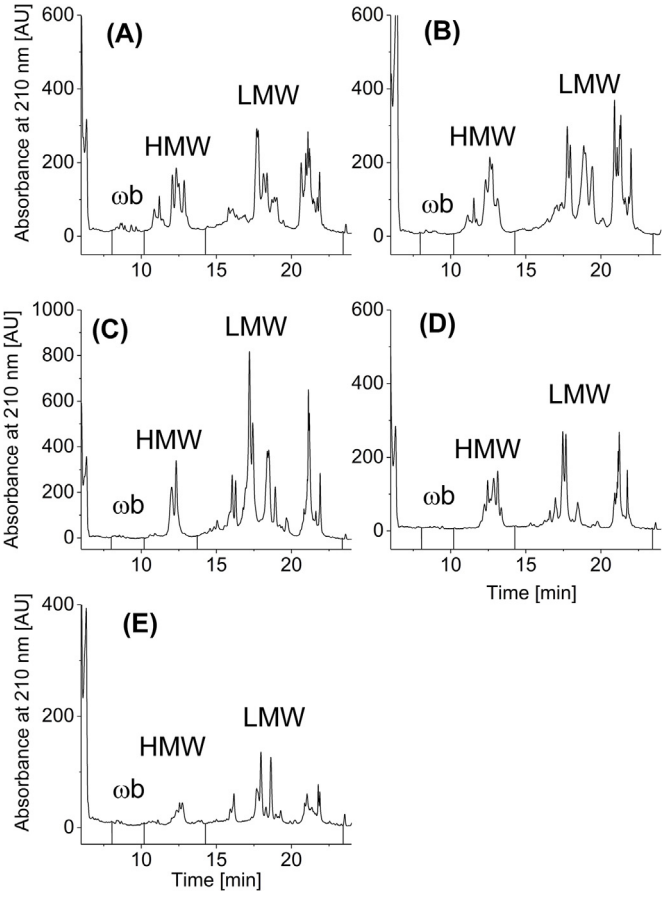

Fig. 2. RP-HPLC profiles of glutenins of **(A)** JB Asano (JBA, common wheat), **(B)** Schwabenkorn (SCH, spelt), **(C)** Lupidur (LUP, durum wheat), **(D)** 9.105/06/01 (EM4, emmer), **(E)** MV Menket (MVM, einkorn); injection volume 20 µL each; ωb, ωb-gliadins; HMW, high-molecular-weight glutenin subunits; LMW, low-molecular-weight glutenin subunits.

results for SDSS and GMP fractions are summarized in Table 1 (Supplementary Table 3). Fractions and subfractions are again expressed as percentages of the sum of total extractable protein in the GMP fractionation (Σ SDSS + GMP). The mean CV of triplicate analysis of SDSS and GMP and of their subfractions was below 9.3%. More than 92% of the extractable proteins were extracted with aqueous SDS-solution (SDSS fraction). The percentage of GMP in common wheat and durum wheat was significantly higher than in spelt, emmer and

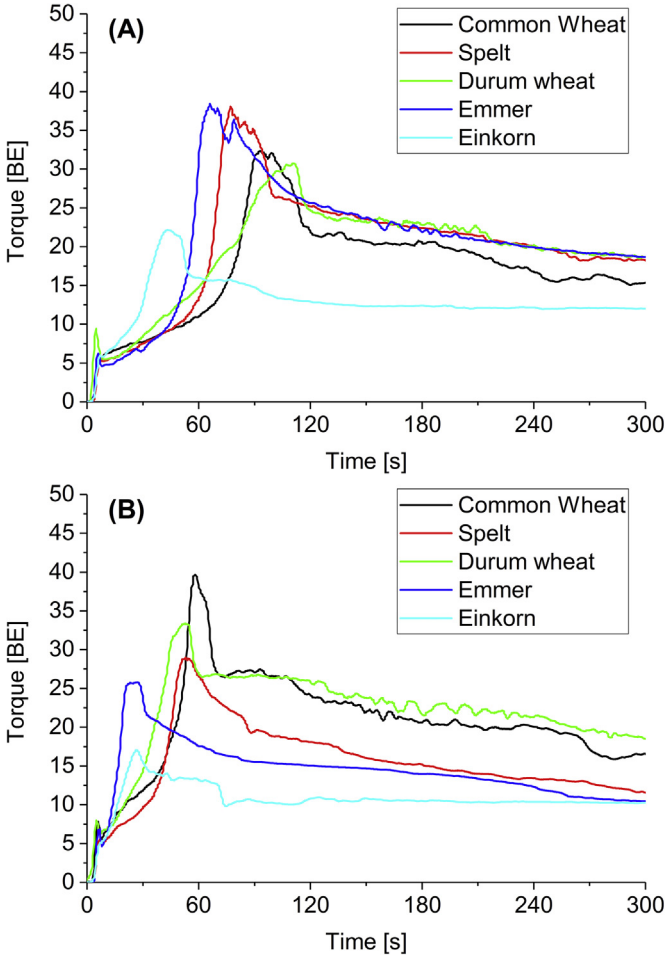

Fig. 3. Gluten aggregation properties (GlutoPeak curves) of one cultivar per wheat species. **(A)** Flours characterized by a slow buildup consistency (long aggregation time) and long-lasting stability at peak consistency (common wheat: Event, EVE; spelt: Franckenkorn, FRA; durum wheat: Logidur, LOG; emmer: CC1E-04058/01, EM1; einkorn: Monlis, MON). **(B)** Flours with a rapid buildup of consistency (short aggregation time) followed by rapid breakdown (common wheat: Lear, LEA; spelt: Badengold, BAG; durum wheat: Auradur, AUR; emmer: 9.105/06/01, EM4; einkorn: M-04033/03, EK3).

einkorn. This was mainly due to the LMW-subfraction of GMP (LMW-GMP), which formed the major portion of GMP. The ratios of LMW-GS/HMW-GS (Osborne fractionation) and LMW-GMP/HMW-GMP were similar for spelt and einkorn, but different for common wheat, durum wheat and emmer. For example, common wheat had the lowest ratio in the Osborne fractionation, but the highest in the GMP fractionation.

3.5. GlutoPeak test

Fig. 3A shows GlutoPeak curves of the five flours that were characterized by long AGT (einkorn: > 30 s; other wheat species: > 60 s), slow build-up, late PMT (> 40 s) and long-lasting stability of the gluten network as indicated by slow breakdown after PMT. The curves shown in Fig. 3B belong to flours with short AGT (einkorn and emmer: < 30 s; common wheat, spelt and durum wheat: < 60 s), early PMT and fast breakdown of the gluten network. Einkorn flours behaved differently compared to flours of the other wheat species with typically short PMT and low MT. This was also confirmed statistically, because PMT and AGT were significantly lower for einkorn compared to common wheat, spelt and durum wheat (Table 1).

3.6. Microbaking tests

First, water absorption, dough development time and dough stability of all 40 samples were determined in a micro-Farinograph (Table 1, Supplementary Table 4). The water absorptions of common and durum wheat were significantly higher than those of spelt, emmer and einkorn. The dough development times of modern wheat species were (with exception of one durum wheat cultivar) longer than those of spelt, emmer and einkorn. In general, emmer flours showed short dough development times of maximal 5 min and low dough stability with a mean of 30 s. Four out of eight einkorn samples also had dough stability times below 1 min.

For the MBT, optimal water addition and kneading time (defined to reach a consistency of 550 BU at 22 °C) were applied for each flour. Typical parameters of the minibreads are shown in Table 1 and Supplementary Table 4. The breads of common wheat, durum wheat and einkorn were almost spherical, because the form factors (width/height) were significantly lower compared to spelt and emmer. In contrast, flours of some spelt and emmer cultivars formed very flat minibreads. Even though the form factors of durum wheat and einkorn breads were comparable to breads from common wheat, the bread volumes were significantly lower. Examples of minibreads are shown in Supplementary Fig. 5 A–E (good baking performance) and Supplementary Fig. 5 F–J (poor baking performance). As expected, the volume of minibreads from common wheat was the highest followed by spelt and then the other three species.

3.7. Statistical data evaluation

Gluten protein content and composition, gluten aggregation behavior as well as dough and baking properties were determined for eight cultivars each of the five wheat species (Supplementary Table 2, 3 and 4). First, analytical and GPT data were correlated pairwise to dough and baking properties to identify analytical parameters which are suitable to predict the baking quality. Nonparametric Spearman correlation coefficients (r_s) suitable for monotonic functions were calculated (Table 2), because most pairs of parameters were not correlated linearly, but logarithmically. In addition, a PCA was performed to consider all data simultaneously.

In general, the form factor was not correlated with any GPT or analytical parameter, but water absorption, bread volume, dough development time and dough stability were correlated with some results of GPT, Osborne fractionation and GMP fractionation. The GPT parameters AGT and PMT were positively correlated with water absorption, dough development time, dough stability and bread volume ($r_s \geq 0.716$) and MT was correlated to all of the above ($r_s \geq 0.716$) except dough stability. In contrast, LOT was not correlated with any of the parameters.

The CP content, the sum of proteins extracted by Osborne fractionation and gluten content also showed no correlation to any of the results for dough and baking properties. In contrast, GLIA and GLUT contents and the GLIA/GLUT ratio were correlated with Farinograph and MBT results. Even though the GLIA content was inversely correlated with bread volume, dough development time and dough stability, individual GLIA types and ω5-/ω1,2- and α-/γ-ratios were not related to any of the dough and baking parameters. In contrast, all GLUT protein types and the LMW-GS/HMW-GS ratio were correlated with bread volume. Corresponding results were obtained for the contents of GMP and the corresponding subfractions (LMW-GMP, HMW-GMP) that were correlated with the bread volume ($r_s \geq 0.586$).

A PCA was performed with all data of Supplementary Tables 2, 3 and 4. Fig. 4 shows the corresponding biplot of the component scores and variable loadings. In Supplementary Fig. 6, the variable loadings were removed for better visibility. The largest differences were detected between common wheat and einkorn, because the data points were

Table 2
Spearman correlation coefficients (r_s) between results for dough properties and microbaking tests and parameters of GPT, Osborne and GMP fractionation.[a]

	Water absorption [%]	Dough development [min]	Dough stability [min]	Bread volume [mL]	Form factor
GPT parameters					
PMT [s]	**0.716*****	**0.831*****	**0.786*****	**0.734*****	- 0.495**
LOT [s]	- 0.255	- 0.146	- 0.075	- 0.284	- 0.059
AGT [s]	**0.732*****	**0.842*****	**0.763*****	**0.738*****	- 0.484**
MT [BE]	**0.795*****	**0.643*****	0.453**	**0.742*****	0.012
CP [g/100 g]	0.337*	0.154	0.027	- 0.203	- 0.081
Osborne fractions					
Σ Osborne [g/100 g]	0.467**	0.235	0.157	0.171	- 0.003
Gluten (GLIA + GLUT) [g/100 g]	0.500**	0.275	0.153	0.164	−0.011
ALGL [%]	- 0.467**	- 0.278	- 0.084	- 0.123	- 0.108
GLIA [%]	- 0.508***	**- 0.627*****	**- 0.650*****	**- 0.700*****	0.393*
GLUT [%]	**0.820*****	**0.800*****	**0.702*****	**0.804*****	- 0.322*
GLIA/GLUT	**- 0.781*****	**- 0.795*****	**- 0.727*****	**- 0.829*****	0.362*
ω5-Gliadins [%]	- 0.285	- 0.370*	- 0.450**	- 0.524***	- 0.081
ω1,2-Gliadins [%]	0.206	0.089	- 0.213	0.295	0.463**
α-Gliadins [%]	- 0.456**	- 0.536***	- 0.382*	**- 0.563*****	0.187
γ-Gliadins [%]	0.133	0.129	- 0.058	0.108	0.355*
ω5-/ω1,2-ratio	- 0.254	- 0.237	- 0.082	- 0.529***	- 0.314*
α-/γ-ratio	- 0.300	- 0.340*	- 0.183	- 0.353*	- 0.076
ωb-Gliadins [%]	**0.673*****	**0.730*****	0.501***	**0.585*****	- 0.264
HMW-GS [%]	**0.685*****	**0.655*****	0.505***	**0.864*****	- 0.082
LMW-GS [%]	**0.836*****	**0.807*****	**0.718*****	**0.738*****	- 0.367*
LMW-GS/HMW-GS	- 0.272	- 0.244	- 0.067	**- 0.619*****	- 0.245
GMP fractions					
Σ (GMP + SDSS) [g/100 g]	0.241	0.010	- 0.088	- 0.179	0.102
SDSS [%]	**- 0.879*****	**- 0.875*****	**- 0.793*****	**- 0.652*****	0.514***
GMP [%]	**0.879*****	**0.875*****	**0.793*****	**0.652*****	- 0.514***
HMW-GMP [%]	**0.843*****	0.763***	**0.562*****	**0.586*****	- 0.322*
LMW-GMP [%]	**0.860*****	**0.862*****	**0.803*****	**0.622*****	- 0.537***
LMW-GMP/HMW-GMP	- 0.327*	- 0.191	0.084	0.135	- 0.155

Bold number: significant correlation (Spearman correlation coefficients (r): $r \leq \pm 0.54$, no correlation; $\pm 0.54 < r \leq \pm 0.67$, weak correlation; $\pm 0.67 < r \leq \pm 0.78$, medium correlation; $\pm 0.78 < r \leq \pm 1.00$, strong correlation; level of significance (p): no asterisk, $p > 0.05$, not significant; *, $p \leq 0.05$, significant; **, $p \leq 0.01$, highly significant; ***, $p \leq 0.001$, very highly significant).
[a] For abbreviations see Table 1.

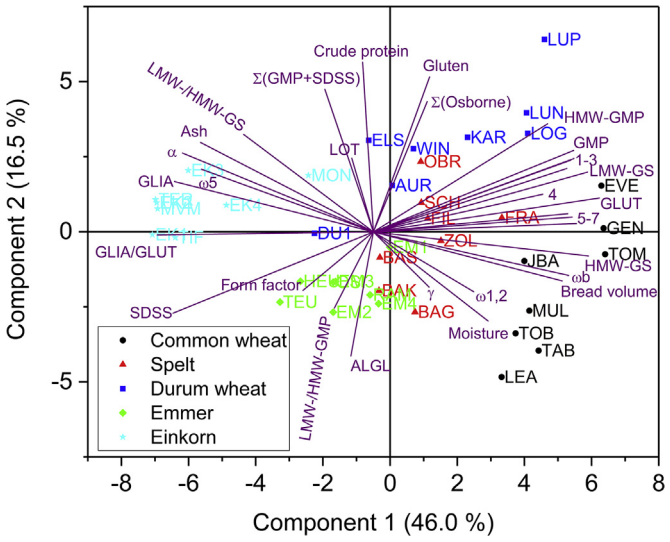

Fig. 4. Principal component analysis biplot of all data (Supplementary Tables 2, 3 and 4). For abbreviation of parameters see Table 1 and for abbreviations of cultivars see Supplementary Table 1. Abbreviation of vectors: 1, low-molecular-weight subfraction of GMP (LMW-GMP); 2, water absorption; 3, dough development time; 4, dough stability; 5, peak maximum time (PMT); 6, maximum torque (MT); 7, aggregation time (AGT).

clearly separated. Furthermore, a tight clustering of emmer cultivars was observed, which demonstrated low variability within the group of emmer cultivars, but three spelt cultivars were located closely to the group of emmer cultivars. Flours of spelt, durum wheat and emmer could not be unambiguously classified due to overlapping positions of the data points in the plot.

In general, the variable loadings indicate which parameters are responsible for the position of data points in the biplot. The loading vectors for CP content, the sum of Osborne fractions and the sum of SDSS and GMP fractions pointed in the same direction, where cultivars of durum wheat, spelt and einkorn were located. The vectors for GLIA, GLIA/GLUT, α-GLIA, ω5-GLIA, SDSS and ALGL pointed to the left side of the plot where einkorn and emmer cultivars were located. In contrast, the vectors for GLUT, GMP, LMW-GS, HMW-GS, LMW-GMP, HMW-GMP, γ-GLIA and ω1,2-GLIA pointed to the right into the direction of common wheat cultivars, as did the vectors belonging to dough properties (water absorption, 2; dough development time, 4; dough stability, 8). The vector for bread volume was exactly in the middle between the eight common wheat cultivars.

4. Discussion

Protein content and composition, gluten aggregation properties as well as dough and bread properties were determined in a unique set of eight cultivars each of common wheat, spelt, durum wheat, emmer and einkorn. Environmental factors typically account for up to two-thirds of the variation in wheat protein content and composition with the

remaining one-third being controlled by genetic factors with additional interaction between the two factors (Shewry, 2009). Grown under standardized conditions at one location in the year 2013 to control for environmental factors, this set of cultivars fulfills all requirements for a direct comparison of data between genotypes, as demanded recently (Shewry, 2018). As such, the results of these experiments are new in the literature.

The ash contents were typical for wholemeal flours of each wheat species (Brandolini et al., 2008; Pagnotta et al., 2009; Ruibal-Mendieta et al., 2005) as were the results for CP contents (Longin et al., 2015). The qualitative differences in the ALGL, GLIA and GLUT chromatograms were ascribed to variation in protein expression patterns largely determined by genetic factors, especially due to lack of the D genome in durum wheat and emmer and both B and D genomes in einkorn. The chromatographic profiles were also in agreement with earlier reports (Koenig et al., 2015; Wieser, 2000; Wieser et al., 2009). Typical values for ALGL contents are 9.1–19.0% in white flour (Thanhaeuser et al., 2014). Due to the use of wholemeal flours in this study, the proportions of ALGL (16.7–32.0%) were generally higher and consequently the proportions of gluten (68.0–83.3%) lower compared to white flour. The GLIA/GLUT ratio defines the balance between elastic and viscous properties of wheat dough. Typical values for common wheat are between 1.5 and 3.1 (Wieser et al., 1998) and were confirmed in the current study (2.0–3.2). The GLIA/GLUT ratios of spelt and einkorn were also in accordance with the literature (Koenig et al., 2015; Wieser et al., 2009). Only two durum wheat and seven emmer samples had been analyzed so far (Wieser and Koehler, 2009; Wieser et al., 2003; Wieser, 2000) resulting in GLIA/GLUT ratios from 3.1 to 7.6 and the values found in the present study were also in agreement.

Considering the contents of gluten protein types, significant differences were observed between the five species, with einkorn being most different to the other four wheat species, because all gluten protein types except LMW-GS had either higher (ω5-, ωb- and α-GLIA) or lower (ω1,2- and γ-GLIA and HMW-GS) concentrations. While the contents of both HMW-GS and LMW-GS decreased from hexaploid to tetraploid and diploid species, the magnitudes were different, because the LMW-GS/HMW-GS ratio increased. These changes in gluten protein composition can be attributed to breeding for higher GLUT and HMW-GS contents for improved baking quality (Kiszonas and Morris, 2018). When comparing both fractionation procedures (modified Osborne vs. SDSS/GMP fractionation) higher contents of total extractable proteins were obtained after SDSS/GMP fractionation, which may be explained by the higher disaggregating potential of the solvent containing 1% SDS. When comparing both LMW-GS/HMW-GS and LMW-GMP/HMW-GMP ratios, the results were in accordance with Thanhaeuser et al. (2014) who reported higher LMW-GMP/HMW-GMP ratios compared to LMW-GS/HMW-GS ratios for common wheat. The same was true for emmer, but both ratios were comparable for spelt and einkorn and the opposite was found for durum wheat. The most likely explanations seem to be the two different extraction and HPLC separation procedures as well as differences in the gluten network-forming capability of the flours from the five wheat species, but further investigations are necessary to clarify this aspect.

The GPT has been used as a fast and easy method to predict the baking performance of white flours from common wheat and durum wheat (Bouachra et al., 2017; Huen et al., 2017; Marti et al., 2015a, 2015b; Marti et al., 2014). Its application was recently extended to wholemeal flours (Malegori et al., 2018), but to the best of our knowledge, our study is the first to report comparative GPT data for all five wheat species. The analysis of dough properties in the Farinograph was done according to the protocol for white flours of common wheat (Wieser and Kieffer, 2001) that defines the optimal dough consistency as 550 BU at 22 °C and 60 rpm to give maximal volume in the MBT. Here, we applied this protocol to wholemeal flours of the five wheat species to ensure comparability of the results between the flours. However, it may be that wholemeal flours, especially of emmer or einkorn, might give better volume at other target values for optimal dough consistency. Under the conditions used here, the flours of ancient wheat species showed poor mixing characteristics, as has been observed before (Løje et al., 2003). Adapting dough handling and baking parameters to the flour characteristics of ancient wheat species is likely to result in higher bread volumes, but this was not the focus of this study.

Spearman correlations and PCA were used to identify analytical parameters to predict the baking quality (high bread volume, low form factor, long-lasting dough stability) of common wheat, spelt, durum wheat, emmer and einkorn wholemeal flours (Table 2). None of the analyzed parameters was suitable to predict the form factor, but several parameters were significantly correlated to bread volume and dough stability. The CP of flours from different wheat species was unsuitable as a predictor for bread volume, as has been reported earlier (Kazman, 2010). In contrast, the contents of polymeric protein fractions such as GLUT, HMW-GS and GMP were correlated to the bread volume and can therefore be used as predictors of baking quality not only for common wheat (Thanhaeuser et al., 2014; Weegels et al., 1996), but also for the other four wheat species. The parameters PMT, AGT and MT obtained by GPT also showed significant correlations to the bread volume. Consequently, the fast and easy GPT is a useful tool to predict the baking performance of flours from all five wheat species and may serve as an alternative to the time-consuming and labor-intensive MBT.

Cultivars of spelt, emmer and einkorn with good baking performance within the respective wheat species were identified based on the proximity of their positions to those of common wheat in the PCA biplot. For example, einkorn Monlis (MON) yielded the highest bread volume, had the longest AGT, the lowest GLIA/GLUT-ratio and the highest GLUT- and GMP-content of all einkorn cultivars (Supplementary Tables 2, 3, and 4). Corresponding observations were true for spelt Franckenkorn (FRA) and emmer CC1E-04058/01 (EM1). Breads from einkorn MON and spelt FRA were additionally characterized by a low form factor, i.e., they formed spherical breads. On the other hand, flour of emmer EM1 gave large, but flat breads in spite of the above “positive” analytical parameters. To summarize, PCA was useful to select cultivars from other wheat species that had similar properties as common wheat in terms of low GLIA/GLUT ratio, high GLUT, HMW-GS and GMP contents and long AGT.

5. Conclusion

Five wheat species (eight cultivars each) grown under the same geographic and climatic conditions (year and area) were analyzed and characterized with various analytical and rheological techniques. Because of the standardized growing conditions (except for species-specific fertilization), significant differences in protein content and composition were due to species and cultivar. These differences significantly affected the baking quality (bread volume, dough stability). Different protein parameters showed weak to strong correlation coefficients to the baking quality. Especially HMW polymeric gluten proteins (GLUT, GMP and their subfractions) were responsible for high bread volume. PCA with all parameters was suitable to identify spelt, emmer and einkorn cultivars that yielded high bread volume. The GPT was found to be a fast and easy alternative to the time consuming and labor intensive MBT to estimate the bread volume of wholemeal flours from the wheat species common wheat, spelt, durum wheat, emmer and einkorn. At least one spelt, emmer and einkorn cultivar each with a favorable gluten protein composition for good baking performance were identified based on the robust dataset presented here. The predictive value of the analytical parameters to estimate bread volumes identified in this study will be further assessed in a larger sample set. The potential health benefits of ancient compared to modern wheats also merit in-depth investigations to utilize ancient wheats for the promotion of biological and food diversity.

Notes

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jcs.2018.08.012.

Abbreviations Used

AGT	aggregation time
ALGL	albumins/globulins
ANOVA	analysis of variance
BE	Brabender equivalents
CP	crude protein
CV	coefficient of variation
GLIA	gliadins
GLUT	glutenins
GMP	glutenin macropolymer
GP-HPLC	gel-permeation high-performance liquid chromatography
GPT	GlutoPeak test
HMW-GS	high-molecular-weight glutenin subunits
LMW-GS	low-molecular-weight glutenin subunits
LOT	lift off time
MBT	microbaking test
MT	maximum torque
PCA	principal component analysis
PMT	peak maximum time
RP-HPLC	reversed-phase high-performance liquid chromatography
SDS	sodium dodecyl sulfate
SDSS	sodium dodecyl sulfate soluble
SPBI	State Plant Breeding Institute, University of Hohenheim, Stuttgart, Germany
TFA	trifluoroacetic acid

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3.2 Targeted LC–MS/MS reveals similar contents of α-amylase/trypsin-inhibitors as putative triggers of nonceliac gluten sensitivity in all wheat species except einkorn

In this study, suitable marker peptides for the quantitation of ATIs in different wheat species and cultivars were identified. For this purpose, ATIs were extracted from common wheat, spelt, durum wheat, emmer and einkorn. After reduction with TCEP and alkylation with CAA, the proteins were hydrolysed with trypsin. The peptides were analysed by LC-MS/MS and the data were evaluated by untargeted searches using a protein database for green plants. Sabrina Geisslitz performed the experiments and selected five suitable marker peptides for the quantitation of five ATIs (sum of 0.19 and 0.53, 0.28, CM2, CM3 and CM16). These peptides were purchased in unlabelled form and in stable isotope labelled form (^{13}C and ^{15}N). The latter ones were used as internal standards in the targeted LC-MS/MS method. The method in the SRM mode was developed by Sabrina Geisslitz. Further, Sabrina Geisslitz validated the new method by determining the limit of detection (LOD), the limit of quantitation (LOQ) and precision.

The validation of accuracy was afflicted with difficulties. Usually, reference materials with a defined analyte content are investigated with a newly developed method and the results are compared with the theoretical content. But in this case, no reference material was available. Therefore, a commercially available protein mix with the name ‘α-amylase-inhibitor from wheat’ was analysed with the new method and with an alternative LC-MS/MS system in DDA mode. On the one hand, the ATI content analysed with the new method was calculated by comparison with the internal standard. On the other hand, the DDA data were evaluated by an untargeted search using the wheat proteome and the protein amounts were estimated by the iBAQ algorithm. Both methods and data evaluations revealed comparable results. Sabrina Geisslitz designed the experiments, approached suitable collaboration partners and evaluated the data for the validation of accuracy.

The sample set of 3.1 was analysed for ATI contents with the newly developed method. Sabrina Geisslitz performed all experiments and evaluated the LC-MS/MS data. The ATI content in the einkorn samples was very low or even below the LOD. Spelt and emmer contained higher amounts of ATIs than common wheat and durum wheat. The relative distribution of ATIs (CM-types to 0.19, 0.28 and 0.53) was different in hexaploid wheats compared to that of tetraploid wheats. No correlation between the ATI content and ALGL or protein content was observed. This confirmed the necessity of the new targeted LC-MS/MS method for the quantitation of ATIs. Sabrina Geisslitz wrote the manuscript, performed all laboratory work, designed all figures and revised the manuscript according to the reviewer comments.

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Targeted LC−MS/MS Reveals Similar Contents of α-Amylase/Trypsin-Inhibitors as Putative Triggers of Nonceliac Gluten Sensitivity in All Wheat Species except Einkorn

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Supporting Information

ABSTRACT: Amylase/trypsin-inhibitors (ATIs) are putative triggers of nonceliac gluten sensitivity, but contents of ATIs in different wheat species were not available. Therefore, the predominant ATIs 0.19 + 0.53, 0.28, CM2, CM3, and CM16 in eight cultivars each of common wheat, durum wheat, spelt, emmer, and einkorn grown under the same environmental conditions were quantitated by targeted liquid chromatography-tandem mass spectrometry (LC−MS/MS) and stable isotope dilution assays using specific marker peptides as internal standards. The results were compared to a label-free untargeted LC−MS/MS analysis, in which protein concentrations were determined by intensity based absolute quantitation. Both approaches yielded similar results. Spelt and emmer had higher ATI contents than common wheat, with durum wheat in between. Only three of eight einkorn cultivars contained ATIs in very low concentrations. The distribution of ATI types was characteristic for hexaploid, tetraploid, and diploid wheat species and suitable as species-specific fingerprint. The results point to a better tolerability of einkorn for NCGS patients, because of very low total ATI contents.

KEYWORDS: *α-amylase/trypsin-inhibitor, modern and ancient wheats, mass spectrometry, stable isotope dilution assay, nonceliac gluten sensitivity*

INTRODUCTION

Wheat proteins are classically divided into four fractions, namely albumins, soluble in water, globulins, soluble in 0.5 mol/L sodium chloride, gliadins, soluble in 70% aqueous ethanol, and insoluble glutenins.[1] Some wheat proteins contain amino acid sequences that may trigger celiac disease, wheat allergy and nonceliac gluten sensitivity (NCGS) in susceptible individuals.[2] With a reported prevalence of 0.6−6%,[3,4] NCGS is characterized by gastrointestinal complaints (e.g., abdominal pain, diarrhea, bloating) and extraintestinal symptoms (e.g., headache, chronic fatigue, depression), which disappear on a gluten-free or -reduced diet.[3,5,6] Different constituents of gluten-containing cereals such as fermentable oligo-, di-, and monosaccharides and polyols (FODMAPs), gluten and α-amylase/trypsin inhibitors (ATIs) were suggested as triggers of NCGS. ATIs have been shown to activate the toll-like receptor 4 TLR4-MD2-CD14 complex causing secretion of pro-inflammatory chemokines and cytokines.[7,8] This dose-dependent activation of innate immunity may lead to the symptoms typical of NCGS as well as worsening of pre-existing inflammatory reactions.[8,9]

Genome sequencing of common wheat revealed up to 19 different ATI-encoding genes,[10] of which 13 types have evidence at protein level in common wheat (UniProtKB: 0.19, 0.28, 0.53, CM1, CM2, CM3, CM16, CM17, CMX1/3, CMX2, wheat subtilisin inhibitor, Bowman-Birk type trypsin inhibitor, and chymotrypsin inhibitor WCI). Known as wheat allergens, ATIs account for as much as 2−4% of the protein in common wheat (*Triticum aestivum* L., hexaploid) with a relative distribution of 50% of CM-types, 33% of 0.19 and 17% of 0.28.[11] ATIs are salt-soluble plant-defense proteins located in the seed endosperm and the content of CM3 was influenced both by genetic and environmental factors.[12] The nine to ten cysteine residues form four to five intramolecular disulfide bonds, leading to a compact three-dimensional structure that is responsible for the resistance of ATIs to digestive enzymes of insects and mammals.[13,14] Within ATIs, 0.19 and CM3 from common wheat had the highest bioactivity on TLR4-bearing monocytes, whereas ATI extracts from the "ancient" (hulled) wheat species spelt (*T. spelta* L., hexaploid), emmer (*T. dicoccum* L., tetraploid), and einkorn (*T. monococcum* L., diploid) only reached 30−70% of the activity of common wheat.[7] Furthermore, liquid chromatography-tandem mass spectrometry (LC−MS/MS) showed that einkorn contained very low amounts of ATIs or even none.[15] Although ancient wheats are only produced in small quantities due to low grain yields,[16] they have gained increasing popularity in the last decades due to plant resilience, promotion of biodiversity, and positive consumer perceptions of regional, organic, and healthy.

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The low bioactivity of ancient wheats and the proposed absence of ATIs in einkorn lead to the hypothesis that the ancient wheats spelt, emmer, and einkorn may be better tolerated by NCGS patients, because of lower ATI contents compared to the "modern" (naked) species common and durum wheat (*T. durum* L., tetraploid). To confirm this hypothesis, we aimed to develop a targeted LC−MS/MS method using stable isotope labeled peptides as internal standards (stable isotope dilution assay, SIDA) to quantitate the predominant ATIs 0.19, 0.28, 0.53, CM2, CM3, and CM16 in grains of a set of eight well-characterized common wheat, durum wheat, spelt, emmer, and einkorn cultivars, respectively, grown under the same environmental conditions. To confirm the absolute ATI concentrations determined by SIDA, we also performed a global, label-free and untargeted LC−MS/MS analysis and applied the intensity based absolute quantitation algorithm (iBAQ) to estimate absolute protein abundances for the ATIs.

MATERIALS AND METHODS

Reagents. All reagents were of analytical grade or higher and purchased from VWR Merck (Darmstadt, Germany), Serva (Heidelberg, Germany), LECO (Kirchheim, Germany) or Sigma-Aldrich (Steinheim, Germany). Water was deionized by a water purification system Arium 611VF (Sartorius, Goettingen, Germany). Trypsin (TPCK-treated) and the ATI reference were from Sigma-Aldrich (α-Amylase Inhibitor from *Triticum aestivum* (wheat seed), type I). Unlabeled (marker peptides P1−P5) and stable isotope labeled (internal standards IS1-IS5) peptides (Table S-1 of the Supporting Information, SI) were synthesized by GenScript (Piscataway, NJ, U.S.A.). Each heavy peptide contained at least two [^{13}C]- and [^{15}N]-labeled amino acids (Table S-1). For stock solutions (1 mg/mL in water or dimethyl sulfoxide), the peptides were solubilized according to the manufacturer's guidelines and stored at −80 °C prior to use. The purities of IS1-IS5 were analyzed by ^{13}C quantitative nuclear magnetic resonance spectroscopy (^{13}C qNMR).[17] The purities of P1−P5 were analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC) and UV-detection at 210 nm after alkylation. HPLC peak areas of IS1−IS5 were used as calibrants for P1−P5, respectively.

Grain Samples. Eight cultivars each of common wheat, spelt, durum wheat, emmer and einkorn were cultivated by the State Plant Breeding Institute, University of Hohenheim (Stuttgart, Germany) at Seligenstadt, Germany, and harvested in 2013 (Table S-2).[16] The grains were milled into wholemeal flours using a cross-beater mill (Perten Instruments, Hamburg, Germany). To prepare the common wheat flour mix, grains of 15 cultivars (the eight cultivars above plus seven cultivars from the same growing area) were mixed in equal weights prior to milling. Contents of water, ash, crude protein, and albumins/globulins were already reported by Geisslitz et al.[18]

Identification of ATI Marker Peptides. The extraction of ATIs from five flours of common wheat and one flour of durum wheat, spelt, emmer, and einkorn, respectively, was based on Zevallos et al.[7] and Junker et al.[8] followed by reduction, alkylation, tryptic digestion and untargeted LC−MS/MS-Iontrap analysis as described by Rombouts et al.[19] (for details, see SI).

Sample Preparation for Targeted LC−MS/MS and SIDA. The parameters extraction time, temperature, and solvent, as well as conditions for reduction, alkylation and digestion were optimized using the common wheat flour mix (see SI). This resulted in the following final procedure: Flour (50 mg) was stirred twice with ammonium bicarbonate (Abic) solution (0.5 mL, 50 mmol/L, pH 7.8) for 30 min at 22 °C. After every extraction step the suspensions were centrifuged for 25 min at 3750*g* and the supernatants combined. The extracts were evaporated to dryness and the residue was dissolved in Tris-HCl (320 μL, 0.5 mol/L, pH 8.5) and 1-propanol (320 μL). The IS (50 μL of the mixed solution, 20 μg/mL) were added and reduction was performed by adding tris(2-carboxyethyl)phosphine (TCEP) (50 μL, 0.05 mol/L TCEP in 0.5 mol/L Tris-HCl, pH 8.5) and incubation for 30 min at 60 °C. Cysteine residues were alkylated with chloroacetamide (CAA) (100 μL, 0.5 mol/L CAA in 0.5 mol/L Tris-HCl, pH 8.5) for 45 min at 37 °C in the dark followed by lyophilization. Tryptic hydrolysis (0.5 mL, enzyme-to-substrate ratio 1:50, 0.04 mol/L urea in 0.1 mol/L Tris-HCl, pH 7.8) was performed for 24 h at 37 °C in the dark. The reaction was stopped with 2 μL trifluoroacetic acid. The solution was evaporated to dryness. The residue was dissolved in 1 mL of 0.1% formic acid (FA) and the solution filtered through a 0.45 mm membrane.

Response Lines. Two solutions (100 μg/mL of each peptide), solution 1 with P1−P5 and solution 2 with IS1−IS5, were prepared from the stock solutions. An aliquot of each solution 1 and 2 was reduced with TCEP and alkylated with CAA as described for grain samples. Alkylated solutions 1 and 2 (20 μg/mL of each peptide) were mixed in molar ratios n(P)/n(IS) between 9.1 and 0.1 (9 + 1, 4 + 1, 3 + 1, 1 + 1, 1 + 3, 1 + 4, and 1 + 9) for calibration.

Targeted LC−MS/MS and SIDA. An UltiMate 3000 HPLC system (Dionex, Idstein, Germany) coupled to a triple-stage quadrupole mass spectrometer (TSQ Vantage, ThermoFisher Scientific, Bremen, Germany) was used. An Aqua-C_{18} column (50 × 2 mm, 5 μm, 12.5 nm, Phenomenex, Aschaffenburg, Germany) was used for peptide separation with the following LC conditions: solvent A, FA (0.1%, v/v) in water, solvent B, FA (0.1%, v/v) in acetonitrile; gradient, 0−5 min 10% B, 5−20 min 10−90% B, 20−23 min 90% B, 23−25 min 90−10% B, 25−40 min 10% B; flow rate, 0.2 mL/min; injection volume, 10 μL; column temperature, 22 °C. The ion source was operated in the ESI positive mode and the following source parameters were set: spray voltage, 4500 V; vaporizer temperature, 50 °C; sheath gas pressure, 40 arbitrary units (au); aux gas pressure, 5 au; capillary temperature, 300 °C.[20] Selected reaction monitoring (SRM) was used to analyze the transitions from precursor to product ions using experimentally optimized collision energies (Table S-1).

Data Analysis of SIDA. SRM peak area integration was performed using Skyline 4.1 (MacCoss Lab Software, University of Washington, Seattle, WA, U.S.A.)[21] with manual verification of automated peak integration. The data are publicly available on Panorama Public[22] (https://panoramaweb.org/V8iLUY.url). The ratios between the three transitions (precursor−product ion pairs) were confirmed to be the same for the response lines and the flour samples (±5% absolute deviation, Table S-3), allowing the use of averaged peak ratios from the three transitions, respectively. These constant ratios were additionally used as identification criteria for the marker peptide signals. Response lines (Table S-3) were plotted by linear regression of the peak area ratios A(P1−P5)/A(IS1−IS5) against the molar ratios n(P1−P5)/n(IS1−IS5). All quantitations were performed from three biological replicates with one injection. Contents of ATIs were calculated by multiplying the peptide content with the respective factor ($M_{Protein}/M_{Peptide}$) using the molecular mass of the protein without the signal peptide (Table S-1). For CM2 and CM16, the protein contents were calculated separately from P3/P3c and P5/P5c and summed up. Both ratios were constant at about 95/5 for P3/P3c and 50/50 for P5/P5c. P3c and P5c occurred because of water loss at N-terminal glutamine or glutamic acid after dissolving P3 and P5.

Precision. Repeatability precision was evaluated by analysis of six biological replicates (n = 6) of the common wheat flour mix and intermediate precision with six biological replicates on 3 days (same analyst, same instrument), respectively (n = 18).[23]

Limit of Detection and Limit of Quantitation. To determine the limits of detection (LOD) and quantitation (LOQ), P1−P5 and IS1−IS5 (500 ng, 250 ng, 50 ng, and 25 ng absolute) were added to cassava starch (100 mg) as analyte-free matrix (n = 6). Sample preparation and analysis were the same as described above (extraction volume 2 × 1 mL). The last spike-on step, where the identification criteria (correct ratios of the three transitions) were fulfilled, was used to calculate LOD and LOQ. LOD was defined as three times the standard deviation and LOQ as ten times the standard deviation of this last step.[24] LOD and LOQ were verified by adding P1−P5 and

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Table 1. Repeatability, Intermediate Precision, LOD, LOQ, and Recoveries of Spike, Dilution, and Near LOQ Experiments

			precision (%)[b]		sensitivity (μg/g)[c]		recovery (%)[d]		
no.	amino acid sequence[a]	ATI-type	repeatability	intermediate	LOD	LOQ	spike	dilution	near LOQ
P1	LQCNGSQVPEAVLR	0.19 + 0.53	1.8	2.2	0.5	1.5	93.5	99.8	105.3
P2	LQCVGSQVPEAVLR	0.28	3.9	6.8	0.1	0.3	77.9	97.4	90.5
P3	EYVAQQTCGVGIVGSPVSTEPGNTPR	CM2	3.4	5.2	0.9	2.7	76.3	96.3	113.6
P3c	qYVAQQTCGVGIVGSPVSTEPGNTPR	CM2	11.0	27.6	6.8	20.5	29.1	<LOD	78.3
P4	SGNVGESGLIDLPGCPR	CM3	5.3	6.7	0.2	0.7	120.9	92.3	78.9
P5	QQCCGELANIPQQCR	CM16	3.3	5.2	1.6	4.7	85.2	101.9	80.7
P5c	qQCCGELANIPQQCR	CM16	3.9	4.1	0.9	2.9	99.7	97.0	107.5

[a]q, pyroglutamyl. [b]Precision in common wheat flours, repeatability: six biological replicates on 1 day (n = 6), intermediate: six biological replicates on 3 days (n = 18). [c]Limits of detection of peptides in cassava starch, LOD, limit of detection, LOQ, limit of quantitation. [d]Spike, spiking of P1−P5 to common wheat flour (spiking level: + 75%); dilution, common wheat flour diluted with cassava starch (1 + 9; dilution level: −90%); near LOQ, spiking of P1−P5 to cassava starch (spiking level: P1, P2, and P4 < 1% and P3 and P5 < 10% of normal content in common wheat).

IS1−IS5 six times to cassava starch in the concentrations calculated for LOD and LOQ and calculation of recovery near LOQ (Table 1). In a second experiment, the common wheat flour mix was diluted 1 + 9, 1 + 49, and 1 + 99 with cassava starch in triplicates and each sample (n = 9) was analyzed and evaluated as described above.

Recovery. Recovery was determined by means of (a) spiking experiments of P1−P5 to the common wheat flour mix and (b) dilution of the common wheat flour mix with cassava starch. For the spiking experiments, a mixed solution of P1−P5 was prepared to spike to +75% of P1−P5. Twelve samples of the common wheat flour mix (50 mg) were extracted two times with Abic buffer, and the supernatants were lyophilized. Then, IS1−IS5 were added to all samples, and the mixed solution of P1−P5 (100 μL) was added to six of the 12 samples followed by sample treatment as described. Recovery was calculated by comparison of the theoretical amounts with the difference between samples with and without addition of P1−P5. For the dilution experiments, the recovery was calculated from the common wheat flour/cassava starch sample (1 + 9).

Sample Preparation of ATI Reference and Flour Samples for Untargeted nanoLC−MS/MS (iBAQ). The protein content of the ATI reference was determined by RP-HPLC−UV according to Geisslitz et al.[18] For in-gel digestion, the ATI reference was dissolved in ultrapure water, incubated with sample buffer, reduced, alkylated, and run on a 4−12% NuPAGE gel prior to in-gel tryptic digestion following standard procedures.[25] The resulting peptides were lyophilized, dissolved in 0.1% FA and analyzed by nanoLC−MS/MS-Orbitrap. Flours (50 mg) of two cultivars of each wheat species were treated as described for targeted LC−MS/MS and SIDA. The peptide concentrations were determined by Nanodrop (One, Thermo Scientific, Madison, U.S.A.) and 0.5 μg total peptide amount was injected per nanoLC−MS/MS-Orbitrap run.

Untargeted nanoLC−MS/MS (iBAQ). An Eksigent nanoLC-Ultra 1D+ system (Eksigent Technologies, Dublin, CA, U.S.A.) was coupled online to a LTQ-Orbitrap Velos mass spectrometer (ThermoFisher Scientific). Using a flow rate of 5 μL/min of solvent A_1 (0.1% FA in water), peptides were loaded onto a trap column (ReproSil-Pur 120 ODS-3, 5 μm, 2 cm × 75 μm, Dr. Maisch, Ammerbuch-Entringen, Germany). Subsequently, peptides were separated on an analytical column (ReproSil-Gold 120 C_{18}, 3 μm, 40 cm × 75 μm, Dr. Maisch) using a flow rate of 300 nL/min and a gradient from 2% to 32% solvent B (solvent A: 0.1% FA and 5% DMSO in water, solvent B: 0.1% FA and 5% DMSO in acetonitrile) in 110 min. The eluate from the analytical column was sprayed via a stainless steel emitter (ThermoFisher Scientific) into the MS at a source voltage of 2.2 kV. The transfer capillary was heated to 275 °C. The Orbitrap Velos was set to data-dependent acquisition in positive ion mode, automatically selecting the 20 most intense precursor ions from the preceding full MS (MS1) spectrum with an isolation width of 2.0 m/z for fragmentation using CID at 35% normalized collision energy and subsequent identification by MS/MS (MS2). MS1 (360−1300 m/z) spectra were acquired in the Orbitrap using a resolution of 60,000 (at 400 m/z) and MS2 spectra in the ion trap. Dynamic exclusion was set to 60 s. The peptides and proteins in ATI reference and flour samples were identified and relatively quantitated using MaxQuant (version 1.5.3.30) by searching the MS data against a wheat protein reference database derived from UniprotKB (004565_triticum_aestivum_uniprot-proteome%3AUP000019116, download 11.05.17, protein entries 136867) using the search engine Andromeda.[26] Variable modifications included oxidation of methionine and N-terminal protein acetylation. Carbamidomethylation on cysteines was specified as fixed modification and trypsin as proteolytic enzyme with up to two allowed missed cleavage sites. Match-between-runs (matching time window 0.7 min, alignment time window 20 min) was enabled, and the results were filtered for a minimal length of seven amino acids and 1% peptide and protein false discovery rate. To investigate the differences in concentration for the identified proteins within one sample, we estimated absolute protein intensities using the iBAQ algorithm implemented within MaxQuant.[27] A total sum normalization of iBAQ protein intensities between samples was performed to correct for different total protein injection amounts.

Statistics. Principal component analysis (PCA), Grubbs' test for outliers and one-way analysis of variance (ANOVA) with Tukey's test were performed with Origin 2017 (OriginLab, Northampton, Massachusetts, U.S.A.).

RESULTS AND DISCUSSION

Identification of ATI Marker Peptides. The first step to develop a targeted LC−MS/MS SIDA method (abbreviated as SIDA in the following) to quantitate the five major ATIs in common wheat, spelt, durum wheat, emmer, and einkorn was to identify ATI marker peptides that are present in all wheat species. ATIs were extracted from wholemeal flours of each wheat species, reduced, alkylated and hydrolyzed with trypsin followed by untargeted LC−MS/MS-Iontrap analysis. In total, 19 peptides were identified for the ATIs 0.19, 0.28, 0.53, CM2, CM3, CM16, and CM17 in common wheat, durum wheat, spelt, and emmer, but not in einkorn (Table S-4). The peptides were unique for each different ATI type, except for P1 that occurred in 0.19 and 0.53. Furthermore, the database search showed that some of the marker peptides also occur in other cereal species such as rye and barley. No unique peptide was identified for 0.19, because the peptides all belonged to uncharacterized ATI-like proteins. One peptide that was only present in 0.19 and not in 0.53 was not suitable either, because it was not present in durum wheat and emmer. Thus, 0.19 and 0.53 could not be differentiated and the content of P1 represents the sum of both. The marker peptide for CM17, which was found in common wheat and spelt, could not be synthesized. The final five marker peptides P1−P5 (Table S-1)

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J. Agric. Food Chem. 2018, 66, 12395−12403

were chosen, because of high scores in all wheat species, reproducible tryptic cleavage, good MS response, and high stability during storage at −80 °C.

Targeted LC−MS/MS and SIDA. To develop the SIDA, full-scan mass spectra of P1−P5 and IS1−IS5 were acquired to define suitable precursor ions for targeted LC−MS/MS. In general the 2+ charge states of the precursor ions had the highest intensity and were therefore selected except for P3 and IS3 (3+). Next, the selected precursor ions were fragmented to define the three most abundant transitions for SRM, including at least one m/z higher than the m/z of the precursor ion for improved selectivity. The collision energies of the three selected SRM transitions were experimentally optimized to obtain the highest peak area of the product ions (Table S-1). The purities of the unalkylated IS were determined by ^{13}C qNMR. The purity of IS3 was very low (13.0%) in contrast to the other IS (IS1, 80.6%; IS2, 69.3%; IS4, 68.5%; IS5, 72.0%) (Table S-3). The purities of alkylated P1 to P5 were 90.1%, 91.5%, 28.7%, 81.0% and 83.2%, respectively. These values were taken into account for all calculations. The intercepts of all response lines were close to 0.0 and the slopes were between 0.7 and 1.2, with the exception of P3c/IS3c and P5/IS5 (0.6). The response lines were reanalyzed weekly together with the samples and only slight variations were observed (Table S-3). Ideally, the slope should be 1.0 in SIDA, but the ranges observed here were consistent with earlier reports.[20]

Optimization of Sample Preparation for SIDA. Details of the performed sample optimization can be found in the SI. Each parameter was systematically varied and the procedure resulting in higher absolute peak areas of marker peptides was chosen. A common wheat flour mix consisting of 15 cultivars was used to even out cultivar-specific variations. First, the ATI extraction overnight at 4 °C[7] was shortened and simplified to two times 30 min at 22 °C without significant changes in peak areas. A third extraction step was not necessary, because over 97% of ATIs were extracted in the first and second step. Reducing the sample weight from 500 mg to 50 mg led to higher peak areas (+100%) and smaller standard deviations (−50%) due to better homogenization by the stirring bar in the sample vessels. The sample weight of 50 mg appeared to be the best compromise between a homogeneous distribution of ATIs in the flour and low amounts of IS required, which is why no lower sample weight was tested. Instead of the Abic buffer, solutions based on phosphate buffer, 60% ethanol (v/v), and 50% 1-propanol (pH 7.6, v/v) under reducing conditions (glutenin solution) were tested. The peak areas were similar for P1 and P4 with both Abic and phosphate buffer, but the Abic buffer resulted in higher peak areas for P2 (+20%), P3 (+15%), and P5 (+70%). Stepwise extraction of the flour first with Abic buffer, followed by 60% ethanol and then glutenin solution showed that ≥90% of P1−P5 were detected in the Abic solution, < 8% in 60% ethanol and <2% in the glutenin solution. This confirmed the exhaustive extraction of ATIs using Abic buffer.[7] The use of CAA for alkylation gave slightly higher peak areas (≤+10%) compared to iodoacetamide, so that CAA was chosen. The average efficiency of alkylation was >99%. To ensure complete tryptic hydrolysis, different incubation times (2, 4, 6, 18, 21, and 24 h) were tested. Peak areas increased (≥70%) between 2 and 18 h, but then a plateau was reached (≤ ± 8%) between 18 and 24 h. An incubation time of 24 h was chosen.[19]

Precision, LOD, LOQ, and Recovery. Precision, LOD, LOQ, and recovery of the SIDA were evaluated (Table 1) using the final method. Except for P3c, very good values for repeatability (1.8−5.3%) and intermediate precision (2.2−6.8%) were obtained. The comparatively poor precision of P3c was due to contents close to the LOQ. Wheat starch, vital gluten and cassava starch were considered as possible matrices to determine LOD and LOQ. Targeted LC−MS/MS showed the absence of marker peptides and signal interferences only in cassava starch and excluded wheat starch and vital gluten. The marker peptides were detected with high sensitivity, resulting in an LOD between 0.1 and 1.6 μg/g and an LOQ between 0.3 and 4.7 μg/g. The only outlier was A3c with an LOD of 6.8 μg/g and an LOQ of 20.5 μg/g. These limits agreed well with those reported for gluten peptides from common wheat.[20] LOD and LOQ were additionally confirmed by spiking the calculated concentrations of LOD and LOQ to cassava starch. The concentrations at LOD still fulfilled the identification criteria, because the three ratios of precursor to product ions were constant. Furthermore, concentrations at LOQ also showed acceptable recoveries (78.3−113.6%, Table 1). Additionally, the dilution experiments of common wheat flour with cassava starch confirmed the detection limits. It was possible to detect P1, P2, and P4 in the 1 + 99 (flour + starch) mix and the other marker peptides in the 1 + 9 mix. The concentrations of P1, P2, and P4 matched the LOD in the 1 + 99 mix and those of P3 and P5 matched the LOQ in the 1 + 9 mix. The recovery in the 1 + 9 mix (92.3−101.9%, Table 1) substantiated the good performance of the extraction and the developed SIDA. The recovery, proven by spiking experiments to common wheat, revealed good ranges for the majority of marker peptides (76.3−120.9%, Table 1). The poor recovery (29.1%) of A3c was again due to very low concentrations in common wheat.

Comparison of SIDA with Untargeted nanoLC−MS/MS-Orbitrap (iBAQ). To confirm the quantitative accuracy of the SIDA results, we performed a label-free and untargeted nanoLC−MS/MS analysis and applied the iBAQ algorithm to estimate absolute protein abundances. A key advantage of this algorithm, which is implemented within MaxQuant, is that iBAQ intensities of different proteins can directly be compared to each other within one sample.[27] Because MaxQuant uses protein masses with signal peptide from the UniProtKB database, both SIDA and iBAQ peptide contents were converted to protein concentrations with the appropriate factor including the signal peptide for better comparability. First, a commercially available ATI reference, which consisted of different ATIs and other proteins, was analyzed by the established SIDA. The ATI reference was directly dissolved, reduced, alkylated and hydrolyzed with trypsin. The IS were added prior to reduction. The advantage of SIDA is that losses during the preparation, e.g. filtration, are considered, but the usage of labeled peptides does not compensate for incomplete digestion or peptide modifications. The efficiency of digestion was checked by LC-QTOF-MS and SDS-PAGE. In both cases no molecular masses typical of ATIs were detected after enzymatic hydrolysis (data not shown). The total ATI content (sum of 0.19 + 0.53, 0.28, CM2, CM3, and CM16) was 188.2 mg/g based on SIDA. The most abundant ATIs were 0.19 + 0.53 with 79.3 mg/g and the least abundant one was CM2 with 8.5 mg/g (0.28: 48.4 mg/g; CM3: 31.3 mg/g and CM16: 20.7 mg/g). The ATI reference consisted of 38% protein and 62% other ingredients such as starch, minerals and other small molecules. Considering the total protein content in the ATI reference of 380.0 mg/g determined by RP-HPLC-UV, 49.5%

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J. Agric. Food Chem. 2018, 66, 12395−12403

of proteins belonged to the analyzed ATIs (188.2 mg/g of 380.0 mg/g). The distribution of the different ATIs (20.9% 0.19 + 0.53, 12.7% 0.28, 2.2% CM2, 8.2% CM3, and 5.5% CM16; 50.5% other proteins) in the ATI reference was hard to compare to that in real grain samples,[7,13] because the preparation procedure is unknown. Nevertheless, the contents of 0.19 + 0.53 and CM3 (58.8%) made up more than 50% of the total ATI content, as has been reported earlier (Figure 1).[7]

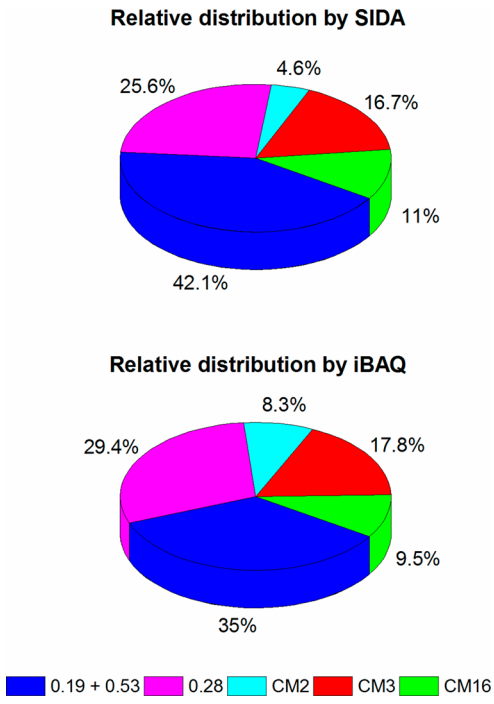

Figure 1. Comparison of relative concentrations of ATIs 0.19 + 0.53, 0.28, CM2, CM3, and CM16 in the ATI reference determined by SIDA and iBAQ.

The proportion of the other ATIs were lower (0.28:25.6%; CM2:4.6%; CM16:11.0%). Then, all iBAQ protein intensities of all identified proteins were summed up, and the percentages of single ATIs were calculated considering this normalized total iBAQ intensity. The predominant ATIs were 0.19 + 0.53 with 17.7% followed by 0.28 (14.9%), CM3 (9.0%), CM16 (4.8%), and CM2 (4.2%). In total, 50.6% of identified proteins belonged to the ATIs of interest by iBAQ (other identified proteins: nonspecific lipid transfer protein, β-amylase, thioredoxin H, and others), which agreed perfectly with the 49.5% determined by SIDA. Some differences in the distribution of ATIs were detected (Figure 1), especially for CM2 (SIDA: 4.6%, iBAQ: 8.3%). Small differences were obtained for 0.19 + 0.53, which was higher by SIDA (+15%), and for 0.28, which was higher by iBAQ (+14%), but the contents of CM3 and CM16 agreed very well. Reasons for the differences could be the low accuracy of the iBAQ algorithm, because it is assumed that the distribution of peptide intensities per protein is constant between all proteins, which is not true for all proteins.

All in all, the results of iBAQ and SIDA appeared to be comparable for the ATI reference and so this comparison was expanded to two cultivars per wheat species (Figure 2). Einkorn is not shown, because the iBAQ intensities were at least 1000-fold lower than those of the other wheat species, SIDA results were below the LOD and no peptides were identified in Skyline. The percentages of 0.19 + 0.53 were perfectly comparable for both methods in all samples, but some differences between iBAQ and SIDA were detected for the other ATIs. Higher percentages of CM2 and lower percentages of CM16 were detected in durum wheat and emmer by iBAQ compared to SIDA, while the values for 0.28 were higher in all wheat species. The values for CM2 and CM16 matched well for common wheat and spelt, and those of CM3 for durum wheat and emmer. Compared to untargeted LC−MS/MS, SIDA is less affected by sample complexity and background and typically has lower LODs, wider dynamic range and excellent repeatability and reproducibility, because of nonredundant targeted data acquisition.[28] However, one major drawback is that everything except the predefined marker peptides is filtered out,[29] even if there may be naturally

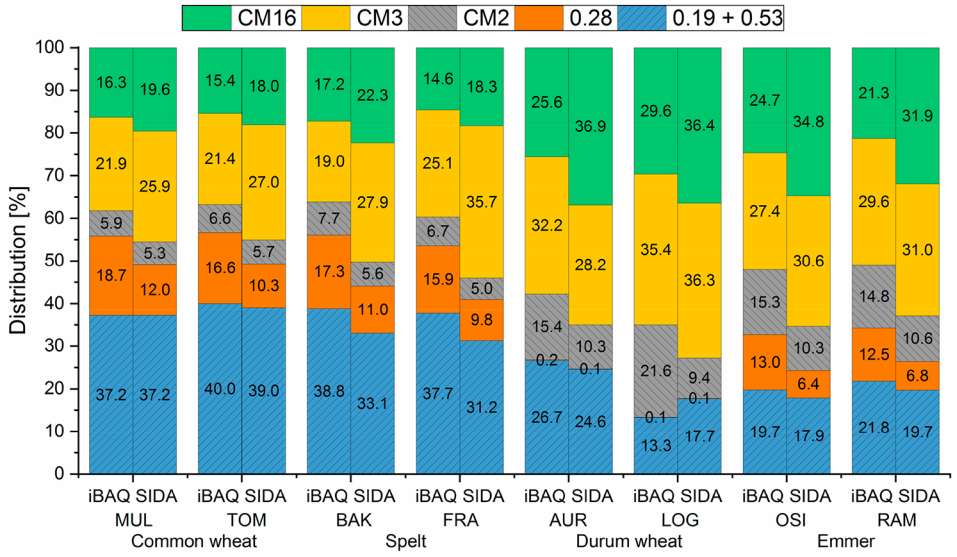

Figure 2. Comparison of the distribution of ATI types of two cultivars (cv.) each of common wheat, spelt, durum wheat and emmer analyzed by iBAQ and SIDA. AUR, cv. Auradur, BAK, cv. Badenkrone, FRA, cv. Franckenkorn, LOG, cv. Logidur, MUL, cv. Mulan, OSI, cv. Osiris, RAM, cv. Ramses, TOM, cv. Tommi.

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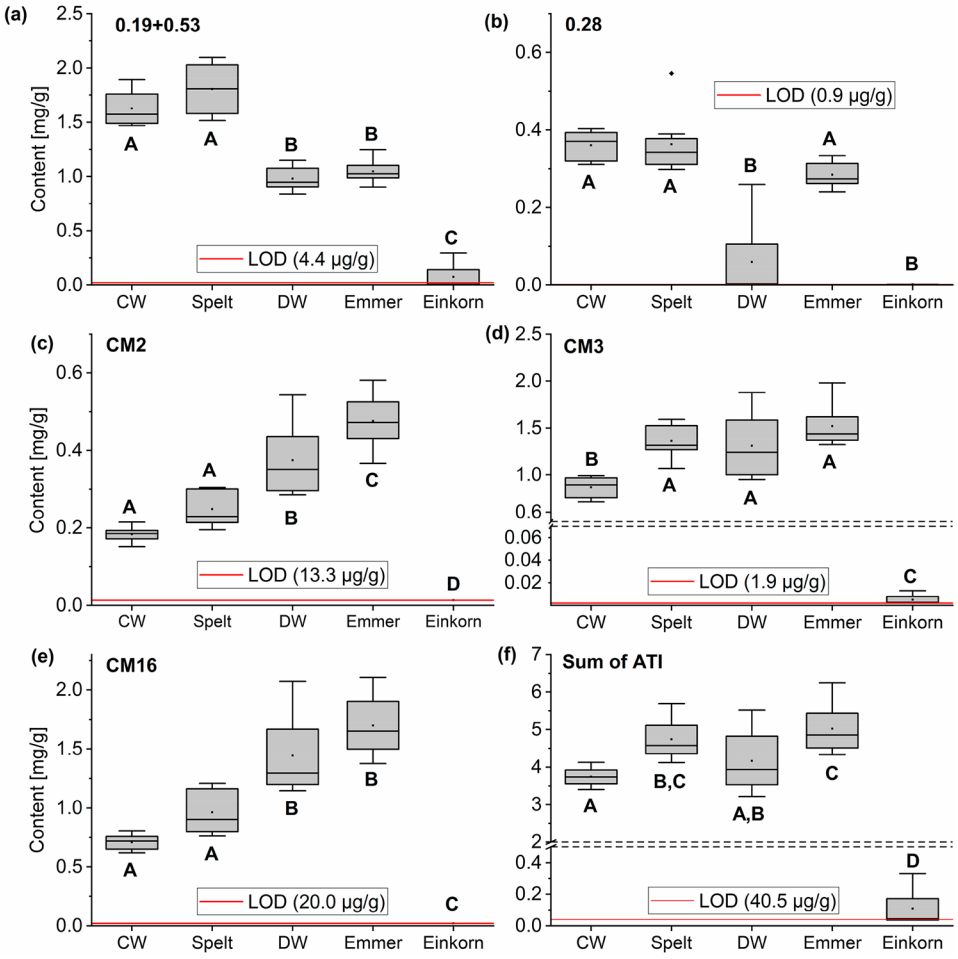

Figure 3. Contents of (a) 0.19 + 0.53, (b) 0.28, (c) CM2, (d) CM3, (e) CM16, and (f) the sum of ATI types of eight cultivars per wheat species. Data are shown as box plots with median (line in the box), average (point in the box), outliers (points outside the box), minima and maxima (whiskers), and limit of detection (LOD, red line).

occurring variations resulting in amino acid substitutions or deletions. These are in turn picked up by iBAQ, so that both methods are complementary, even though iBAQ also has drawbacks, e.g., due to irreproducible ion selection during data-dependent acquisition.[30] Due to the overall good agreement of SIDA and iBAQ, the quantitative accuracy of SIDA was substantiated.

SIDA Quantitation of ATIs in Different Wheat Species. The ATI contents of flours of eight cultivars per wheat species (40 samples grown at the same location), which had already been characterized in detail for protein composition,[18] were analyzed by SIDA (Figure 3, Table S-5).

The contents of ATIs 0.19 + 0.53 were significantly higher in common wheat (1.47−1.89 mg/g) and spelt (1.51−2.08 mg/g) than in durum wheat (0.84−1.15 mg/g) and emmer (0.90−1.25 mg/g), and they were detectable in three out of eight einkorn cultivars, but with low contents (0.12−0.29 mg/g). Common wheat (0.31−0.40 mg/g), spelt (0.30−0.55 mg/g), and emmer (0.24−0.33 mg/g) had similar contents of ATI 0.28. Within durum wheat, two out of eight cultivars (LUN and WIN, 0.21 mg/g and 0.26 mg/g) had comparable contents of ATI 0.28 to common wheat, spelt and emmer, but the content was near the LOD or even below (<6.6 µg/g) in the other six cultivars. The analysis of two of these six durum wheat cultivars (AUR and LOG) by iBAQ (Figure 2) confirmed the same low content of 0.28. Therefore, all peptides of 0.28 were not detectable (<LOD), including the marker peptide. ATI 0.28 was detected in three out of eight einkorn cultivars, but below the LOQ (<2.6 µg/g). The content of CM2 was highest in emmer (0.37−0.58 mg/g), followed by durum wheat (0.29−0.54 mg/g), then common wheat (0.17−0.21 mg/g) and spelt (0.20−0.30 mg/g) and einkorn (<13.3 µg/g, LOD). The content of CM3 was significantly higher in spelt (1.07−1.59 mg/g), durum wheat (0.95−1.88 mg/g) and emmer (1.32−1.98 mg/g) than in common wheat (0.77−0.98 mg/g). CM3 was detected in four out of eight einkorn cultivars, but in very low concentrations near the LOQ (7.8−12.9 µg/g) or even below. CM16 was more abundant in durum wheat (1.15−2.07 mg/g) and emmer (1.38−2.10 mg/g) than in common wheat (0.62−0.81 mg/g) and spelt (0.76−1.21 mg/g), while the contents were < LOD in einkorn. The total ATI content was 3.4−4.1 mg/g in common wheat, 4.1−5.7 mg/g in spelt, 3.2−5.5 mg/g in durum wheat, 4.4−6.3 mg/g in emmer and between the LOD (40.5 µg/g) and 0.3 mg/g in einkorn. The ATI contents in common wheat were in accordance with the 2−4% of wheat proteins determined by 2D-gel electrophoresis.[13] Assuming a typical protein content of 10−15% in common wheat flours, this results in an absolute predicted ATI content of about 2−6 mg/g, which is in agreement with the contents detected in this study. The total ATI content of einkorn was below 10% in comparison to the other wheat species and no ATIs were

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detected in three einkorn cultivars. A study on gene expression of ATIs suggested that ATIs are expressed in low amounts or not at all in einkorn and another study reported very low MS-intensities of CM2, CM3, and 0.28 and even no signal for 0.19.[15,31] We can confirm the absence or very low amounts of the five ATIs in einkorn, especially of the most bioactive ATIs 0.19 and CM3.[7] This observation was corroborated by SDS-PAGE of flour and ATI extracts (Figure S-1). The ATI contents of the eight durum wheat cultivars showed a higher variability compared to the other wheat species and so only few significant differences to the other wheat species were detected. Such high variability among durum samples had also been found for CM3 contents, with ranges from 0.22−1.11 mg/g.[12] In contrast, the total ATI content of spelt and emmer was significantly higher than that of common wheat. Thus, the hypothesis that ancient wheat species (einkorn, emmer and spelt) have lower ATI contents than modern wheat species (common wheat and durum wheat) was partly confirmed and partly refuted. The eight einkorn cultivars did have significantly lower ATI contents than the other wheat species, but the ATI contents of the eight cultivars of spelt and emmer were even significantly higher than those of common wheat. Interestingly, ATI extracts of spelt and emmer showed lower biological activity in cell assays compared to those of common wheat.[7] However, our results on ATI concentrations determined by SIDA are hard to compare to those of biological activity, because different flours were used and ATI contents are known to be affected by both genetics and environment.[12] Our study is the first to report ATI contents in a set of 40 well-characterized samples grown at one location in one year to minimize environmental effects and thus enable a direct comparison between species and also individual cultivars.

Statistical Analysis. Linear regression was performed between contents of total ATI, crude protein, and albumins/globulins (Table S-5).[17] Considering all samples (n = 40), no correlation was observed between total ATI and albumins/globulins (r = −0.096) or crude protein contents (r = −0.036). Because the total ATI content in einkorn was below or near the LOD, linear regression was also performed without einkorn (n = 32), but this gave no correlation either (albumins/globulins : r = −0.155; crude protein: r = 0.045). There were also no correlations between total ATI contents and albumins/globulins or crude protein contents within each wheat species (n = 8, r < 0.3). These findings were recently confirmed for 22 common wheat cultivars.[32] Therefore, it was not possible to predict total ATI contents from protein or albumins/globulins contents, confirming the necessity and high relevance of our new SIDA.

A PCA based on the contents of each individual ATI and total ATI of the 40 samples was performed to assess whether these variables could be used to differentiate between common wheat, spelt, durum wheat, emmer and einkorn (Figure 4). Both principal components together accounted for 95.0% of data variability. All variables were positively correlated to component 1 and the CM-ATIs were correlated negatively to component 2. In contrast, ATIs 0.19, 0.28, and 0.53 were positively correlated to component 2. A distinct clustering of einkorn cultivars in the opposite direction of the vector belonging to the total ATI content was observed due to their very low ATI contents. Common wheat and spelt cultivars were located together in the upper rectangle and durum wheat and emmer cultivars in the lower rectangle of the plot. Therefore, it was possible to differentiate between the

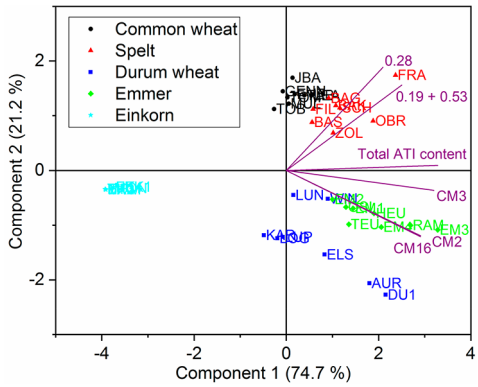

Figure 4. Principal component analysis biplot of data for total ATI and individual ATI contents in eight cultivars each of common wheat, spelt, durum wheat, emmer, and einkorn.

hexaploid wheat species common wheat and spelt and the tetraploid wheat species durum wheat and emmer using their positions in the PCA plot. A closer look at the distribution of ATIs revealed that the ratio of CM-ATIs to the sum of 0.19 + 0.53 and 0.28 was about 50:50 (43.4−56.7% for CM-ATIs) in the hexaploid wheat species and about 75:25 (70.2−79.9% for CM-ATIs) in the tetraploid wheat species.

Taken together, the targeted LC−MS/MS method based on SIDA for the absolute quantitation of five ATIs in different wheat species performed well in terms of precision, detection limits, and recovery. The analysis of ATI contents in five wheat species and eight cultivars each grown under the same environmental conditions revealed that einkorn contained very low amounts of ATIs. In contrast, spelt and emmer had higher overall contents of ATIs than common wheat, with durum wheat in between. The results point to a better tolerability of einkorn for NCGS patients, because of very low overall ATI contents. However, this cannot be stated for spelt, because total ATI contents were higher compared to common wheat, including CM3 and 0.19 as most bioactive ATIs. Further in-depth investigations using well-defined grain samples such as the ones used here are necessary to correlate ATI contents and in vitro and in vivo bioactivity to allow a precise assessment of tolerability of certain wheat species and cultivars for NCGS patients.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.8b04411.

Expanded experimental procedures, Tables S-1 to S-5, Figure S-1, LC−MS/MS data on Panorama Public (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

Abic, ammonium bicarbonate; ANOVA, one-way analysis of variance; ATI, α-amylase/trypsin inhibitor; ^{13}C qNMR, ^{13}C quantitative nuclear magnetic resonance spectroscopy; CAA, chloroacetamide; FA, formic acid; FODMAPs, fermentable oligo-, di-, and monosaccharides and polyols; iBAQ, intensity based absolute quantitation algorithm; LOD, limit of detection; LOQ, limit of quantitation; NCGS, nonceliac gluten sensitivity; PCA, principal component analysis; SIDA, stable isotope dilution assay; SRM, selected reaction monitoring; TCEP, tris(2-carboxyethyl)phosphine

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4 General discussion

Five wheat species were classified into modern (common and durum wheat) and ancient wheat species (spelt, emmer and einkorn). The background of this classification was that einkorn, emmer and spelt were cultivated in high amounts in the past and are nowadays cultivated in very low amounts. The modern wheats common wheat and durum wheat are cultivated and used in very high and moderately high amounts today, respectively.

In this study, the sample set consisted of eight cultivars each of common wheat, spelt, durum wheat, emmer and einkorn, which were cultivated at the same location. The advantage of this sample set was that all samples were grown under the same conditions and the reported differences were due to the genetic background (species/cultivar) and not due to geographic or environmental effects. The variation in e.g. protein content, gluten protein composition and ATI content is influenced for up to two-thirds by environmental factors and one-third by genetic factors and the interaction between the two factors [4.1]. The data set fulfilled all requirements for the direct comparison of all observed differences between genotypes as it has been demanded recently [4.2].

The aim of this study was divided into two parts. In the first part, the focus was on the gluten protein composition and the influence of these proteins on the baking quality. In the second part, the focus was on the quantitation of ATIs. ATIs are the potential triggers of NCGS. It was reported that ancient wheats might be better tolerated by NCGS patients, which led to the hypothesis that ancient wheats might have lower ATI contents than modern ones.

4.1 Gluten protein composition and aggregation properties as predictors for bread volume of common wheat, spelt, durum wheat, emmer and einkorn

4.1.1 Properties of breads from modern and ancient wheats

The bread volume and the form factor (width/height) were analysed by a laser-based scanner. Breads made of common wheat and spelt flour had higher bread volumes than those made of durum wheat, emmer and einkorn. However, the breads of common wheat, durum wheat and einkorn were more spherical than those of spelt and emmer, which were flatter. In this study, the doughs made of wholemeal flours of all five wheat species were kneaded according to the standard parameters for white flours with the optimal dough consistency at 550 BU and 22 °C. This was done to ensure comparability of the results between the flours. However, optimised parameters during dough mixing and resting might surely give higher bread volumes especially for emmer and einkorn.

4.1.2 Protein composition of modern and ancient wheats

The proteins of the five wheat species were extracted both according to the modified Osborne fractionation and the GMP fractionation. The resulting Osborne fractions (ALGL, GLIA and GLUT) were analysed by RP-HPLC (separation according to polarity) and the SDSS and GMP fractions by GP-HPLC (separation according to molecular weight).

Higher contents of GLIA were observed for spelt, emmer and einkorn than for common wheat. This was not the case for the ALGL content in spelt, emmer and einkorn. A possible reason is that single proteins of the ALGL fractions were more enriched in ancient wheats, which lead to higher single peaks and detector saturation. The chromatographic pattern of the ALGL fraction showed the greatest differences in the elution time range (7.5 – 9.0 min) of ATIs. This time range was characterised by low peak intensity and a low diversity of the peak pattern for all einkorn cultivars. Considering the lower injection volume for spelt and emmer than that for common wheat, the peak intensity was higher in this elution time range for spelt and emmer than that for common wheat. Even if the elution period of ATIs was identified by comparison with the commercially available reference protein (α-amylase-inhibitor from wheat), no accurate and reliable quantitation of ATIs was possible by RP-HPLC. This was confirmed by the missing correlation of ALGL contents (RP-HPLC) and ATI contents (LC-MS/MS) ($r = -0.096$). There are approaches to quantify ATIs by RP-HPLC after ultracentrifugation with molecular weight cut-off membranes [4.3]. This approach has the disadvantages that all proteins with molecular weights lower than the cut-off are quantitated and high losses may occur during ultrafiltration due to high protein retention at the membrane.

Differences in the patterns of ω5- and ω1,2-gliadins of einkorn cultivars compared to those of the other wheat species were detected. The ω5-gliadins were enriched in einkorn, whereas the ω1,2-gliadins were more abundant in common wheat, spelt, durum wheat and emmer. The ratio of ω5- to ω1,2-gliadins was not correlated to the bread volume and has, therefore, no influence on the baking quality. The reason for this could be the absence of cysteine residues in these GLIA types, so that polymeric aggregates cannot be formed and complex network formation does not occur [4.4].

The ωb-gliadins were only present in common wheat and were nearly absent in spelt, durum wheat, emmer and einkorn. Due to the relatively low ωb-gliadin content and low difference between the wheat species, statements about their influence on the baking quality were hard to make. The pattern of the HMW-GS differed between hexaploid, tetraploid and diploid wheats. The chromatograms of hexaploid wheats had the highest number of peaks followed by the tetraploid wheats and the diploid wheat with the lowest number. This is obvious because the number of HMW-GS types is encoded on the genome (AA, BB and DD) [4.5]. Einkorn only

has the A genome (one HMW-GS), the tetraploid wheats have the A and B genomes (up to three HMW-GS) and the hexaploid wheats have the A, B and D genome (up to five HMW-GS).

The separation efficiency of the GP-HPLC experiments was poorer than that of RP-HPLC. The chromatograms of GP-HPLC showed lower numbers of peaks, which were not clearly separated and distinguishable compared to RP-HPLC. For the GP-HPLC analysis, the composition of the elution solvents was changed from SDS (1 %) containing phosphate buffer (0.05 mol/L, pH 6.9) as described by Thanhaeuser et al. (2014) to 50 % acetonitrile in water [4.6]. The SDS phosphate buffer had the disadvantage of column blockage due to salt crystals, which lead to the necessity of long washing periods of the column. As described in literature, 50 % acetonitrile was suitable for the separation of proteins and contrary to the description in literature did not lead to retention time shifts (Figure 15) [4.7]. The benefits using 50 % acetonitrile for longer column lifetimes and fewer HPLC downtimes outweighed the slightly poorer peak separation

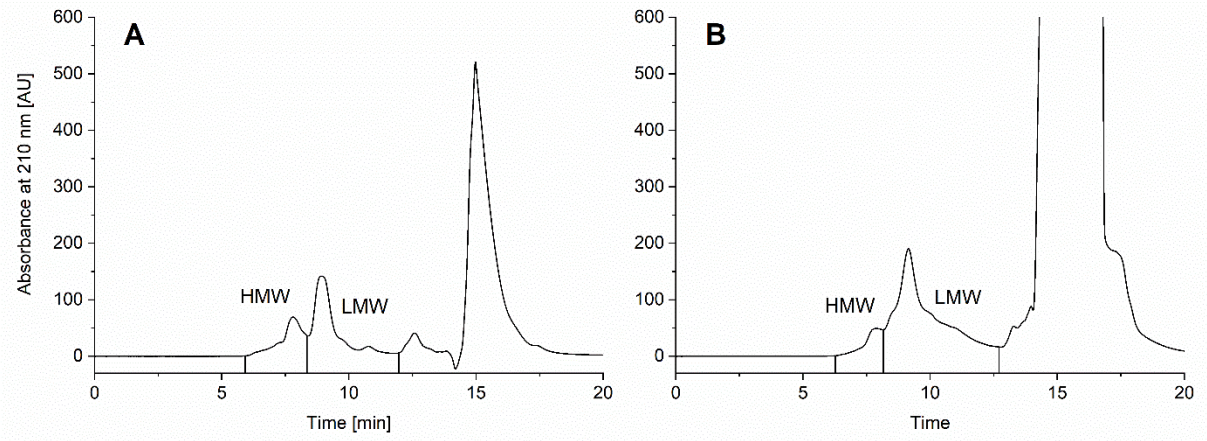

Figure 15: Comparison of injections of glutenin macropolymer (GMP) fractions (own data). A, the elution solvent contained 1 % sodium dodecyl sulphate (SDS) and phosphate (0.05 mol/L); B, the elution solvent was a mixture of 50 % acetonitrile in water. The peak patterns of both chromatograms were very similar. Differences in the ratio between the first and the second peak are due to different concentrations and different common wheat cultivars. The highest peaks were caused by the reducing agent dithiothreitol (DTT), respectively. HMW, high-molecular-weight; LMW, low-molecular-weight.

4.1.3 Gluten protein composition as predictor of the baking quality of modern and ancient wheats

In general, the form factor was not correlated with any parameter of the analytical part (e.g. GLIA, GLUT, HMW-GS and GMP contents). Thus, the form factor and the shape of the breads were not predictable by any of these parameters. The reasons for this might be that not only the composition of gluten proteins is responsible for the flat bread shape especially of spelt and emmer, but other factors such as mixing time, rest period or an uneven formation of the starch-gluten-network.

However, the bread volume was correlated with many analytical parameters. HMW-GS (r = 0.864), GLIA/GLUT (r = - 0.829) and GLUT (r = 0.804) showed the highest correlation coefficients to the bread volume. In contrast, no correlation was observed between the bread volume and the protein content (r = - 0.203), ALGL (r = - 0.123) and gluten content (r = 0.164). For this reason, not the amount of total proteins or the amount of gluten proteins was responsible for a high bread volume, but the composition of the gluten proteins. High contents of GLUT, HMW-GS and GMP (r = 0.652) led to high bread volumes. In contrast to this, high amounts of GLIA (r = - 0.700) led to low bread volumes, which is expressed by a negative correlation. This is displayed in the ratio of GLIA/GLUT. A high GLIA/GLUT and therefore a surplus of GLIA tends to give low bread volumes. As shown in Figure 10, the gliadins are the softeners for the glutenins [4.8]. A high content of GLIA and a low content of GLUT (high GLIA/GLUT) are the reason for softer doughs with more difficult handling properties during the baking process. Compared to common wheat (100 %), emmer had on average only 58 % GLUT and 53 % HMW-GS and einkorn only 44 % GLUT and 27 % HMW-GS. The GLUT and HMW-GS contents are important parts for the formation of the gluten network. The low amounts of GLUT and HMW-GS in emmer and einkorn were the reason for weaker and poorer gluten network formation and low bread volume.

4.1.4 Gluten protein aggregation properties as predictors of the baking quality of modern and ancient wheats

The GPT was intensively used to analyse the gluten aggregation properties of white flours of the modern wheat species common and durum wheat [e.g. 4.9, 4.10]. Further, the application was expanded to wholemeal flours of common wheat [4.11]. Our comparative study first described its use for the characterisation of gluten protein aggregation properties of the five wheat species common wheat, spelt, durum wheat, emmer and einkorn. The GPT was suitable for the application to wholemeal flours of the ancient wheats spelt, emmer and einkorn without restriction. All einkorn cultivars showed an increase of the curve even if the peak was not as high as that of the other wheat species. However, it was possible to evaluate all einkorn curves.

The parameters MT, AGT, LOT and PMT characterise the gluten aggregation properties of flour (Figure 9). AGT is the most important evaluation parameter besides MT [4.12]. Both parameters show the formation and degradation of the gluten network during the test. The good correlation between AGT and GLUT (r = 0.842) and HMW-GS content (r = 0.716), as well as between PMT and GLUT (r = 0.835) and HMW-GS content (r = 0.694) showed that especially proteins with high molecular weights are responsible for the formation of the gluten network. With the exception of LOT, MT (r = 0.742), AGT (r = 0.738) and PMT (r = 0.734) showed high correlations to the bread volume. Consequently, the GPT is a useful tool to predict the baking quality of wholemeal flours from all five wheat species. The GPT is a fast, easy and

cheap alternative to the time-consuming and labour-intensive MBT. However, if further information about gluten content and composition (types and subunits) are required or are of interest, the analytical methods by means of Osborne and GMP fractionation are preferably. In contrast to the cheap, fast and easy GPT, for both fractionations a HPLC system is required and the gluten extraction is more time-consuming and labour-intensive than GPT analysis. Thus, the application of GPT or analytical methods depends on the fundamental question.

4.1.5 Identification of cultivars with good baking performance

Cultivars of the ancient wheats spelt, emmer and einkorn with good baking performance were identified by means of PCA [4.13]. These cultivars were located closer to the common wheat cultivars than the other cultivars of the respective wheat species. This procedure was applied because all common wheat cultivars generally had high bread volumes and good baking quality. Within the respective wheat species, the spelt cultivar “Franckenkorn”, the emmer cultivar “CC1E-04058/01” and the einkorn cultivar “Monlis” were located most closely to common wheat. For example, Monlis had the highest bread volume, the longest AGT, the lowest GLIA/GLUT and the highest GLUT and GMP content of all einkorn cultivars. Similar observations were true for Franckenkorn and CC1E-04058/01. Monlis and Franckenkorn additionally formed spherical breads. This was not true for CC1E-04058/01, which had a flat bread shape. Therefore, the PCA was not suitable to identify cultivars with good form factors and spherical breads. Nevertheless, PCA of the investigated parameters was appropriate to select cultivars from spelt, emmer and einkorn, which had similar properties to common wheat. It has to be stated that it is not required to carry out all methods to identify cultivars with good baking properties. All three methods including GPT, Osborne fractionation and GMP fractionation are suitable predictors for the baking quality of common wheat, spelt, durum wheat, emmer and einkorn.

4.2 Targeted LC–MS/MS reveals similar contents of α-amylase/trypsin-inhibitors as putative triggers of nonceliac gluten sensitivity in all wheat species except einkorn

4.2.1 Identification of marker peptides for the quantitation of ATIs

In the second part of the study, a targeted LC-MS/MS SIDA method in the SRM mode was developed for the quantitation of ATIs in common wheat, spelt, durum wheat, emmer and einkorn. First, marker peptides had to be identified based on different criteria. ATI extracts of five common wheat species and one cultivar each of spelt, durum wheat, emmer and einkorn were hydrolysed with trypsin and peptides were identified by database searches after untargeted LC-MS/MS. These experiments were performed on a HCT-Ultra PTM ion trap mass spectrometer (Bruker Daltonics), which is a system with low resolution compared to e.g. Orbitrap™ (Thermo Fisher) or TripleTOF® 6600 (Sciex). However, at the time of marker peptide

identification no high resolution MS system was available. Therefore, the peptide identification was afflicted with difficulties and had lower accuracy as it would have been with high resolution systems.

The most important criterion for the marker peptides was that they should be present in all five wheat species. In general, peptides of the ATIs 0.19, 0.28, 0.53, CM2, CM3, CM16 and CM17 were identified with high scores. The score describes the probability of correct identification and should be higher than 40 [4.14]. No ATI peptides were identified in the einkorn cultivar Tifi by untargeted LC-MS/MS. No CM17 peptides were detected in durum wheat and emmer. The remaining peptides were consistently identified with a few exceptions in common wheat, spelt, durum wheat and emmer.

In the next step, marker peptides for the use as internal standards were selected. The peptides should have a length between eight and 25 amino acids. Further, they should not contain amino acids, which can be chemically modified (e.g. methionine and tryptophan are oxidised, *N*-terminal glutamic acid and glutamine form pyroglutamate and cysteine forms intra- and interchain disulphide bonds or is oxidised) [4.15]. ATIs are small proteins containing between 120 and 150 amino acids and a high amount of up to ten cysteine residues. It was not possible to fulfil all named criteria. Due to cost effectiveness only one marker peptide was chosen per ATI (Table 4.1). All selected peptides contained a cysteine residue, because cysteine was present in all identified peptides. However, a reduction and alkylation step was included in the workflow to reduce the disulphide bonds and alkylate the cysteine residues to avoid the formation of new disulphide bonds or cysteine oxidation. Peptide (P) 1 was only suitable to quantitate the sum of ATI 0.19 and ATI 0.53. Five peptides were identified for the ATI 0.19, of which four were also found in ATI 0.53. The only peptide of ATI 0.53, which was not present in ATI 0.19, was not detected in the sample. Thus, it was not possible to distinguish between ATI 0.19 and ATI 0.53. P3 had a length of 26 amino acids. The other potential peptide for CM2 was shorter than 25 amino acids, but was only present in durum wheat and emmer and was not detected in the other three wheat species. P3 and P5 contained glutamic acid and glutamine at the *N*-terminus, which formed pyroglutamate in solution. To avoid too low concentrations during quantitation, the SRM parameters of the pyroglutamate forms (P3c and P5c) were included in the MS parameters. Last, the selected peptide for CM17 could not be synthesised and was not considered in the final method.

Table 4.1: Selection of marker peptides with their amino acid sequence for the quantitation of α-amylase/trypsin-inhibitors (ATIs) in common wheat, spelt, durum wheat, emmer and einkorn.

Abbreviation	ATI	Amino acid sequence
P1	0.19+0.53	LQCNGSQVPEAVLR
P2	0.28	LQCVGSQVPEAVLR
P3	CM2	EYVAQQTCGVGIVGSPVSTEPGNTPR
P4	CM3	SGNVGESGLIDLPGCPR
P5	CM16	QQCCGELANIPQQCR

One important criterion for the selection of marker peptides is that the peptides are unique within the proteome of the investigated samples. This was only true for P3, which was only present in common wheat. P1 was present in more than 200 entries in the UniProtKB database and P2 in more than 100 entries. These entries included other cereal species such as rye and barley and other wheat species, e.g. wild emmer, wild einkorn and durum wheat. P4 and P5 were present in six other entries, respectively. P4 was present in durum wheat and goat grass, and P5 in rye, barley and *T. macha*. A UniProtKB search with the search terms “amylase inhibitor” and “triticum” resulted in 357 entries, of which 65 are present in common wheat, four in durum wheat, 195 in wild emmer, eight in wild einkorn and none in spelt (status March 2019). This underlined the difficulty of selecting unique peptides. However, it has to be stated that the selected peptides are not present in other organisms e.g. yeast, human, or bacteria. Only 17 entries of the above named search had the status ‘reviewed’, which means that the proteins are not only predicted due to genetic analysis but have evidence on protein level. The four entries of durum, 195 entries for wild emmer and eight entries of wild einkorn had the status ‘unreviewed’ and might be present in the genome, but might not be expressed as proteins. At this time, a more detailed characterisation of the ATIs of spelt, durum wheat, emmer and einkorn was not possible due to the use of a low resolution MS system and the lack of reliable reference protein sequences in the database.

4.2.2 Validation of the new targeted LC-MS/MS method for the quantitation of ATIs

Good values for repeatability (1.8 – 5.3 %; six samples on the same day) and intermediate precision (2.2 – 6.8 %; three days with six samples each) showed the high precision of the newly developed method. These experiments were performed with a mixture of 15 common wheat wholemeal flours. An alternative would have been to expand this experiment to mixtures of spelt, durum wheat and emmer cultivars, because the wheat species differ in their protein content and protein composition [4.16]. However, the contents of the ATI containing ALGL fraction did not differ significantly. The data obtained for common wheat was, therefore, deemed to be transferable to the other wheat species.

The evaluation of limits of detection is usually performed with analyte-free matrices. These matrices should ideally have the same properties and composition as the analysed materials.

Flours of common wheat, spelt, durum wheat and emmer were not suitable due to high ATI contents. Further, einkorn flour was not suitable as well because some cultivars contained very low ATI amounts. Flours of rye and barley showed signals, which were identified as ATI peptides. Flours made of rice, potato starch and wheat starch were not suitable due to interferences in the elution region of the analytes. Flour of cassava starch was the only matrix, which had no interferences and analyte signals. The properties of cassava starch and wheat flour were not absolutely comparable because cassava starch has a much lower protein content than wheat flour. On the other hand no other tested matrix was suitable for the evaluation of the limit of detection. The low values for LOD (0.1 – 1.6 µg/g) and LOQ (0.3 – 4.7 µg/g) of the marker peptides showed the high sensitivity of the method. Additionally, the LOD and LOQ were checked by spiking the marker peptides to cassava starch in the calculated concentrations.

Another important validation parameter is the recovery of the analytes. The recovery is tested either by spiking the analytes to an analyte-free matrix or as standard addition to an analyte containing matrix. The most important challenge in this case is that in real samples the analytes are present in their protein form and in the spiking solution the analytes are present as peptides. At this point, the extractability of the ATIs plays a major role. Different extraction buffers (e.g. based on ammonium bicarbonate or phosphate, or GLIA and GLUT extraction solvents) were tested. Ammonium bicarbonate buffer yielded higher amounts of ATIs than phosphate buffer. Extraction with GLIA and GLUT extraction solution generated only an increase of 10 % after extraction with ammonium bicarbonate solution. Further, the interferences in the MS analysis were higher with ethanol (GLIA) or 1-propanol (GLUT) extraction.

In this study, different approaches were performed to evaluate the recovery. Spiking experiments revealed recoveries between 29.1 % and 120.9 %. The range was quite high due to the above named problem that peptides were used for spiking whereas the analytes are present as proteins in the samples. Secondly, dilution experiments were performed. Common wheat flour was diluted 1+9 with cassava starch. The very good values for recovery (92.3 – 101.9 %) showed the high extraction efficiency of the optimised method.

To evaluate the quantitative accuracy of the newly developed targeted method, the results of this method were compared to label-free and untargeted nanoLC-MS/MS analysis. The nanoLC-MS/MS data were evaluated by the iBAQ algorithm. This algorithm estimates absolute protein abundances and is implemented within the software MaxQuant. The key advantage of the iBAQ algorithm is that iBAQ intensities of different proteins are directly comparable to each other within one sample [4.17]. In contrast, the key advantage of SIDA is that SIDA

compensates losses during preparation. However, the usage of labelled peptides as internal standard does not consider incomplete digestion or peptide modifications that can be observed by untargeted analysis. Untargeted analysis monitors all obtained peptides e.g. with or without modification (oxidation, glycosylation) or peptides with missed cleavages. In contrast, LC-MS/MS SRM experiments are limited to the selected transition, which leads to higher precision, higher repeatability and lower limits of detection compared to untargeted analysis [4.18]. First, contents of a commercially available ATI reference were compared with both methods, which revealed similar contents of the investigated ATIs 0.19, 0.28, 0.53, CM2, CM3 and CM16.

Next, the comparison between both methods was expanded to two cultivars per wheat species. The iBAQ intensities of ATIs were 1000fold lower in einkorn samples than those in the other wheat species. This confirmed the absence of all ATI peptides and thus, not only the marker peptide was not detectable. The same was true for ATI 0.28 in the two durum wheat cultivars, in which no ATI 0.28 peptide was detectable by untargeted analysis. The relative results of both methods were in agreement, which showed the quantitative accuracy of the LC-MS/MS SIDA method.

4.2.3 Quantitation of ATIs in modern and ancient wheats

ATIs were extracted with ammonium bicarbonate solution from wholemeal flour of eight cultivars each of common wheat, spelt, durum wheat, emmer and einkorn cultivated at the same location. After addition of the internal standards, the disulphide bonds were reduced with TCEP and the cysteine residues were alkylated with CAA. The proteins were hydrolysed with trypsin and the peptides were analysed by the new targeted LC-MS/MS SRM method. The hypothesis proposed at the beginning of the study was that the ancient wheats spelt, emmer and einkorn might have a lower ATI content than the modern wheats common wheat and durum wheat. ATIs are the potential triggers of NCGS. It was reported that ancient wheats are better tolerated by NCGS patients.

On the one hand, the study confirmed this hypothesis for einkorn. The levels of the ATIs 0.19, 0.28, 0.53, CM2, CM3 and CM16 were at most 10 % of that of the other wheat species. It was confirmed that the gene for ATIs might be silenced or expressed in very low amounts in einkorn [4.19]. Further, it was suggested that a reduction of the daily ATI intake to about 5 – 10 % of the average daily intake may lead to improvement of NCGS symptoms [4.20]. The replacement of common wheat with einkorn could have benefits for NCGS patients, but einkorn has generally lower grain yield and poorer baking properties than common wheat [4.16, 4.21].

On the other hand, the study revealed that the hypothesis was not true for spelt and emmer. The level of the total ATI content (sum of 0.19, 0.28, 0.53, CM2, CM3 and CM16) was significantly higher for spelt and emmer than for common wheat. At this point it has to be stated

that the correlation between ATI concentration and ATI bioactivity on human cells is not yet known. It was reported that flours of emmer and spelt have a lower bioactivity on cells than common wheat [4.20]. Different samples were analysed for the ATI content and the ATI bioactivity and thus, both studies are hard to compare. Further research is needed to answer the questions about the possible correlation between ATI content and ATI bioactivity.

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5 Future research

In this work, one location and eight cultivars per wheat species were investigated for ATI contents, gluten protein composition and baking performance. To expand the findings, the sample set should be expanded to more locations, to more cultivation years and to more cultivars of each wheat species. The photometric Bradford assay is a fast and easy alternative to the quantitation of GLIA and GLUT by RP-HPLC. Because GLIA, GLUT and the ratio between both are good predictors for good baking performance, the Bradford assay could be used to predict the baking quality of a very large sample set by high throughput analysis. If more locations and/or more cultivation years were investigated for GLIA and GLUT, the effects of both genetic and environmental factors could be revealed.

The HMW-GS were identified to have a very high effect on the baking quality in all five wheat species. Usually, different types of HMW-GS are characterised and identified by SDS-PAGE. As a more reliable, but more labour-intensive alternative, the identification of HMW-GS by LC-MS/MS would be an option. With methods based on LC-MS/MS, a lot more information about the proteins are generated than by UV-detection. With LC-MS/MS, differences in the amino acid sequence of different HMW-GS could be identified to improve the knowledge of structure-function-relationships. For example, modifications (e.g. amino acid substitution) might lead to modified baking quality. The amino acid sequences of common wheat HMW-GS are already identified, but the ones in the ancient wheats are still poorly characterised. LC-MS/MS analysis would reveal the differences and similarities in the amino acid sequences of HMW-GS in the various wheat species. Further, the unknown amino acid sequence of the ancient wheats could be identified by de-novo sequencing of highly purified HMW-GS.

With the newly developed targeted LC-MS/MS method, five ATIs can be quantitated in five wheat species. For each ATI type, only one marker peptide was used. The use of more marker peptides per protein (ideally two to three), leads to more accurate results due to possible amino acid modifications (e.g. amino acid exchange or post-translational modifications). If one marker peptide is affected, the second one balances the modification. Further, alternative, more sensitive mass spectrometers (e.g. Orbitrap™, Thermo Fisher, or QTRAP® 6500, Sciex) might increase the precision and sensitivity. The new method should be expanded to quantitate all known 13 ATIs in common wheat and not only the most predominant five. Secondly, the method should be optimised for the ancient wheat species by adding other ATIs, which are specific for the ancient wheats.

This work laid the foundations to identify many new questions, which will be answered in the future.

6 Summary

The wheat species common wheat, spelt, durum wheat, emmer and einkorn can be classified either according to their genome (hexaploid, tetraploid and diploid) or according to their cultivation in ancient and modern times. Common wheat and spelt are hexaploid wheats, durum wheat and emmer are tetraploid wheats and einkorn is a diploid wheat species. The ancient wheats einkorn, emmer and spelt were cultivated a long time ago, but today, their cultivation and use is negligibly low compared to those of the modern wheats common wheat and durum wheat. The almost complete absence of the ancient wheats is due to their low grain yields and poor baking performance. However, they have been attracting attention during the last decades, because some consumers associate health and sensory benefits with products made of einkorn, emmer and spelt.

The aim was to identify prediction parameters for the baking quality for all five wheat species and to identify ancient wheats with good baking quality. In the second part, the focus was on the characterisation and quantitation of ATIs. ATIs are the potential triggers of NCGS. NCGS patients reported a better tolerability of ancient wheats and improvement of symptoms typical of NCGS by replacing modern wheats with ancient wheats in their nutrition. Thus, the hypothesis was proposed that ancient wheats might have a lower ATI content than modern ones.

The sample set in this study consisted of eight cultivars each of common wheat, spelt, durum wheat, emmer and einkorn, which were cultivated at the same location under the same climatic conditions. Thus, geographic and climatic conditions (e.g. soil, temperature, rainfall) are expected to have only a low effect on the detected differences and mostly the genetic effect of the different wheat species was observable. The study was divided into two parts. In the first part, the focus was on the gluten protein composition of the five wheat species and its influence on the baking quality, the second peart was dedicated to the quantitation of ATIs.

The gluten protein composition was analysed by modified Osborne fractionation and by GMP fractionation followed by measurement of the respective fractions by RP- or GP-HPLC. Common wheat had the lowest GLIA content compared to the other wheat species. The mean GLIA content in relation to the total protein content increased from common wheat (53.3 %), to spelt (61.2 %), durum wheat (61.9 %), emmer (63.5 %) and einkorn (66.7 %). The GLUT content showed the opposite behaviour. Emmer (12.6 %) and einkorn (9.2 %) had the lowest mean GLUT content and common wheat the highest one (21.4 %). This led to significant differences in the GLIA/GLUT ratios, which were significantly higher for emmer (3.6 – 6.7) and einkorn (4.2 – 12.0) than for common wheat (2.0 – 3.2) with spelt (2.8 – 4.0) and durum wheat (2.2 – 5.3) in between. The behaviour of the mean GMP content was similar to the GLUT

content. The mean GMP content increased from einkorn (2.7 %) to emmer (3.4 %), spelt (4.6 %), durum wheat (7.6 %) and common wheat (7.8 %).

It is generally accepted that gluten proteins have a very high effect on the baking performance, because the gluten proteins influence positively the formation of a viscoelastic dough with a high gas holding capacity. Common wheat shows very good baking performance due to a well-balanced GLIA/GLUT ratio. The higher GLIA/GLUT ratios of the ancient wheats spelt, emmer and einkorn might lead to poor baking performance and low bread volumes. To confirm this, breads of flours of all five wheat species were baked by the MBT. Bread volume and bread shape were analysed by a laser-based scanner. Breads of common wheat had the highest mean bread volume (35.1 mL) followed by spelt (31.4 mL), durum wheat (23.4 mL), emmer (22.2 mL) and einkorn (19.6 mL). Breads of common wheat, durum wheat and einkorn were spherical, which is shown by the form factor (width divided by height) close to 2 (1.3 – 2.4). Breads of spelt and emmer had a flat shape, which was shown by form factors higher than 2 (2.4 – 3.2).

Correlation analysis between the results of MBT, the Osborne and GMP fractionation identified parameters for the prediction of the baking quality of all five wheat species. The form factor was not correlated with any parameter. This showed that the form factor is not only influenced by gluten proteins, but by other factors e.g. the starch-protein network. In contrast, good correlation coefficients were observed between bread volume and GLUT ($r = 0.804$), GLIA/GLUT ($r = -0.829$), GMP ($r = 0.652$) and HMW-GS contents ($r = 0.864$). Thus, especially proteins with high molecular weights are responsible for high bread volumes.

Osborne and GMP fractionation are labour-intensive and need a HPLC system. As alternative to HPLC experiments, the gluten protein aggregation properties were investigated by the GPT. No correlations between the form factor and GPT results were observed. In contrast, the bread volume was correlated with AGT ($r = 0.738$) and PMT ($r = 0.734$). The GPT is a fast and easy method to predict the baking quality of all five wheat species. However, if more detailed data about gluten protein composition is required, the Osborne and/or GMP fractionation is the method of choice for the prediction of the baking quality.

Wheat proteins do not only positively influence the baking quality, but they can cause hypersensitivities such as CD, food allergy or NCGS in susceptible individuals. The hypothesis was proposed that ancient wheat species might have a lower ATI content than modern wheat species. To verify this hypothesis, a targeted LC-MS/MS SIDA method in the SRM mode was developed to quantitate ATIs in common wheat, spelt, durum wheat, emmer and einkorn.

ATIs were extracted with diluted salt solution from the flours. Due to the high number of cysteine residues and disulphide bonds, a reduction and alkylation step was performed. The

proteins were hydrolysed to peptides by tryptic digestion. First, marker peptides were identified by untargeted LC-MS/MS and database searches. In total, 21 peptides were identified for the ATIs 0.19, 0.28, 0.53, CM2, CM3, CM16 and CM17 in common wheat, spelt, durum wheat and emmer, but not in einkorn. One marker peptide each of the ATIs 0.19, 0.28, CM2, CM3 and CM16 was selected as stable isotope labelled standard. The peptide of 0.19 was also present in 0.53 and thus, the sum of both ATIs was determined.

Good values for repeatability (1.8 – 5.3 %; six samples on the same day) and intermediate precision (2.2 – 6.8 %; three days with each of six samples) confirmed the high precision of the newly developed method. The low values for LOD (0.1 – 1.6 µg/g) and LOQ (0.3 – 4.7 µg/g) of the marker peptides showed the high sensitivity of the new method. The quantitative accuracy was evaluated by comparison of the LC-MS/MS SIDA method to an alternative untargeted LC-MS/MS analysis. The protein intensities in the untargeted LC-MS/MS measurements were estimated by the iBAQ algorithm. The relative results of both methods were in agreement, which confirmed the quantitative accuracy of the LC-MS/MS SIDA method.

Except durum wheat and einkorn, all samples of common wheat, spelt, and emmer contained all five marker peptides. Only two of eight durum wheat cultivars contained ATI 0.28. In three of eight einkorn cultivars, the peptides of ATI 0.19, 0.28, 0.53 and CM3 were detected and the content was above the LOQ. The contents of ATI 0.19, 0.28, 0.53 and CM3 in einkorn (0.2 ± 0.1 mg/g) were only 1/20 as compared to the other wheat species. On average the ancient wheats spelt (4.7 ± 0.5 mg/g) and emmer (5.0 ± 0.5 mg/g) had higher ATI contents than the modern wheats common wheat (3.7 ± 0.3 mg/g) and durum wheat (4.2 ± 0.9 mg/g). This showed that the assumption that ancient wheats contain less ATI than modern ones is true for einkorn. On the other hand, the study revealed that the hypothesis was not true for spelt and emmer.

Furthermore, differences in the distribution of ATIs were observed. The percentages of ATI 0.19, 0.28 and 0.53 to CM-types was balanced (1:1) in the hexaploid wheat species common wheat and spelt, and in the tetraploid wheats durum wheat and emmer the CM-types were predominant (1:3). Therefore, hexaploid and tetraploid wheats might be distinguished on the basis of their ATI pattern. Finally, correlations were calculated between ATI contents, protein contents and albumin/globulin (ALGL) contents. The ATI content was neither correlated with protein content ($r = 0.01$) nor with ALGL content ($r = 0.05$). Thus, the content of ATIs cannot be predicted by analysing the protein or ALGL content by means of fast and established methods. Consequently, there is a necessity for the developed method to quantitate ATIs in wheat.

7 Zusammenfassung

Die Weizenarten Weichweizen, Dinkel, Hartweizen, Emmer und Einkorn werden entweder anhand ihres Genoms (hexaploid, tetraploid oder diploid) oder anhand ihres Anbaus in der Vergangenheit oder Gegenwart (alte bzw. moderne Weizenart) eingeteilt. Weichweizen und Dinkel sind hexaploide Weizenarten, Hartweizen und Emmer tetraploide und Einkorn ist eine diploide Weizenart. Die alten Weizenarten Einkorn, Emmer und Dinkel wurden hauptsächlich vor mehreren tausend Jahren angebaut. Ihre Bedeutung hinsichtlich Anbauflächen und wirtschaftlichem Nutzen ist heuzutage gegenüber den modernen Weizenarten Weichweizen und Hartweizen deutlich vermindert. Gründe für die Dominanz des Weichweizens sind geringerer Ertrag und schlechtere Backeigenschaften der alten Weizenarten. Dennoch rücken diese Weizenarten wieder vermehrt in den öffentlichen Fokus, da einige Verbraucher mit den alten Weizenarten gesundheitliche und sensorische Vorteile gegenüber modernen Arten verbinden.

Im ersten Teil der vorliegenden Studie wurde die Verteilung der Kleberproteine in den Weizenarten und deren Einfluss auf die Backqualität untersucht. Im zweiten Teil lag der Schwerpunkt auf der Charakterisierung und Quantifizierung von α-Amylase-Trypsin-Inhibitoren (ATIs). Diese gelten als mögliche Verursacher der Nicht-Zöliakie-Nicht-Weizenallergie-Weizensensitivität (engl. *noncoeliac gluten sensitivity*, NCGS). NCGS Betroffene berichteten über eine bessere Verträglichkeit von Produkten aus alten Weizenarten und, dass der Verzehr solcher Lebensmittel zu einer Linderung von NCGS-Symptomen führt. Aus diesem Grund wurde die Hypothese aufgestellt, dass alte Weizenarten möglicherweise weniger ATIs enthalten als moderne Weizenarten.

Das Probensortiment dieser Studie bestand aus acht Sorten der Weizenarten Weichweizen, Dinkel, Hartweizen, Emmer und Einkorn. Diese wurden jeweils am gleichen Standort unter identischen klimatischen Bedingungen angebaut. Daher war zu erwarten, dass keine geographisch und klimatisch bedingten Variationen (z.B. durch Standort, Bodenbeschaffenheit, Temperatur, Niederschlag) der Kornzusammensetzung auftraten. Die Unterschiede zwischen den Proben waren somit beinahe ausschließlich durch genetische Effekte bedingt.

Die Weizenproteine wurden mittels modifizierter Osborne-Fraktionierung und der Gluteninmakropolymer (GMP-) Fraktionierung extrahiert. Die Messung der Fraktionen erfolgte entweder mittels Umkehrphasen (RP-) Hochleistungsflüssigkeitschromatographie (HPLC) oder Gelpermeations (GP-) HPLC. Weichweizen hatte im Vergleich zu den anderen Weizenarten den geringsten Gliadin (GLIA-) Gehalt. Der durchschnittliche GLIA-Gehalt bezogen auf den Gesamtproteingehalt stieg von Weichweizen (53,3 %) zu Dinkel (61,2 %),

Hartweizen (61,9 %), Emmer (63,9 %) und Einkorn (66,7 %) an. Gegensätzliches wurde für den Glutenin (GLUT-) Gehalt beobachtet. Emmer (12,6 %) und Einkorn (9,2 %) hatten den geringsten und Weichweizen (21,4 %) den höchsten GLUT-Gehalt. Dies führte zu signifikanten Unterschieden im Gliadin-zu-Glutenin Verhältnis (GLIA/GLUT). Die GLIA/GLUT-Verhältnisse waren in Emmer (3,6 – 6,7) und Einkorn (4,2 – 12,0) signifikant höher als in Weichweizen (2,0 – 3,2). Die Verhältnisse bei Dinkel (2,8 – 4,0) und Hartweizen (2,2 – 5,3) lagen zwischen Weichweizen, Emmer und Einkorn. Der GMP-Gehalt verhielt sich über die untersuchten Weizenarten vergleichbar zum GLUT-Gehalt, da dieser von Einkorn (2,7 %) über Emmer (3,4 %), Dinkel (4,6 %) und Hartweizen (7,6 %) zu Weichweizen (7,8 %) deutlich stieg.

Es ist allgemein akzeptiert, dass die Kleberproteine die Backeigenschaften beeinflussen, da Kleberproteine zur Bildung eines viskoelastischen Teigs mit großem Gashaltevermögen beitragen. Weichweizen zeichnet sich durch ein ausgeglichenes GLIA/GLUT-Verhältnis aus, das zu sehr guten Backeigenschaften führt. Die hohen GLIA/GLUT-Verhältnisse der alten Weizenarten führen somit vermutlich zu schlechteren Backeigenschaften und niedrigeren Brotvolumina. Um dies zu bestätigen, wurden Backversuche im 10 g-Maßstab (MBT) durchgeführt. Brotvolumen und -form wurden mittels eines laserbasierten Scanners ermittelt. Weichweizenbrote hatten durchschnittlich die höchsten Brotvolumina (35,1 mL), gefolgt von Dinkel (31,4 mL), Hartweizen (23,4 mL), Emmer (22,2 mL) und Einkorn (19,6 mL). Brote aus Weichweizen, Hartweizen und Einkorn waren kugelförmiger als die Brote aus Dinkel und Emmer. Dies war anhand des Formfaktors (Breite/Höhe) von nahezu 2 (1,3 – 2,4) erkennbar. Dinkel- und Emmerbrote waren deutlich flacher, was sich anhand eines Formfaktors über 2 (2,4 – 3,2) äußerte.

Anhand von Korrelationsanalysen zwischen den Ergebnissen von MBT, Osborne- und GMP-Fraktionierung wurden Parameter identifiziert, die eine Vorhersage der Backqualität der fünf Weizenarten ermöglichen. Der Formfaktor war jedoch mit keinem Parameter korreliert, was zeigt, dass die Brotform nicht nur durch Kleberproteine bestimmt wird, sondern von weiteren Faktoren, wie z.B. dem Stärke-Kleber-Netzwerk, abhängig ist. Im Gegensatz dazu wurden hohe Korrelationskoeffizienten (r) zwischen Brotvolumen und GLUT-Gehalt ($r = 0,804$), GLIA/GLUT-Verhältnis ($r = -0,829$), GMP-Gehalt ($r = 0,652$) und dem Gehalt der hochmolekularen Gluteninuntereinheiten (HMW-GS) ($r = 0,864$) ermittelt. Die zeigt, dass vor allem Proteine mit hohen Molekulargewichten verantwortlich für ein hohes Brotvolumen sind.

Die modifizierte Osborne- und die GMP-Fraktionierung sind mit hohem Arbeits- und Geräteaufwand verbunden. Als Alternative zu HPLC-Experimenten wurde die Aggregationsfähigkeit der Glutenproteine unter Anwendung des GlutoPeak Tests (GPT) bestimmt. Es wurde ebenfalls keine Korrelation zwischen dem Formfaktor und den

Ergebnissen des GPT festgestellt. Im Gegensatz dazu war das Brotvolumen mit der Aggregationszeit (AGT) (r = 0,738) und der Zeit am Peakmaximum (PMT) (r = 0,734) korreliert. Daher ist der GPT eine schnelle und einfache Methode, um die Backqualität aller fünf Weizenarten vorherzusagen. Dagegen liefern jedoch die Osborne- und GMP-Fraktionierung deutlich mehr Aussagen über die Kleberproteinverteilung. Je nach Fragestellung ist entweder der GPT oder eine der beiden Fraktionierungen die Methode der Wahl.

Weizenproteine beeinflussen nicht nur die Backeigenschaften positiv, sondern können auch Unverträglichkeiten, wie Zöliakie, Nahrungsmittelallergie oder NCGS verursachen. Es wurde die Hypothese aufgestellt, dass alte Weizenarten einen geringen ATI-Gehalt aufweisen als moderne Weizenarten. Zur Untersuchung dieser Hypothese wurde eine *targeted* Flüssigkeitschromatographie-Tandem-Massenspektrometrie (LC-MS/MS) Methode im *single reaction monitoring* (SRM) Modus nach dem Prinzip der Stabilisotopenverdünnungsanalyse (SIDA) entwickelt, um ATIs in Weichweizen, Dinkel, Hartweizen, Emmer und Einkorn zu quantifizieren.

Die ATIs wurden aus Mehl mit verdünnter Salzlösung extrahiert. Aufgrund der hohen Anzahl an Cysteinresten und Disulfidbrücken wurden diese zuerst reduziert und anschließend alkyliert. Die extrahierten Proteine wurden mit Trypsin zu Peptiden hydrolisiert. Um Markerpeptide zu identifizieren, wurde eine *untargeted* LC-MS/MS Analyse mit anschließendem Datenbankabgleich durchgeführt. Insgesamt wurden 21 Peptide der ATIs 0.19, 0.28, 0.53, CM2, CM3, CM16 und CM17 in Weichweizen, Dinkel, Hartweizen und Emmer, nicht jedoch in Einkorn, identifiziert. Für die ATIs 0.19, 0.28, CM2, CM3 und CM16 wurde jeweils ein geeignetes Peptid stabilisotopenmarkiert synthetisiert. Das Peptid für 0.19 war zusätzlich in 0.53 vorhanden, sodass hierbei lediglich die Summe der beiden ATIs bestimmbar war.

Sehr gute Werte für die Wiederholbarkeit (1,8 – 5,3 %; sechs Proben am gleichen Tag) und die Laborpräzision (2,2 – 6,8 %; sechs Proben an drei Tagen) bestätigten die hohe Präzision der neuentwickelten Methode. Niedrige Nachweis- (0,1 – 1,6 µg/g) und Bestimmungsgrenzen (0,3 – 4,7 µg/g) der Markerpeptide zeigten die hohe Sensitivität der neuen Methode. Die Richtigkeit wurde durch den Vergleich der neuen LC-MS/MS SIDA Methode mit einer alternativen *untargeted* LC-MS/MS Analyse validiert. Die Proteinintensitäten wurden in der *untargeted* LC-MS/MS Analyse mittels des *intensity based absolute quantitation* (iBAQ) Algorithmus abgeschätzt. Die relativen Ergebnisse beider Methoden waren vergleichbar, was die Richtigkeit der neuen LC-MS/MS SIDA Methode bestätigte.

Mit Ausnahme der Einkorn- und Hartweizenproben enthielten alle Weichweizen-, Dinkel- und Emmerproben alle fünf Markerpeptide. Lediglich zwei der acht Hartweizensorten enthielten das ATI 0.28. In drei der acht Einkornsorten wurden Peptide der ATIs 0.19, 0.28, 0.53 und CM3 detektiert, wobei die Gehalte über der Bestimmungsgrenze lagen. Die Gehalte der ATIs 0.19, 0.28, 0.53 und CM3 betrugen in Einkorn (0,2 ± 0,1 mg/g) lediglich etwa 5 % der Gehalte in den anderen Weizenarten. Durchschnittlich enthielten die alten Weizenarten Dinkel (4,7 ± 0,5 mg/g) und Emmer (5,0 ± 0,5 mg/g) höhere ATI-Gehalte als die modernen Weizenarten Weichweizen (3,7 ± 0,3 mg/g) und Hartweizen (4,2 ± 0,9 mg/g). Dies zeigte, dass die Annahme, alte Weizenarten enthielten weniger ATIs als moderne Weizenarten, im Falle des Einkorns richtig war. Andererseits zeigte diese Studie, dass die Hypothese nicht auf Dinkel und Emmer zutraf.

Weiterhin wurden Unterschiede in der ATI-Verteilung ermittelt. Das Verhältnis der ATIs 0.19, 0.28 und 0.53 zu den CM-Typen war in den hexaploiden Weizenarten Weichweizen und Dinkel ausgeglichen (1:1). Dagegen überwogen in den tretraploiden Weizenarten Hartweizen und Emmer die CM-Typen (3:1). Daher wurde die Vermutung aufgestellt, dass hexaploide und tetraploide Weizenarten auf Basis ihrer ATI-Verteilung unterscheidbar sind. Schlussendlich wurden Korrelationen zwischen ATI-, Gesamtprotein- und Albumin/Globulin- (ALGL) Gehalt berechnet. Der ATI-Gehalt war weder mit dem Gesamtproteingehalt ($r = 0,01$) noch mit dem ALGL-Gehalt ($r = 0,05$) korreliert. Daher ist der ATI-Gehalt nicht durch die Analyse des Gesamtprotein- oder ALGL-Gehalts mit etablieren Methoden vorhersagbar, was die Notwendigkeit der neuen Quantifizierungsmethode bestätigte.

8 Appendix

8.1 Gluten protein composition and aggregation properties as predictors for bread volume of common wheat, spelt, durum wheat, emmer and einkorn

8.1.1 Bibliographic data

Title:	Gluten protein composition and aggregation properties as predictors for bread volume of common wheat, spelt, durum wheat, emmer and einkorn
Authors:	Sabrina Geisslitz, Herbert Wieser, Katharina Anne Scherf, Peter Koehler
Journal:	Journal of Cereal Science
Publication year:	2018
Volume:	83
Pages:	204-212
DOI:	10.1016/j.jcs.2018.08.012
Hyperlink:	http://www.sciencedirect.com/science/article/pii/S0733521018304636

8.1.2 Publication reprint permission

A reprint of the publication „Gluten protein composition and aggregation properties as predictors for bread volume of common wheat, spelt, durum wheat, emmer and einkorn" is at the pages 58-66.

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8.2 Targeted LC–MS/MS reveals similar contents of α-amylase/trypsin-inhibitors as putative triggers of nonceliac gluten sensitivity in all wheat species except einkorn

8.2.1 Bibliographic data

Title:	Targeted LC–MS/MS reveals similar contents of α-amylase/trypsin-inhibitors as putative triggers of nonceliac gluten sensitivity in all wheat species except einkorn
Authors:	Sabrina Geisslitz, Christina Ludwig, Katharina Anne Scherf, Peter Koehler
Journal:	Journal of Agricultural and Food Chemistry
Publication year:	2018
Volume:	66
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Pages:	12395-12403
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Hyperlink:	https://pubs.acs.org/doi/abs/10.1021/acs.jafc.8b04411

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