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Impact of breeding on the protein composition of wheats from 1891 to 2010 and its effect on wheat-related disorders

Darina Pronin

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Vorsitzender:	Prof. Dr. Michael Rychlik
Prüfer der Dissertation:	1. Prof. Dr. Katharina Scherf
	2. Prof. Dr. Matthias Feige

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We keep moving forward, opening new doors and doing new things, because we are curious and curiosity keeps leading us down new paths.

-Walt Disney-

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Abbreviations

AGA	Anti-gliadin antibody
ALGL	Albumins and globulins
APC	Antigen-presenting cell
ATI	α-Amylase/trypsin inhibitor
CCL_2	Chemokine (C-C motif) ligand 2
CD	Celiac disease
DGPA	Deamidated gluten peptides antibody
DTT	Dithiothreitol
ELISA	Enzyme-linked immunosorbent assay
EMA	Endomysial antibody
ESI	Electrospray ionization
FODMAP	Fermentable oligo-, di-, and monosaccharides and polyols
GFD	Gluten-free diet
GLIA	Gliadins
GLUT	Glutenins
GS	Glutenin subunit
GP	Gel permeation
HCA	Hierarchical cluster analysis
HLA	Human leukocyte antigen
HMW	High-molecular-weight
HPLC	High-performance liquid chromatography
IBS	Irritable bowel syndrome
IEL	Intraepithelial lymphocytes
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LC	Liquid chromatography
LMW	Low-molecular-weight
mAb	Monoclonal antibody
MMP	Matrix metalloproteinase
MMW	Medium-molecular-weight
MRM	Multiple reaction monitoring

MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
m/z	Mass-to-charge ratio
NCGS	Non-celiac gluten sensitivity
pAb	Polyclonal antibody
PAGE	Polyacrylamide gel electrophoresis
PCA	Principle component analysis
PWG	Prolamin Working Group
r	Correlation coefficient
RP	Reversed-phase
SDS	Sodium dodecyl sulphate
SIDA	Stable isotope dilution assay
TG2	Tissue transglutaminase
TGA	Anti-tissue transglutaminase antibody
TLR	Toll-like receptor
TNF	Tumor necrosis factor
ULN	Upper limit of normal
WDAI	Wheat dimeric amylase inhibitor
WDEIA	Wheat-dependent exercise-induced anaphylaxis
WMAI	Wheat monomeric amylase inhibitor
WTAI	Wheat tetrameric amylase 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

1.1 Cereals

Cereals are the most important group of cultivated plants worldwide and are grown on about 60% of the agricultural area. Maize, wheat and rice are the three major crops in terms of production and utilization. The one-seeded fruits of cereals (grains) are used as staple food for human consumption, as animal feed and various other uses. According to the most recent data published by the Food and Agriculture Organization of the United Nations (FAO) 1147 x 10^6 tons of maize, 782 x 10^6 tons of rice and 734 x 10^6 tons of wheat were produced worldwide in the year 2018 (http://www.fao.org/faostat/en/#data). Cereals are cultivated forms of the monocotyledonous plants belonging to the *Poaceae* grass family and are further divided into subfamilies, namely *Pooideae* (wheat, rye, barley, oats), *Oryzoideae* (rice) and *Panicoideae* (maize) [1, 2]. The structure of the grains is fairly similar for all cereals. The individual grain consists of the endosperm and the germ, which are enclosed by the bran. The endosperm makes up the largest part of the grain and is the germ´s source of nutrition because it contains about 80% starch and 13% proteins. The germ, as the reproductive part, contains about 34% proteins and 28% lipids [3]. The bran consists of four layers, namely pericarp, testa, nucellar layers, aleurone and is a by-product of milling. The bran is an important source for dietary fiber with contents of up to 63% [4].

1.2 Wheat

1.2.1 Overview

The first cultivation of wheat began about 10 000 years ago during the period of transition from hunting and gathering to farming the food. Since then, wheat has spread worldwide due to its adaptability and high yields, becoming an important crop and a significant source of nutrients for mankind [5]. In the year 2018, a cultivation area of more than 214 x 10^6 ha was used to harvest 734 x 10^6 tons of wheat crops, consequently making wheat one of the top 3 cereal crops. Wheat is considered an important source of energy because of its high content of carbohydrates. Besides the significant amount of carbohydrates (65.4-78.0%, containing fiber) a wheat grain

contains proteins (8.0-19.0%), as well as minor constituents like water (7.8-14.8%), lipids (0.9-3.3%), minerals (1.2-3.0%) and vitamins [6, 7].

The term wheat is used in a broad sense and covers many species of wheat within the genus *Triticum* in the family of *Poaceae*. Cultivated wheat consists of diploid, tetraploid and hexaploid wheat species. Common wheat (*T. aestivum*) accounts for 95 % of wheat production and is therefore the most widely cultivated wheat species. Describing its application, the hexaploid common wheat is also called bread wheat. The remaining 5% are mostly covered by the tetraploid durum wheat [8]. Due to the unique properties of wheat flour and dough, wheat can be processed into a variety of products, like bread, pasta, cookies and pastries. Additionally, wheat is a great source of nutrients and consequently forms the basis for approximately 50% of the total calories consumed by humans [9].

1.2.2 Genetics of wheat species

Today's wheat is the result of crossing species of cereals and wild grasses from over 10 000 years ago during the transition to agriculture in western Asia. The first cultivated wheat species were einkorn, emmer and barley (*Hordeum vulgare*), which originated from the Middle East [5]. From this region, the expansion of farming led to the distribution of domesticated einkorn (*T. monococcum*, genomes A^mA^m) and domesticated emmer (*T. turgidum* ssp. *dicoccon*, genomes AABB) across Asia, Africa and Europe. Wild forms of einkorn and emmer are still found today, this is why domestication by human selection seems clear [10]. By hybridization of a diploid (genome AA) wild einkorn species (*T. urartu*) with the BB genome of an unknown species of a wild grass (presumably *Aegilops*) the tetraploid form of wheat (AABB) has resulted. It is assumed that this tetraploid wild emmer (*T. turgidum* ssp. *dicoccoides*) was domesticated and is now known as emmer [11, 12]. No wild hexaploid species are known, thus common wheat (*T. aestivum*) is a more recent cross, which was most likely formed by a spontaneous hybridization. Common wheat contains three genomes AABBDD that were derived from different ancestors and is also known as hexaploid wheat. The hybridization of the AABB genome from emmer occurred with the DD genome of a wild grass (*Aegilops tauschii*) and led to the formation of hexaploid common wheat. Further hexaploid wheat is spelt (*T. spelta* L.), which was cultivated historically but also grows nowadays. It is unknown in what order common wheat and spelt appeared. Tetraploid

durum wheat (*T. durum* Desf.) is known as a modern cultivar and is mainly used for pasta production [5].

1.2.3 Physiological properties of wheat

Wheat is mainly being cultivated for consumption within the country where it is produced, yet about one-fifth of the wheat yield is exported to other countries where wheat cannot be grown. One reason for the popularity of wheat is its source of energy, since wheat contains high contents of carbohydrates. The wheat grain contains 85% of carbohydrates, of which the major component is starch. Starch is mainly present in the endosperm of the wheat grain but also can be found in the wheat bran, however more likely as a contaminant from the endosperm [13]. Depending on the study, starch accounts for up to 80% of the carbohydrates [14] and is composed of two glucan polymers, namely amylose and amylopectin, which are present in a 1:3 ratio. Amylose consists of α-D-glucose units which are linearly linked by α 1→4 bonds, while amylopectin is a highly branched polymer of α-D-glucose units linked by α 1→4 and α 1→6 bonds [7]. The major part of starch can be digested by enzymes (α-, β-amylases) in the small intestine but a proportion of starch is inaccessible to digestive enzymes and is therefore called resistant starch [15]. Resistant starch (RS) can be classified into five types, however wheat contains only one type, namely the RS1. This type is enclosed in intact cells and is consequently physically not accessible. Further RS types are RS2-RS5 and occur in bananas, potatoes and amylose maize starch. RS2 is starch, which is inaccessible to digestive enzymes due to its structure. However, when heated, its compact structure unfolds and can be digested as a result of starch gelatinization. RS3 is retrograded amylose and starch, which is produced when starch is heated and then cooled. It forms a compact crystal structure that is inaccessible to the digestive enzymes. RS4 is chemically modified or repolymerized starch with modified chain cross-linking and is inaccessible to the digestive enzymes, whereas RS5 is a form of starch, which has been heated with oil, what leads to a new digest-resistant structure [16]. The remaining part of carbohydrates consists of approximately 7% mono-, di-, and oligosaccharides, which are present in the aleurone layer and endosperm and further 12% in cell wall polysaccharides [14]. The latter form the part known as dietary fiber, also including lignin, cellulose, fructans and resistant starch. The HEALTHGRAIN study determined that the majority of dietary fiber of wheat is

concentrated in the bran, so to say in the whole grain and is associated with health benefits [17, 18]. According to the HEALTHGRAIN definition wholegrain is defined as “the intact, ground, cracked or flaked kernel after the removal of inedible parts such as the hull and husk [19]. The most frequent oligosaccharides in wheat are fructo-oligosaccharides and fructans. The latter belong to the dietary fiber fraction and are almost entirely built of D-fructose monomers. Fructans form the group of small fermentable carbohydrates, also called FODMAPs (fermentable, oligo-, di-, and monosaccharides, and polyols). FODMAPs have an impact on the gut and health, since they are barely absorbed in the small intestine but are fermentable in the colon [20]. A low FODMAP diet can reduce the fermentation in the colon and consequently is suggested as a treatment of irritable bowel syndrome (IBS) and inflammatory bowel disease (Crohn’s disease) [21, 22]. Due to its high carbohydrate content wheat is rather considered as a good source of energy, yet it contains further components with a significant nutritional value. These components are proteins, fiber and also lipids. Important minerals such as zinc and iron [23] and also vitamins such as vitamins B and E [7] are present in wheat grains as minor components.

1.2.4 Goals of modern wheat breeding

The cultivation area of wheat occupies the largest part of all cultivated cereals worldwide. The total area accounted for 214 x 10^6 ha and the amount of harvested crops for 734 x 10^6 tons in the year 2018, which makes wheat a staple food. Since the first cultivation of wheat about 10 000 years ago, plant and wheat breeding became an important part of agriculture and has developed significantly. The main breeding goal remained unchanged until today, which is the increase of grain yield [24]. According to the FAO, wheat grain yield has more than tripled over the past 50 years. Interestingly, the harvest area remained unchanged, which means that the yield per unit area has increased. The increase of grain yield as a part of breeding practices is a common outcome of plant development and the emergence of new cultivars. On the technological side, the utilization of fertilizers has been invented in order to ensure the yield and the protein quality of wheat grains. With regard to the development of new cultivars, the adaptation to climate conditions and changes in particular ecological regions have to be considered. This is why different wheat species are suitable to grow in certain areas. While common wheat is suitable for a broad range of agricultural

environments and therefore is being cultivated on 95 % of the wheat growing area, durum wheat and spelt are less adaptable [25]. Durum wheat has a higher tolerance towards heat and arid climate compared to common wheat, which is why durum wheat is rather cultivated in dry and hot areas of the world and accounts for 5% of the total wheat production. Spelt is more likely to be cultivated in cool and hilly European regions but does not contribute significantly to the wheat growing area [26]. During the so called “Green Revolution” between 1950 and 1960 modern and high-yielding varieties have been developed by introducing the height-reducing (Rht) dwarfing genes [27]. By this means short wheat varieties have been implemented, which are less affected by environmental conditions and possible diseases [28]. Due to their reduced height, the plants do not fall down so quickly and consequently the risk of fungal infections is avoided [29]. During the period of the “Green Revolution” the new breeding practice almost doubled the global grain yield. Summarizing, the goals of wheat breeding are high yields, disease resistance, stress tolerance and nutrient efficiency [29].

1.3 Wheat proteins

Depending on the literature source wheat contains about 8-19% proteins [6, 30, 31], whereby the content and the protein accumulation in the grain are strongly influenced by the environmental and genetic effects [32]. Environmental effects cover factors such as climate, temperature, water access, soil properties and fertilization [33], whereas genetic effects comprise the wheat species and cultivars. Wheat proteins are allocated in the different grain parts and in particular: pericarp (5.1%), testa (5.7), aleurone (22.8%) and mostly to the germ (34.1%) [34]. From a nutritional point of view, the proportions of essential amino acids are a determining factor for protein quality. There are 20 amino acids, out of which nine can be considered to be essential because they cannot be synthesized from other compounds by the human body and must be provided by the diet. These are phenylalanine, valine, threonine, tryptophan, methionine, leucine, isoleucine, lysine and histidine [35]. Regarding wheat, essential amino acids are present in sufficient amounts, except for lysine. Lysine is considered the limiting amino acid and its amount is notably higher in wholegrain flour compared to white flour due to low lysine contents in the storage proteins present in the

endosperm [7]. This follows from the fact that storage proteins are rather high in glutamine and proline contents compared to lysine [23].

1.3.1 Osborne protein fractions

As described by T.B. Osborne, cereal proteins are classified into four fractions (albumins, globulins, prolamins and glutelins) based on their solubility [36]. Albumins are water-soluble and globulins are soluble in diluted salt solutions. Prolamins are soluble in aqueous alcohols and glutenins are insoluble in none of the above-mentioned solutions [37]. Usually albumins and globulins are extracted jointly with phosphate buffered saline as a first step of the modified Osborne protein fractionation. The reason for using a buffered solution is to avoid the liberation of ω-gliadins, which are partly water-soluble. Prolamins are extracted out of the residue with a 60% ethanol solution as a second step. Last, the glutelin fraction is extracted out of the residue with aqueous alcohols in the presence of reducing and disaggregating agents. The application of increased temperatures (60°C) and reducing and disaggregating compounds (1% dithiothreitol (DTT) and urea, respectively) are crucial to cleave the interchain disulphide bonds of glutenins. As glutenins are obtained as monomeric subunits after cleavage, they can be extracted with aqueous alcohols (1-propanol). After the last extraction step, the residue contains structural proteins (Figure 1). Prolamins and glutelins are a group of proteins, which have different names dependent on the cereal they are present in. The prolamins are called gliadins (wheat), hordeins (barley), secalins (rye), zeins (mayze), kafirins (sorghum) and avenins (oats). The glutelins are named glutenins (wheat), hordenins (barley) and oryzenins (rice).

1.3.1.1 Albumins and globulins

The albumin/globulin fraction accounts for 20-25% of the grain protein and mostly has a molecular weight lower than 25 kDa. However, some proteins present in this fraction have a molecular weight between 60 and 70 kDa [38]. The albumin/globulin fraction mainly contains metabolic proteins, such as enzymes and enzyme inhibitors [39]. In the first place, enzyme inhibitors, which belong to the family of trypsin- and α-amylase-inhibitors, are involved in plant defense, e.g. against disease and for pest resistance [40]. Nonetheless, α-amylase- and trypsin-inhibitors have been shown to be the main

allergens triggering baker's asthma [41]. Furthermore, α-amylase-trypsin-inhibitors (ATIs) are considered to activate the innate immune response and presumably cause non-celiac gluten sensitivity (NCGS) [42]. Minor allergens present in the albumin/globulin fraction are wheat germ agglutinin, β-amylase and lipid transfer proteins [43]. Further enzymes present in the wheat albumin/globulin fraction are responsible for the hydrolysis of carbohydrates (α- and β-amylase, β-glucosidase, cellulase, arabinoxylanase), proteins (peptidases) and lipids (lipase, lipoxygenase) to ensure the germ nutrient supply during germination [44]. The hydrolysis can be inhibited by enzyme inhibitors, of which ATIs play a major role. In the grain albumins/globulins are mostly present in the outer layers of the bran and the germ. Minor amounts can also be found in the endosperm [44]. Particularly with regard to the amino acid composition, the proteins present in the albumin/globulin fraction show higher nutritional values compared to the proteins present in the gliadin or glutenin fraction. Especially the essential amino acids lysine, tryptophan and methionine are present in relatively high amounts [44, 45].

1.3.1.2 α-Amylase-trypsin-inhibitors (ATIs)

ATIs are associated with evolutionary processes like protection of the wheat grain from insects, mites, mammals and parasites by inhibition of their α-amylase and trypsin enzymes and are expressed in the grain endosperm [43, 46, 47]. They are present in the water- and salt-soluble albumin/globulin fraction and are mono-, di- or tetrameric wheat proteins with molecular weights ranging between 12 and 60 kDa. ATIs can be classified into three major groups, two of which are mainly responsible for the inhibition of α-amylase and the other one for α-amylase and trypsin [48, 49]. The first group contains monomeric inhibitors (WMAI) with a molecular weight of 12 kDa and is referred to ATI 0.28, which is encoded on the short arm of the group 6 chromosomes (6B+6D) [43, 48]. The second group contains dimeric inhibitors (WDAI) with a molecular weight of 24 kDa and includes ATI 0.19 and ATI 0.53, which are encoded on the short arm of the group 3 chromosomes (3B+3D). The third group is called tetrameric inhibitors (WTAI) with a molecular weight of 60 kDa and includes CM1, CM2, CM3, CM16 and CM17 proteins. The name is derived from the fact that these proteins are soluble in chloroform/methanol. Generally, WTAI are structured by one copy of CM1 or CM2, one copy of CM16 or CM17 and two copies of CM3. CM1 and CM2 are encoded on

chromosomes 7B and 7B+7D, respectively. CM3 is encoded on chromosomes 4B+4D, whereas CM16 and CM17 are encoded on chromosomes 4B and 4D, respectively [43, 48, 50].

The listed ATIs have been shown to trigger baker's asthma [43] and further have been suggested as activators of the innate immune response by activating the toll-like receptor 4 complex (TLR4)-MD2-CD14 on monocytes, macrophages and dendritic cells, based on studies of intestinal biopsies. Especially ATIs 0.19 and CM3 have been reported to be major triggers. ATIs are resistant to human intestinal proteolysis and therefore are able to pass the upper part of the intestine as intact proteins. After passing, ATIs are recognized by intestinal mucosal cells and activate the TLR-4 complex (Figure 3). The activation results in a release of proinflammatory chemokines and cytokines interleukin (IL)-8, tumor necrosis factor (TNF)-α and chemokine (C-C motif) ligand 2 (CCL2) and consequently in an activation of the innate immune response and possibly in triggering non-celiac gluten sensitivity (NCGS). It has been noted that the innate immune response triggered by ATIs is dose-dependent and therefore preventable by a reduction of intake [51-53].

1.3.1.3 Gluten proteins

Gliadins and glutenins are storage proteins of the grain and are called gluten proteins. Initially, gluten was defined as the proteinaceous mass remaining after washing wheat dough with water or saline to remove starch and water-soluble compounds. Depending on the washing efficiency, the remaining material contains 75-80% protein based on dry matter [45, 54]. More accurate and according to Codex Standard 118-1979, gluten is the "protein fraction from wheat, rye, barley, oats or their crossbred varieties and derivatives thereof, to which some persons are intolerant and that is insoluble in water and 0.5 mol/L NaCl" [55]. Gluten proteins are only present in the starchy endosperm of the grain and cannot be found in the pericarp or germ. During germination gluten proteins serve as nitrogen- and amino acid storage [56]. The storage proteins account for 70-80% of total grain proteins and the relative amount of gluten proteins depends on the total protein content. The higher the protein content, the higher the proportion of storage proteins. On the contrary, the lower the protein content, the lower the proportion of storage proteins and the higher the proportion of albumins/globulins [44]. Gluten is composed of a complex protein mixture, with either monomeric proteins or

linked by disulfide interchain bonds. Due to their different solubility properties, gluten can be divided into two fractions, the soluble gliadins and insoluble glutenins. The gliadins can be extracted with aqueous alcohols (60-70% ethanol), while glutenin extraction requires aqueous alcohols and additionally reducing and disaggregating agents [37]. Gluten has technofunctional properties since it allows wheat flour to form a strong, cohesive, viscous and elastic dough with gas holding capacity after the addition of water and is therefore of special interest. Gliadins and glutenins contribute to the rheological properties of dough but in different roles. Whereas gliadins are responsible for the viscosity and extensibility of the dough and are considered as softeners [57], glutenins contribute to dough strength and elasticity due to their three-dimensional structure [58]. The mixture of both fractions or more precisely, their ratio, is the key factor determining the technological function of gluten. Thus, the gliadin/glutenin ratio is one of the important factors responsible for the quality and properties of bread [59].

Gliadins and glutenins are classified into protein types and subunits. However, the nomenclature of gluten proteins is inconsistent. There are three possible ways to classify storage proteins, namely based on 1) the amino acid sequence, 2) the molecular weights or 3) the electrophoretic mobility and molecular weights. In the first case, both gliadins and glutenins are called prolamins and are subdivided into sulphur-(S) rich, S-poor and high-molecular-weight (HMW) prolamins according to their protein primary structure [60]. For the second case, gluten proteins (gliadins and glutenins) are classified into three groups according to their molecular weights: 1) HMW-group (67-88 kDa); 2) medium-molecular-weight (MMW)-group (40-50 kDa) and 3) low-molecular weight (LMW)-group (30-42 kDa) [61]. In the third case, the gliadin and glutenin fractions are subdivided into types and subunits. Thus, based on the electrophoretic mobility the gliadins are classified into ω5-, ω1,2-, α- and γ- gliadins, whereas the glutenin fraction is subdivided into high-molecular-weight (HMW-GS) and low-molecular-weight glutenin subunits (LMW-GS) based on different molecular weights [62].

Gluten contains all 20 amino acids, including the nine essential amino acids phenylalanine, valine, threonine, tryptophan, methionine, leucine, isoleucine, lysine and histidine. Yet, the biological value of gluten proteins is rather low with values between 50-60% [63, 64]. This is due to average or below average limiting amounts of essential amino acids. In gluten proteins, methionine, tryptophan and especially lysine are present in rather low amounts (0.5-2.4%, 0.0-0.8% and 0.0-4.0%, respectively) and therefore are limitation factors regarding the nutritional value [23, 39]. On the contrary, two

amino acids, namely glutamine and proline, are present in rather high amounts, and this leads to an imbalanced amino acid composition [65]. Glutamine accounts for 26-53% and proline for 10-29% of the total amino acid content and both contribute to the immunoreactivity of gluten proteins. Generally, proteins are digested and cleaved into peptides and amino acids by human gastric, pancreatic and brush border enzymes. However, human gastrointestinal enzymes are unable to digest proteins before or after proline and glutamine, resulting in large peptides of more than nine amino acid residues [66]. These residues contain CD-active peptides, which reach the small intestine, pass the intestinal epithelium and are detected by T cells of the lamina propria, where they initiate a strong autoimmune reaction in CD patients [67-69]. In a nutshell, gluten is characterized by high amounts of glutamine and proline, which make gluten proteins resistant to cleavage by intestinal peptidases, resulting in CD-active peptides, which in turn trigger a strong immune response.

1.3.1.4 Gliadins

The gliadin fraction is soluble in aqueous alcohols (e.g. 60% ethanol) and mainly consists of monomeric proteins with molecular weights from 28 to 55 kDa, which contribute to the extensibility and viscosity of wheat flour dough. Based on their electrophoretic mobility gliadin proteins can be subdivided into ω5-, ω1,2-, α- and γ-gliadins [39, 45]. According to genetic studies, gliadins are encoded on the chromosomes 1A, 1B, 1D and 6A, 6B, 6D. It has been reported that ω-gliadins and the majority of γ-gliadins are located on the short arms of the 1A, 1B, 1D chromosomes, namely Gli-A1, Gli-B1 and Gli-D1 [70]. On the contrary, α-gliadins are encoded on the short arm of the chromosomes 6A, 6B and 6D, namely Gli-A2, Gli-B2 and Gli-D2 [31]. Gliadins are characterized by exceptionally high amounts of the amino acids glutamine and proline and at the same time by low amounts of nutritionally important amino acids, especially lysine, tryptophan and methionine are present in rather poor quantities.

The ω-gliadins are predominated by proline, glutamine and phenylalanine, which constitute about 80 % of the total amino acid amount present in ω-gliadins [71]. Commonly, ω-gliadins are further classified into ω5- and ω1,2-gliadins and belong to the MMW-group. The ω5-gliadins consist of around 420 amino acid residues and therefore have a molecular weight of approximately 50 kDa. The ω1,2-gliadins are composed of 320-380 amino acid residues and have a molecular weight of roughly

40 kDa. With one exception, ω-gliadins are poor in sulfur because they do not contain cysteine and methionine and consequently they lack cross-linking via disulfide bridges [45, 71]. The exception, which is called ωb-gliadins, is a modified sequence containing a cysteine residue at the C-terminal end [72]. The primary structure of ω-gliadins is characterized by a short non-repetitive N-terminal domain, a large repetitive domain with mainly glutamine, proline, phenylalanine and a non-repetitive C-terminal domain. The secondary structure of ω-gliadins contains many ß-loops with repeating sequences which are rich in glutamine and proline [45, 73]. The repetitive domain is characterized by repetitive units QQPQQPFP for ω1,2- and QQQFP for ω5-gliadins [66]. In contrast to ω-gliadins, α-gliadins show lower contents of proline, phenylalanine and in some cases also of glutamine and glutamic acid. Compared to α-gliadins, the γ-gliadins contain more proline, phenylalanine, methionine and lysine. Nonetheless, proline (11-22%) and glutamine (28-36%) are the predominant amino acids in α- and γ-gliadins, followed by high amounts of leucine (5-9%) and valine (5-8%) [66]. Both, α- and γ-gliadins, are rich in sulfur, since they contain cysteine residues and therefore have the ability to form intramolecular disulfide bonds [71]. The sequences of α- and γ-gliadins are composed of roughly 300 amino acid residues and both protein types are similar in their molecular weight (28-35 kDa) [66]. The N- and C-terminal domains of α- and γ-gliadins show clear differences in terms of repetitive sequences and their frequency. The N-terminal domain is characterized by sequences, which are abundant in glutamine, proline, phenylalanine and tyrosine but which also are unique for each protein type. Typical repetitive units for α-gliadins are peptides such as QPQPFPQQPYP, which are replicated up to five times and also can show modifications of the residues. In contrast, γ-gliadins show modified (additional inserts of amino acids) sequences of the repetitive unit QPQQPFP, which are repeated up to 16 times. Both, α- and γ-gliadins are homologous in terms of their C-terminal domains. This domain consists of non-repetitive units, with fewer amounts of glutamine and proline compared to the N-terminal domain. Located in the C-terminal domain and with some exceptions, α-gliadins contain six cysteines, which form three homologous intramolecular disulfide bonds, whereas γ-gliadins contain eight cysteines, which form four homologous intrachain crosslinks [45, 74]. Studies of the secondary structure have shown that the N-terminal domain of α- and γ-gliadins is characterized by ß-loops, similar to the ω-gliadins. In contrast to the ω-gliadins the C-terminal domain includes α-helices and ß-sheet structures [75].

1.3.1.5 Glutenins

The glutenin fraction is only soluble in aqueous alcohols when treated with increased temperatures, disaggregating and reducing agents due to the presence of interchain disulfide bonds. The latter are responsible for the elasticity of the dough and are classified into HMW-GS (67-88 kDa) and LMW-GS (32-40 kDa) [62]. LMW-GS cover a large part of gluten proteins. As a predominant protein type, LMW-GS account for 20% of total gluten proteins and are similar to α- and γ-gliadins in terms of molecular weight and amino acid residues [76]. This follows from the fact that α- and γ-gliadins belong to the LMW-group with molecular weights ranging between 28 and 35 kDa. Likewise, the LMW-GS contain a repetitive N-terminal and a non-repetitive C-terminal domain. The N-terminal domain contains many recurring sequences such as QQQPPFS, which are rich in glutamine and proline. LMW-GS possess eight cysteines and six of them are in homologous positions as in the α- and γ-gliadins [74]. For this reason, it is assumed that LMW-GS are linked by intramolecular disulfide bonds. The two remaining cysteine residues, which are present in the N- and C-terminal domains number 1 and 4, do not form intramolecular disulfide bonds, probably due to steric reasons. Thus, these two cysteines can form intermolecular disulfide bonds with different gluten proteins [45].
The HMW-GS are among the minor components within the gluten proteins and account for roughly 10%. They are composed of around 600-800 amino acid residues with molecular weights ranging between 70 and 90 kDa. The predominant amino acids are glutamine (26-36%), glycine (16-20%) and proline (10-15%) [66]. HMW-GS can be further divided into two groups, namely the x- and y-type, based on their molecular weights and numbers and composition of the repetitive units. In the x-type, proteins with molecular weights of 83-88 kDa can be found, whereas in the y-type proteins with molecular weights of 67-74 kDa are present. The coding genomes (A, B and D genome), the type (x or y) and mobility in SDS-PAGE (numbers 1-12) determine the nomenclature of single HMW-GS. The HMW-GS comprise three structural domains: a non-repetitive N-terminal domain (A) containing 80-105 amino acids, a repetitive domain (B) with approximately 480-700 amino acids and a C-terminal domain (C) with 42 amino acids [71]. The domains A and C are characterized by the presence of numerous charged amino acids (glutamic acid, arginine) and almost all occurring cysteine residues. The B domain contains high amounts of glutamine, glycine and proline and is characterized by repetetive hexapeptides (QQPGQG) as the backbone, complemented by inserted hexapeptides (e.g. YYPTSP) and tripeptides (e.g. QQP or

QPQ). The biggest difference between the x- and y-type are the A and B domains. The HMW-GS of the x-type, except subunit Dx5, have four cysteine residues, three of which are located in domain A and one in domain C [77]. Two of three cysteine residues present in the A domain are linked by intrachain disulfide bonds, while the third residue of domain A and also the one present in domain C are linked by interchain disulfide bonds. The HMW-GS Dx5 has an additional cysteine residue in the B domain, probably derived from another interchain bond. The HMW-GS of the y-type have five cysteine residues in the A domain and one cysteine residue each in the B and C domain. Consequently, the y-type contains more cysteine residues in total than the x-type HMW-GS [78].

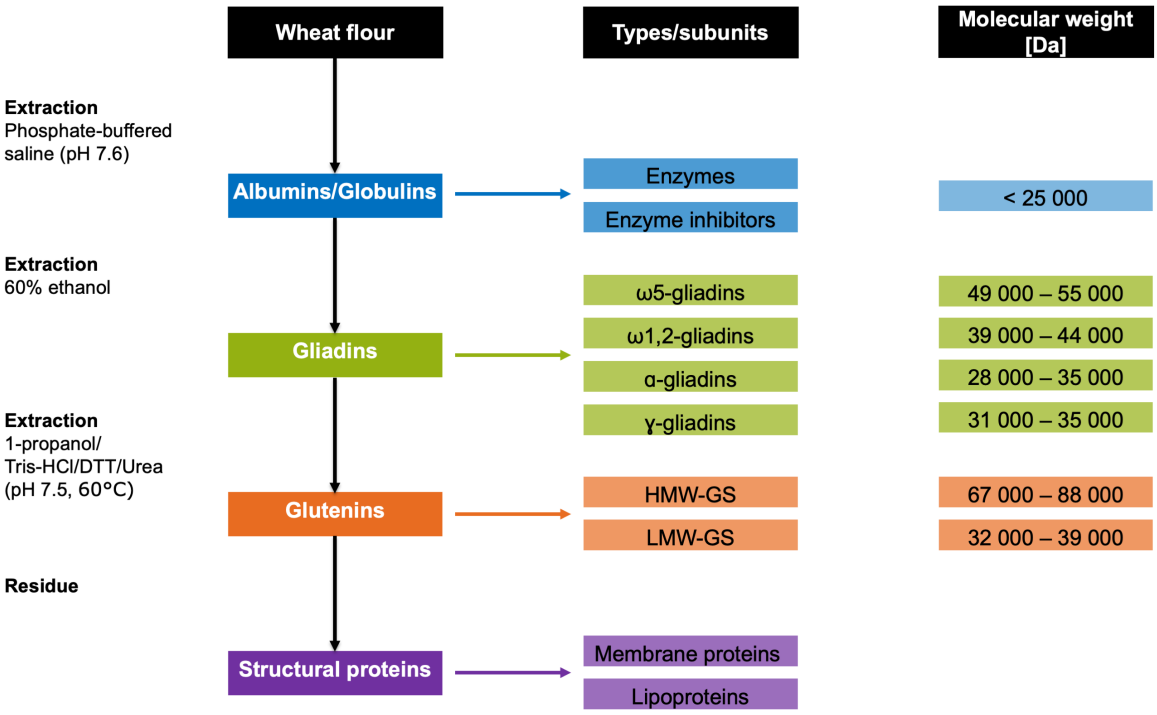

Figure 1: Modified Osborne protein fractionation based on the solubility of wheat proteins. The wheat flour is treated with phosphate buffered saline to obtain the albumin/globulin fraction. The gliadin fraction is obtained with 60% ethanol and the glutenin fraction with buffered 1-propanol under reducing conditions and at 60°C. The albumin/globulin fraction consists of enzymes and enzyme inhibitors, the gliadin fraction of ω5-, ω1,2-, α- and γ-gliadins and the glutenin fraction of high- and low-molecular-weight glutenin subunits (HMW- and LMW-GS). The according molecular weights are presented for all protein types and subunits [45]. DTT, dithiothreitol; Tris, Tris(hydroxymethyl)aminomethane.

1.4 Wheat-related disorders/induced sensitivities

Despite the fact that wheat constituents provide nutritional and physiological benefits, they may cause undesirable effects in predisposed individuals. The ingestion of wheat can trigger a variability of diseases including allergies and other immunological reactions in humans. In this context, the incidence of wheat-related disorders increased greatly over the past 50 years, which constitutes an epidemiologically relevant phenomenon. The global prevalence is estimated to be up to 5% and is therefore subject of current research [79]. Wheat dependent disorders are also called gluten-related disorders and according to the "Oslo definitions" include conditions, which are related to gluten [80]. Depending on the pathomechanism, the disorders are classified into allergic reactions, autoimmunogenic responses and innate immune responses (Figure 2) [81]. Allergic reactions are immunoglobulin E (IgE)-mediated and comprise, amongst others, food allergy and wheat-dependent exercise-induced anaphylaxis (WDEIA), whereas autoimmune responses are IgA- and IgG-mediated and comprise celiac disease (CD) as most important representative. The third type of symptomatic responses to wheat ingestion is the so-called non-celiac gluten sensitivity (NCGS) and is probably mediated by the activation of the innate immune response [82]. Wheat allergy (WA) is an adverse reaction to wheat proteins and is associated with the production of IgE antibodies. Commonly, WA appears minutes or hours after gluten exposure and is characterized by four types: 1) immediate food allergy, 2) respiratory allergy (baker's asthma), 3) WDEIA and 4) contact urticaria [81, 83]. The most investigated and well-known disorder is the inflammatory disease of the digestive tract, also called CD. CD affects about 1% of the population and is triggered by gluten-derived peptides in genetically predisposed individuals [84, 85]. Affected individuals carry the HLA-DQ2 and/or -DQ8 genotypes, which were shown to be strongly associated with the development of the disease [86]. CD is characterized by inflammation of the small intestine and villous atrophy, with the result of nutrient malabsorption. Concerning clinical manifestations, the symptoms of CD are described as intestinal (e.g. bloating, diarrhea) and extra-intestinal (e.g. skin rash, anemia) [81].

The only available therapy for affected patients is a lifelong gluten-free diet. Besides WA and CD, there are responses after wheat consumption in which neither allergic nor autoimmunogenic mechanisms are involved. This disorder is the so-called NCGS and has a prevalence of up to 6% of the population [81, 87]. NCGS symptoms usually appear

shortly after wheat ingestion or up to a few days later and are intestinal (e.g. bloating, abdominal pain, diarrhea) and extra-intestinal (e.g. headache, foggy mind and muscle pain) [88]. NCGS is least well-defined among wheat induced disorders because the pathogenic mechanism is poorly understood, and the triggering factors are still unclear. So far, the diagnosis is made by exclusion of CD, WA and irritable bowel syndrome (IBS). The current opinion is that gluten proteins, ATIs and FODMAPs might possibly contribute to NCGS symptoms [87, 89, 90].

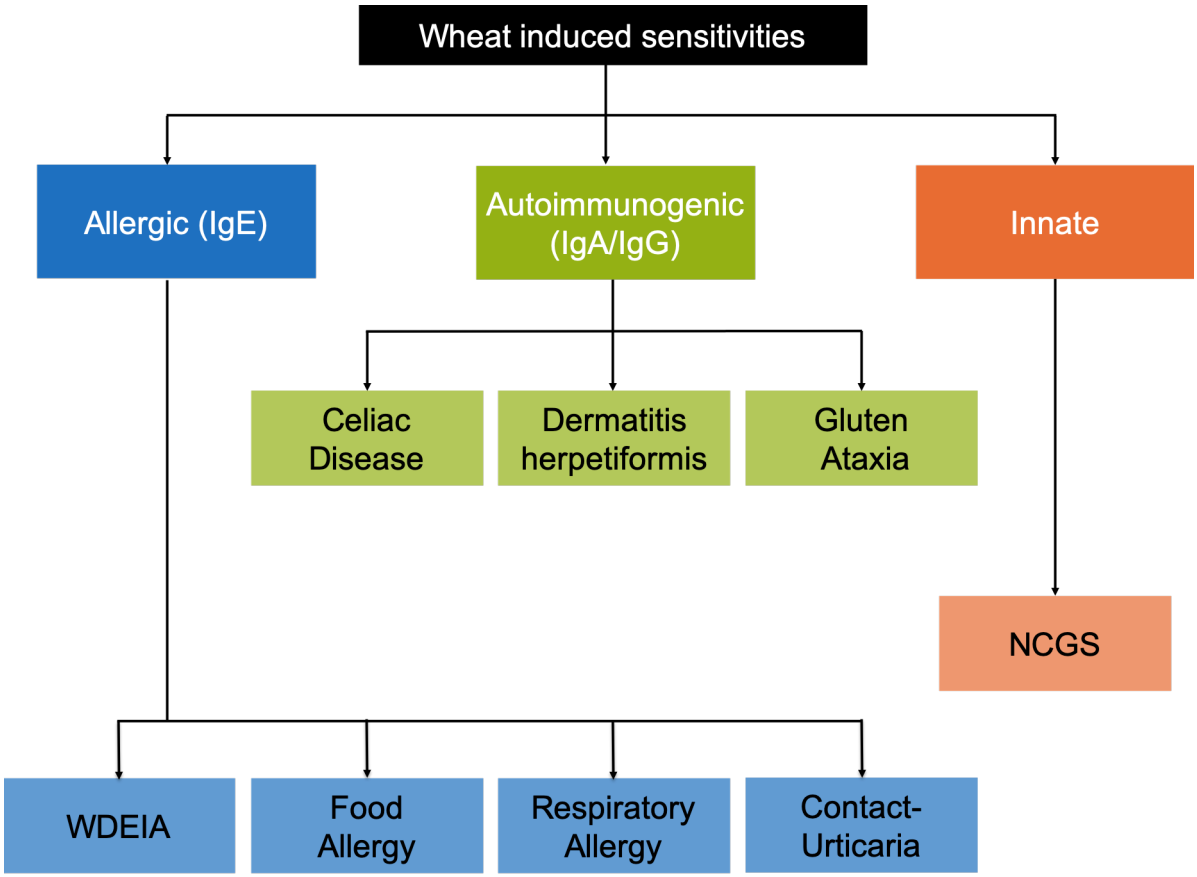

Figure 2: Overview of wheat induced sensitivities according to their pathomechanism. The classification is modified from Sapone et.al, 2012 [81]. Ig, Immunoglobulin; NCGS, Non-celiac gluten sensitivity; WDEIA, wheat-dependent exercise-induced anaphylaxis.

1.4.1 Celiac disease

1.4.1.1 Overview

According to the “Oslo definitions“ CD is “a chronic small intestinal immune-mediated enteropathy precipitated by exposure to dietary gluten in genetically predisposed individuals” [80]. In the field of cereal chemistry, gluten is defined as the remaining rubbery mass after washing wheat dough with water or salt solution to remove starch

granules and water-soluble constituents [45]. In contrast, in the field of CD, gluten is defined as “a protein fraction from wheat, rye, barley, oats or their crossbred varieties and derivatives thereof, to which some persons are intolerant and that is insoluble in water and 0.5 mol/L NaCl” [55]. The autoantigen in CD is tissue transglutaminase (TG2) of the small intestine in complex with gluten ingested through food. Since only the HLA molecules DQ2 and DQ8 can bind gluten peptides and present them to the immune system, only individuals who carry these HLA characteristics are affected. Initially, CD was described as a rare childhood disease and the diagnosis was mainly based on clinical symptoms such as diarrhea, abdominal pain, malabsorption and weight loss. An early study from the 1950s showed that the outcome of CD-like symptoms in Great Britain was between 1:5000 and 1:10000 [91]. Subsequently, when improved diagnostic techniques, including intestinal biopsy and serological tests, had been developed, the awareness of the disease increased on the one hand and on the other, it was shown that CD affects individuals at any age [92, 93]. In South America, North Africa and Asia, CD has been considered as rare but epidemiological studies showed that the disease was and still is underdiagnosed due to lack of diagnostic possibilities [94]. Nowadays, the prevalence in Western populations lies between 1:70 and 1:200, which implies approximately 1% prevalence on average, which means that CD is rather a frequent disorder [95]. In the past, CD has been considered a rare disease primarily affecting individuals of European origin, however it has been shown that CD is a common condition in different continents, with a worldwide mean prevalence of 0.9% [96]. According to the most recent findings, the frequencies of CD show significant regional differences, with values ranging between 5.6% among the Saharawi refugees of Berber-Arabic origin in North Africa [97], 2.0% in Finland, 0.7% in the US and 0.3% in Germany [85].

1.4.1.2 Changes in the prevalence over the past decades

Several studies have reported a significant increase of the incidence of CD and also NCGS over the past 50 years [87, 98-102]. In 2007, Lohi et al. reported that the prevalence of CD between 1978 to 2001 has doubled in Finland [98]. In another study from 2009, Rubio-Tapia et al. showed that the prevalence of undiagnosed celiac disease in the United States increased 4-fold during the past 50 years [99]. The outcome of the study was supported by recent investigations by Ludvigsson et al. (2013), who reported that

the incidence of CD in North America further increased between 2000 and 2010 [100]. A significant increase was also proposed by a study conducted by Grode et al. (2018), who investigated the prevalence of diagnosed CD in Denmark between 1986 and 2016 [101]. The reasons behind the increase can only be partly explained by better diagnostics and increased awareness, hence environmental factors have been suggested as a major cause [98]. Beyond that, new baking techniques with minimized fermentation time as a result of growing population and consequently growing demand for wheat products, have been suggested to contribute to the increase [103]. Other possible causes are an increased use of vital gluten in order to improve baking properties [104, 105] and also changes in the protein composition and use of wheat varieties that might have a greater amount of immunogenic peptides due to modern wheat breeding practices [81, 106]. Several studies have been conducted to elucidate the influence of breeding of the past 100 years on the protein contents of wheat. A study performed by Hucl et al. (2015) reported an increase of protein contents [107], whereas two studies from the US did not find any significant changes [104, 108]. In contrast, studies from the US [109], UK [110] and Germany [111] reported that the protein contents decreased over the last century. Modern wheat breeding practices focus on high yields, improvement of disease resistances and an efficient use of fertilizers [29]. There is evidence that modern agricultural techniques and consequently the selection criteria for breeding might have resulted in an elevated immunostimulatory potential of modern wheat cultivars compared to old wheats and landraces [102, 104, 112]. Several studies were performed to clarify whether the immunostimulatory potential of wheat has changed over the last century. Overall the results were inconclusive, since some studies in fact showed that the contents of CD-active peptides increased [112-114], whereas other studies did not find any changes [115-117].

1.4.1.3 Celiac disease – a multifactorial disorder

The development of CD is characterized by a combination of genetic and environmental factors [118]. The predisposition to CD is based on the major histocompatibility class II genes, which encode the human leukocyte antigens (HLA)-DQ2 and -DQ8 on chromosome 6. Either HLA-DQ2 or HLA-DQ8 is expressed in predisposed individuals, hence approximately 90-95 % of CD patients carry DQ2 alleles, while the remaining patients carry DQ8 alleles. HLA-DQ molecules are

proteins, which are heterodimeric receptors with α- and β-chains. HLA-DQ are expressed on the surface of antigen-presenting cells (APCs), which in turn are responsible for binding the gluten peptides in order to present them to CD4+ T cells. HLA-DQ2 proteins have two isoforms, namely DQ2.5 and DQ2.2. The HLA-DQ2.5 heterodimer is encoded in a cis- and in a trans-form. The cis-form is characterized by two genes: DQA1*05 (α-chain) and DQB1*02 (β-chain), which are located on the same DR3-DQ2 haplotype, whereas the trans-form shows the same genes but located on DR5-DQ7 and DR7-DQ2, respectively. HLA-DQ2.5 is known as a very high predisposition factor for CD, followed by DQ8 (high predisposition) and DQ2.2 (low predisposition) [86, 119]. This can be explained by the fact that HLA-DQ2.5 receptors are able to bind numerous gluten peptides, which contain a number of proline residues, that make these proteins resistant to proteolytic degradation. On the contrary, DQ8 and DQ2.2 only bind few selected gluten epitopes, which contain fewer proline residues and are therefore less resistant to degradation [120, 121]. Approximately 30% of the population express HLA-DQ2/8 but only 1% of the population develops CD. This is why the absence of these alleles is an indicator to exclude CD but on the other hand their presence is not sufficient to develop CD [86]. Thus, environmental factors are also associated with the onset of CD. Since CD-related symptoms disappear on a gluten-free diet, gluten and also the amount of gluten consumption are the primary environmental factors. Further environmental factors that provoke a loss of tolerance to gluten are infections (hepatitis C, adenovirus 12, rotavirus) [122], changes of intestinal microbiota [123] and the so-called hygiene hypothesis, which postulates that a high level of hygiene has led to an increase of immune-mediated hypersensitivities [124]. It has also been discussed that the way of childbirth (natural or Cesarean section), the duration of breastfeeding and the timing of gluten introduction to the diet of infants may be associated with CD development [125-127]. On the contrary, other studies have shown that the age of gluten introduction and breastfeeding neither influenced, nor prevented the development of CD [128].

1.4.1.4 Manifestations of celiac disease

Celiac disease is a frequent gluten-induced autoimmune disorder, which affects approximately 1% of the global population. The classical form of CD with typical symptoms, such as chronic diarrhea and abdominal pain represents only the tip of the

so called “celiac-iceberg” [129]. The iceberg can be subdivided into three sections: 1) symptomatic, 2) asymptomatic (silent) and 3) potential. All manifestations have two things in common, namely the genetic predisposition (HLA-DQ2/8) and the occurrence of CD-specific IgA and IgG antibodies. The tip of the iceberg or the symptomatic section, represents CD patients, which show classical intestinal and/or extraintestinal symptoms, mainly caused by malabsorption of nutrients. Intestinal symptoms include diarrhea, vomiting and abdominal pain, whereas extraintestinal symptoms are characterized by decreased bone mineral density, iron-deficiency anemia and dental enamel defects [130]. Classically, CD is characterized by a damaged mucosa of the upper small intestine (duodenum, proximal jejunum) and is consequently accompanied with crypt hyperplasia, increased infiltration of intraepithelial lymphocytes (IELs) and villous atrophy [131]. Patients with classical symptoms and a flat mucosa can be diagnosed by biopsy and show an improvement of the mucosal structure and a reduction of the symptoms when maintaining a strict gluten-free diet (GFD). Only a small part of individuals shows symptomatic CD, whereas the majority of patients are among two big groups of silent or potential CD. Silent CD is characterized by atypical, minimal or even missing indications for a disorder. Usually, typical CD symptoms are absent, but patients show the typical villous atrophy. By screening at-risk populations (e.g. first-degree relatives of CD patients), patients with silent CD are diagnosed. The last section of the “celiac-iceberg” include CD patients with potential CD. This means that these individuals neither show a damaged mucosa, nor typical symptoms. However, these patients are diagnosed due to the presence of CD-specific IgA and IgG antibodies. Patients belonging to the silent and potential sectors are exposed to higher risks of developing long-term consequences such as osteoporosis, infertility, anemia, or malignancy in case of undiagnosed CD [80].

1.4.1.5 Pathomechanism

The pathomechanism of CD is initiated by the innate and adaptive immune responses in the lamina propria, which is a part of the intestinal lymphatic tissue. Both mechanisms are presented in Figure 3. Usually, human peptidases are able to digest proteins from the diet into small peptides and single amino acids. The inability of human gastric (pepsin), pancreatic (trypsin, chymotrypsin, elastase, carboxypeptidase) and brush border enzymes to digest the proline- and glutamine-rich

gluten peptides is an important precondition for the development of CD. The resulting peptides are relatively large with at least nine amino acid residues. These peptides are characterized by a high number of proline residues with a left-handed polyproline II helical conformation and numerous glutamine residues, which are deamidated or transamidated by human tissue transglutaminase (TG2) resulting in increased immunogenicity [132, 133]. It has been reported that more than 1000 CD-active deamidated or native gluten peptides present in wheat gliadins and glutenins and beyond that in rye secalins, barley hordeins and oat avenins have been identified [69]. In this context, a distinction is made between CD-toxic and/or- immunogenic. The majority of the peptides is described as immunogenic and is considered to induce the adaptive immune response. Seven peptides are characterized as CD-toxic based on in vivo or organ culture studies and five are described as CD-toxic and CD-immunogenic based on T-cell proliferation assays [134, 135]. Generally, gluten is digested similarly by both healthy individuals and CD-patients, however compared to healthy individuals, CD-patients show an intestinal epithelium permeable for macromolecules. Due to the permeability gluten peptides pass the small intestinal epithelium of CD-patients. The uptake of the undigested peptide fragments has not yet been conclusively clarified. It has been considered that defects in the epithelial terminal ridge network ("tight junctions") are responsible for the uptake. The intercellular tight junctions are modulated by a protein called zonulin and the upregulation of zonulin might lead to increased intestinal permeability [94, 136]. After passing through the mucous membrane of the small intestine gluten peptides with QXP or QXXJ motifs (X, any amino acid, J, hydrophobic amino acid) are either transamidated by the calcium-dependent TG2 or are deamidated (conversion of neutral glutamine residues to negatively charged glutamic acid residues). In contrast, peptides with QP and QXXP motifs mostly remain unchanged [137, 138]. This process changes the antigenic potential of the gliadin peptides by optimizing their binding to the HLA-DQ2/8 heterodimers, which are expressed on the cell surface of antigen presenting cells (APCs) [139]. In general, the binding affinity of gluten peptides to HLA-DQ2 or -DQ8 depends on their deamidation positions. In case of deamidation at positions 4, 6 and 7 the binding to HLA-DQ2 is preferred, whereas HLA-DQ8 prefers deamidation at positions 1 and 9 [120]. The peptide-DQ2/8-complex is presented to the T cell receptors by the APCs and subsequently the CD4+-T cells are activated. Essentially, after the activation of CD4+-T cells there are two subsequent pathways: the pro-inflammatory Th1-pathway and the

anti-inflammatory Th2-pathway. In the first case, the gluten specific CD4+ T cells activate the secretion of pro-inflammatory cytokines, such as interferon- (IFN)-γ and TNF-α, which induce the expression of mucosa-damaging matrix metalloproteinases (MMP) in macrophages and fibroblasts and cause mucosal damage directly. On the other hand, activated CD4+-T cells trigger the production of B-cells and also their differentiation into plasma cells, which in turn produce IgA and IgG antibodies against gluten (AGA), endomysium (EMA) and TG2. These antibodies are detected in laboratory diagnostics [86]. Some toxic gluten peptides can activate the innate immune response due to their weak HLA-DQ binding capacity, which is not sufficient to activate the adaptive immune response [126]. These peptides initiate the secretion of the cytokine interleukin (IL)-15, which is generated by cells from the epithelium and the lamina propria and plays a central role in activating both, the adaptive and the innate immune responses [140, 141]. By activating enterocytes, macrophages and dendritic cells, IL-15 is secreted and leads; on the one hand, to a stimulation of IELs to express the receptor NKG2D, and, on the other hand, to stimulation of epithelial cells to express MICA (major histocompatibility complex class I chain-related molecule A). The subsequent binding of MICA to NKG2D leads to a damage of the epithelium (villous atrophy) by IELs [142] [139]. It has been shown that the non-immunodominant α-gliadin peptide LGQQQPFPPQQPY triggers the innate immune response [143]. In addition, ATIs from cereals are also associated with the activation of the innate immune response [144].

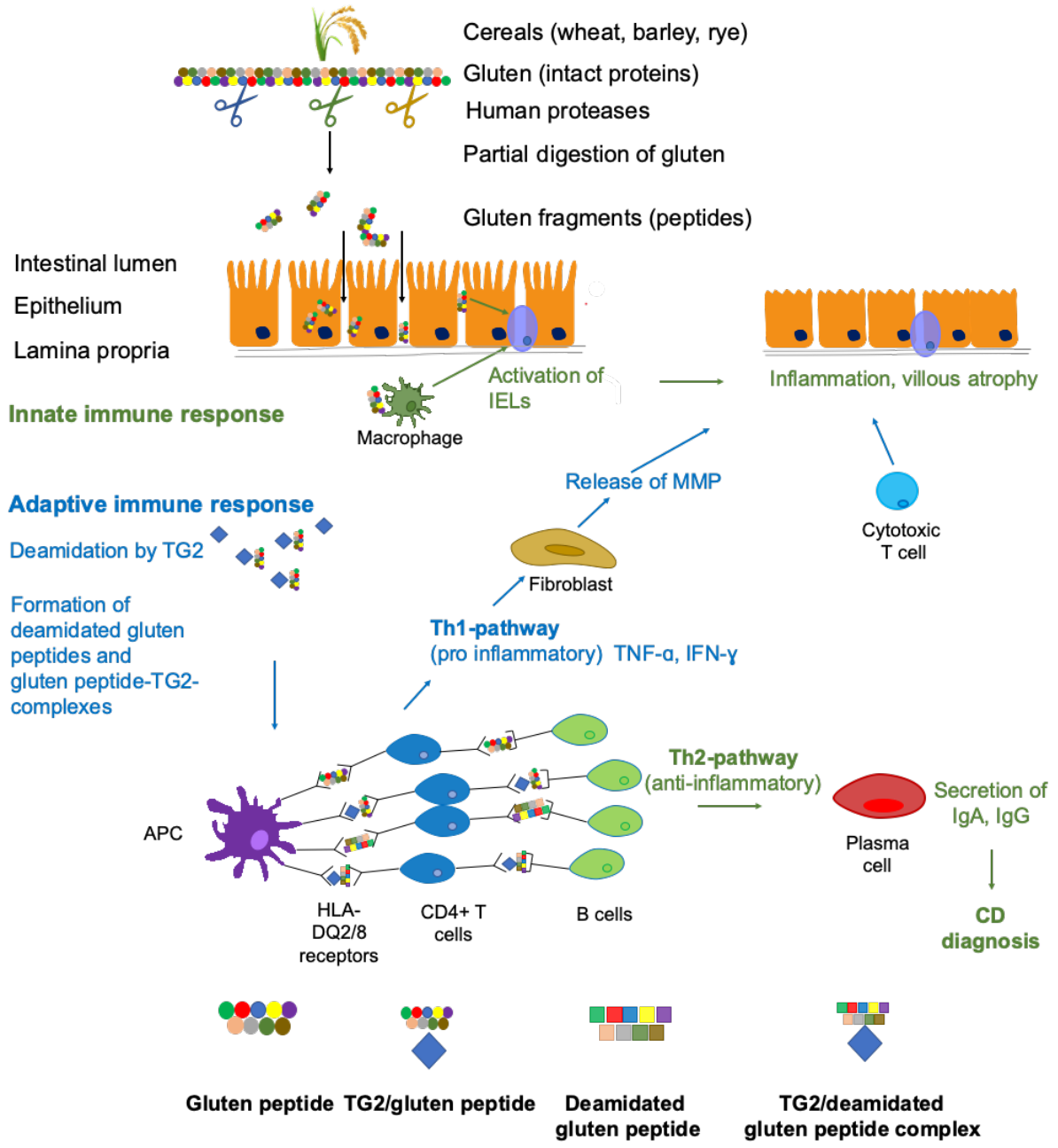

Figure 3: Celiac disease pathomechanism including the adaptive and innate immune responses. (APC, antigen-presenting cell; IELs, intraepithelial lymphocytes; IFN-γ, interferon-γ; MMP, matrix metalloproteinase; TG2, tissue transglutaminase; TNF-α, tumor necrosis factor- α). Figure modified from [67, 142].

1.4.1.6 Diagnosis

The pathophysiology of CD shows that it is a genetically determined, T cell-mediated, chronic inflammatory process directed against small intestinal tissue. Thus, the diagnostic spectrum includes serological, genetic and histological examinations. The European Society for Pediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN) suggests that a reliable diagnosis depends on symptoms, histology and

serology. According to their guidelines and a newly developed diagnostic algorithm for children, symptomatic patients should undergo a serological test. Serological tests are based on screening for CD-specific markers, which are total serum IgA, IgA transglutaminase antibodies (TGA), IgA deamidated gluten peptides antibodies (DGPA) and IgA anti-endomysial antibodies (EMA). At current stage of research, the screening of total serum IgA and IgA-antibodies against TGA are the preferred assays, since a high sensitivity and specificity in CD-diagnostics can be achieved [145]. It has to be noted that a small part of CD patients has an IgA deficiency. In these cases, the IgA antibody determinations are false negative and therefore cannot be used for evaluation. Therefore, the total IgA levels should always be determined in parallel. If an IgA deficiency is known, the determination of the respective IgG antibodies should be performed. In addition to the examination for IgG EMA and TGA antibodies, the examination of IgG DGPA is recommended [146]. However, in cases, in which IgA TGA are negative and total IgA is normal, CD is unlikely. If the IgA TGA levels are lower than 10 × the upper limit of normal (ULN), several biopsies are needed. Patients who are positive for IgA TGA and show levels higher than 10 × ULN, should further be tested for IgA EMA and HLA DQ2/8. If the tests are positive for EMA antibodies and HLA-DQ2 or HLA-DQ8, CD can be confirmed and a GFD is required. If the tests are negative, a biopsy is necessary [145, 147]. A small intestinal biopsy is usually performed after serological tests to determine the grade of villous atrophy according to the Marsh-Oberhuber classification. Based on that classification two parameters are detected: the number of IELs and the ratio of villous height to crypt depth. The latter differentiates different types of mucosal damage, ranging from normal intestinal mucosa, partial and up to total villous atrophy [148, 149]. It has been shown that the biopsy of the intestinal bulb, additionally to the biopsy of the duodenum, provides more accurate CD diagnosis [150]. However, the damage of the intestinal mucosa also can occur locally, therefore the determination of IELs should be performed [142].

The detection of the CD-predisposing alleles HLA-DQ2 and -DQ8 on the one hand serves to identify patients at risk and on the other hand, to exclude CD. This is possible because the HLA characteristics are a mandatory requirement for the development of CD. However, a positive HLA-DQ2 and -DQ8 test only is not suitable to confirm CD, because these alleles are very common in the Western population [119].

1.4.1.7 Celiac disease active epitopes

The proteins present in cereals are classified into albumins/globulins, gliadins and glutenins based on their solubility. Albumins/globulins are known to contain enzymes and enzyme inhibitors, of which the most prominent representatives are the ATIs. Albumins and globulins are most likely not involved in the pathomechanism of CD but are suggested to trigger NCGS [144]. Gliadins and glutenins and their corresponding types and subunits are able to trigger CD, since they contain repetitive units including modifications of the residues, namely QQQFP (ω5- gliadins), QQPQQPFP (ω1,2-gliadins), QPQPFPQQPYP (α-gliadins), QPQQPFP (γ-gliadins), QQPGQ, QQP, QPQ, YYPTSP (HMW-GS), QQQPPFS (LMW-GS) [66, 75]. More than 1000 peptides from storage proteins are known as CD-toxic and/or immunogenic. These peptides are at least nine amino acid residues long, resistant to human gastrointestinal digestion, can reach the lamina propria and build a complex with HLA-DQ2/8 [69]. The incomplete digestion of gliadins leads to a fragment of 33 amino acids (LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF), which is also known as the 33-mer peptide [151]. The 33-mer peptide only occurs in the α2-gliadin of hexaploid wheats, which is encoded on the D-genome. Since the D-genome is absent in tetraploid emmer and durum wheat and diploid einkorn, these species do not contain the 33-mer [152]. This large peptide has six partly overlapping epitopes, in particular PFPQPQLPY (DQ2.5-glia-α1a , one repetitive unit), PYPQPQLPY (DQ2.5-glia-α1b , two repetitive units) and PQPQLPYPQ (DQ2.5-glia-α2, three repetitive units) [69, 153] and therefore is considered to be the most immunogenic peptide [68, 154, 155].

The 33-mer plays an important role in the field of CD, since it was used as a model peptide to determine the capacity of gluten-degrading enzymes [156] and also to study the CD mechanism [157]. Interestingly, commercially available ELISA kits used the 33-mer peptide to raise two monoclonal antibodies (A1 und G12), which even more underlines the relevance of this peptide [158].
As already mentioned, the 33-mer peptide contains six overlapping T cell epitopes but beside these, there are further HLA-DQ2/8 epitopes, which are recognized by CD4+ T cells of CD patients. These epitopes are presented based on their 9-mer core region in Table 1 and are modified from Sollid et al., (2020)[159].

Table 1: Celiac disease relevant T cell epitopes, presented as the 9-mer core region

Epitope	Peptide binding register*									Reference
	1	2	3	4	5	6	7	8	9	
DQ2.5-limited										
DQ2.5-glia-α1a	P	F	P	Q	P	**E**	L	P	Y	153
DQ2.5-glia-α1b	P	Y	P	Q	P	**E**	L	P	Y	160
DQ2.5-glia-α2	P	Q	P	**E**	L	P	Y	P	Q	153
DQ2.5-glia-α3	F	R	P	**E**	Q	P	Y	P	Q	161
DQ2.5-glia-γ1	P	Q	Q	S	F	P	**E**	Q	Q	162
DQ2.5-glia-γ2	I	Q	P	**E**	Q	P	A	Q	L	137, 154
DQ2.5-glia-γ3	Q	Q	P	**E**	Q	P	Y	P	Q	160
DQ2.5-glia-γ4a	S	Q	P	**E**	Q	**E**	F	P	Q	160
DQ2.5-glia-γ4b	P	Q	P	**E**	Q	**E**	F	P	Q	154
DQ2.5-glia-γ4c	Q	Q	P	**E**	Q	P	F	P	Q	160
DQ2.5-glia-γ4d	P	Q	P	**E**	Q	P	F	C	Q	163
DQ2.5-glia-γ4e	L	Q	P	**E**	Q	P	F	P	Q	163
DQ2.5-glia-γ5	Q	Q	P	F	P	**E**	Q	P	Q	160
DQ2.5-glia-ω1	P	F	P	Q	P	**E**	Q	P	F	164
DQ2.5-glia-ω2	P	Q	P	**E**	Q	P	F	P	W	164
DQ2.5-glut-L1	P	F	S	**E**	Q	**E**	Q	P	V	161
DQ2.5-glut-L2	F	S	Q	Q	Q	**E**	S	P	F	161, 165
DQ2.2-limited										
DQ2.2-glut-L1	P	F	S	**E**	Q	**E**	Q	P	V	166
DQ2.2-glia-α1	Q	G	S	V	Q	P	Q	Q	L	167
DQ2.2-glia-α2	Q	Y	S	Q	P	**E**	Q	P	I	167
DQ8-limited										
DQ8-glia-α1	**E**	G	S	F	Q	P	S	Q	**E**	168
DQ8-glia-γ1a	**E**	Q	P	Q	Q	P	F	P	Q	169
DQ8-glia-γ1b	**E**	Q	P	Q	Q	P	Y	P	**E**	169
DQ8-glia-γ2	P	Q	Q	S	F	P	**E**	Q	**E**	170
DQ8-glut-H1	Q	G	Y	Y	P	T	S	P	Q	168
DQ8.5- limited										
DQ8.5-glia-α1	**E**	G	S	F	Q	P	S	Q	**E**	171
DQ8.5-glia-γ1	P	Q	Q	S	F	P	**E**	Q	**E**	171
DQ8.5-glut-H1	Q	G	Y	Y	P	T	S	P	Q	171

* Glutamate residues (E), which were deamidated by TG2 are shown in bold. Glutamine (Q) residues also targeted by TG2 are underlined.

1.4.2 Non celiac gluten sensitivity

Non-celiac gluten sensitivity (NCGS) is frequently discussed in the scientific community. Since the accurate pathomechanism is still unclear and the triggering factors as well as reliable diagnostic parameters are missing, NCGS has been discussed controversially [87, 172, 173]. NCGS is an umbrella term and further names, including non-celiac wheat sensitivity, nonallergy wheat sensitivity and wheat sensitivity are commonly used in the literature [53, 87, 174]. According to the 'Oslo definition', NCGS is characterized 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" [80]. Depending on the study, the prevalence of NCGS is estimated at 0.55 - 6% [81, 175-177]. A relatively high prevalence of 13% was reported by a study conducted in the UK, but it must be taken into account that the diagnosis was implemented by the participants themselves and only 1% of the study participants had to undergo a medical examination [178]. Due to a considerable overlap with IBS and lack of NCGS-and IBS-biomarkers, obtaining precise numbers on the prevalence is challenging. According to the studies carried out in Denmark and Northern Norway, the prevalence of IBS in the adult population is 16%–25% [179, 180]. In an extensive survey by Carroccio et al. (2012), it has been shown that individuals with IBS symptoms, according to the Rome II criteria, additionally suffer from NCGS and therefore the prevalence might be even higher. Additionally, it has been suggested that individuals suffering from NCGS can be classified into two categories, the one with CD-like symptoms and the second with food allergy-like symptoms [181]. The symptoms of NCGS can be intestinal (bloating, abdominal pain and diarrhea) or extraintestinal (tiredness, headache, anxiety, foggy mind and muscle pain) and appear hours or days after consumption of wheat or gluten-containing products. Especially the intestinal symptoms are often part of the IBS or CD clinical picture [182] [88]. Once patients are on a GFD, the symptoms disappear. Nonetheless, it is uncertain to what extent the GFD should be followed, due to the lack of knowledge about the triggers of the sensitivity. It is anticipated that a reduction of 5-10% of the total wheat consumption is sufficient for a significant improvement [53]. As long as the causative factors are not elucidated in detail, a GFD or a gluten-reduced diet are currently the only available treatments. The unknown pathomechanism and biomarkers, lead to another major drawback, namely the diagnosis, which is made by exclusion of CD, wheat allergy and IBS. In contrast to

CD, patients who suffer from NCGS neither show villous atrophy and TG2 or EMA nor IgE antibodies, which are indicators for CD or wheat allergy [182]. It has further been noticed that no correlation exists between the antibodies and the expression of HLA-DQ2 or -DQ8 genes [87, 181].

Therefore, the medical examination of NCGS patients should show a negative CD serology, a normal small intestinal mucosa and negative results in IgE-based assays [183]. After exclusion, a two-step procedure based on the gastrointestinal symptom rating scale (GSRS) is carried out to confirm NCGS. The first step includes a weekly rating of symptoms during a period of a six-week GFD, followed by a six-week gluten-containing diet. NCGS can be excluded if the symptoms do not improve after the six-week trial. In case of an improvement, the second step is carried out, which comprises a double-blind placebo-controlled challenge over the period of three weeks. The implementation is as follows: week 1) gluten-containing (recommended amount: 8 g) diet or placebo; week 2) strict GFD and week 3) gluten-containing diet or placebo. Based on the variation of at least 30% between the main symptoms of both diets, a positive result is discriminated from a negative result [184]. However, this procedure is difficult to perform in routine clinical practice and second, a reintroduction of gluten into the diet is often refused by patients due to signs of discomfort. The pathomechanism of NCGS is not revealed yet, and there are several options in terms of triggering factors. Wheat constituents, such as gluten proteins, ATIs and also FODMAPs are considered potentially harmful. The latter comprise short chain oligosaccharides of fructose (fructans) and galactooligosaccharides (stachyose, raffinose), disaccharides (lactose), monosaccharides (fructose) and sugar alcohols (polyols, such as sorbitol, mannitol, xylitol and maltitol). FODMAPs are completely or partially fermented in the large intestine by gut microbiota due to their resistance towards digestion and absorption in the small intestine. The fermentation usually shows positive effects on intestinal integrity and gut health, but the accompanying formation of gas can cause intestinal symptoms typical for IBS. It has been demonstrated that FODMAPs do not trigger extra-intestinal symptoms typical for NCGS and consequently are unlikely to contribute to NCGS [87, 90]. Gluten is assumed to be harmful for NCGS patients because symptoms disappear on a GFD and what is more, a double-blind randomized placebo-controlled study of well characterized NCGS patients (CD-excluded) not only showed that a GFD diet improves symptoms but also that the reintroduction of gluten into the diet leads to a reappearance of the symptoms [185]. However, in contrast to CD, gluten

peptides do not exhibit an intestinal inflammatory activity in NCGS patients. Furthermore, no differences in markers of the adaptive immune response were observed [186]. Interestingly, the levels of the toll-like receptor (TLR) 2, TLR4 and also α- and β-IELs were increased, whereas the amount of regulatory T cells was decreased. These are all markers, which induce the innate immune response. ATIs have been found to trigger the innate immune response in intestinal monocytes, macrophages and dendritic cells by passing the upper part of the intestine as intact proteins due to their resistance towards intestinal proteolysis (especially trypsin) [187]. Studies in animal models have shown that ATI induce inflammatory processes in the intestine and thus interfere with the integrity of the intestinal barrier [53]. ATIs are known to engage the TLR4-MD2-CD14 complex, which results in release of pro-inflammatory cytokines and chemokines IL-8, TNF-α and CCL2. The activation of the immune response is avoidable, when reducing the intake of ATIs, since their inducing-activity is dose-dependent [51-53]. For more information on ATIs see 1.3.1.2.

1.5 Methods for protein characterization

1.5.1 Immunological methods

Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assays (ELISA) are most widely applied for the determination of gluten in food products, especially for gluten-free products, which must not exceed the threshold of 20 mg/kg [55]. ELISA is characterized by a high sensitivity and specificity, hence more than 20 ELISA kits for gluten determination were developed and are available on the market. Due to their different calibration standards, sample extraction solutions and procedures, application of different antibodies (mono- or polyclonal) and specificity towards peptide sequences, different kits can be hardly compared. Generally, a distinction is made between two types of ELISA methods, namely sandwich and competitive ELISA [39]. Sandwich ELISA is applied for intact and rather large antigens (e.g. gluten proteins) due to the specification of the antigens of having at least two antigen binding sites (epitopes) to allow at least two different antibodies to bind. In contrast, competitive ELISA is used for the analysis of small-sized antigens (gluten peptides), because this method requires the presence of only one epitope. In gluten analysis, sandwich ELISA is used in raw materials (e.g. flour),

whereas competitive ELISA is suitable for hydrolyzed or fermented products (e.g. sourdough, beer). The method principle of sandwich ELISA is as follows (Figure 4): a monoclonal (mAb) or polyclonal (pAb) capture antibody with known concentration is immobilized on a microtiter plate. A solution with the antigen (gluten) is added and forms an antibody-antigen-complex after incubation (Step 1). Next, the enzyme-labelled detection antibody is added, which binds to the second binding site of the antigen, so the name-giving sandwich is complete (Step 2). After incubation, a substrate for the enzyme is added, which binds to the labelled antibody and catalyzes an enzymatic color reaction (Step 3). The resulting colored end product can be measured spectrophotometrically. Between each step the microtiter plate should be washed several times to remove the excess of antigen and unbound detection antibodies. The concentration of the antigen can be determined based on a calibration curve of a gluten reference material because the concentration is directly proportional to the absorbance in the sample. In case of a competitive ELISA, antigens are coated on the microtiter plate. These antigens are incubated with enzyme-labelled antibodies, which compete for the limited binding sites at the bound antigens and the unlabeled antigens from the sample. Once this reaction has reached equilibrium, unbound antigens and enzyme-labelled antibodies are removed by washing and an enzymatic substrate is added. The more antigen is present in the sample, the less conjugated antibody can be bound to the immobilized antigen. The substrate generates a color reaction that can be measured spectrophotometrically. For competitive ELISA, the absorbance is inversely proportional to the quantity of antigen in the sample. One of the oldest assays commercially available for gluten determination in raw and processed foods was developed by Skerritt and Hill in the 1990s [188]. This ELISA is a sandwich ELISA, with a sensitivity of 20 to 160 mg gluten/kg. This assay uses monoclonal antibodies, which were raised against ω-gliadins and react with the QQGYYP epitope [189]. In 2003, the Mendez Group in Madrid developed a sandwich ELISA based on the monoclonal R5 antibody, which was raised against ω-secalins from rye and recognizes the epitopes QQPFP, QQQPF, LQPFP, and QLPFP in CD-toxic sequences of cereal prolamins [190]. A major drawback of the R5 ELISA is its limited reactivity towards glutelins [191]. The R5-ELISA is calibrated with the reference material PWG gliadin and also can be obtained as a competitive assay, which in turn is calibrated with a mixture of peptic-tryptic hydrolysates from cereal prolamins [192]. Another frequently used assay is based on the G12 mAbs, which were raised against

the 33-mer peptide of α-gliadin. The G12 method is a sandwich ELISA and is calibrated with a gluten extract and recognizes QPQLPY and QPQLPF sequences, that occur in α-gliadins [193].

A new sandwich ELISA assay for the quantification of wheat, rye, and barley gluten in oat and oat products has been developed most recently. This assay is based on four different mAbs including the R5 mAb and four different wheat cultivars as calibration. It is able to recognize intact prolamins from wheat, rye and barley and also LMW-GS from wheat and HMW-GS and HMW-secalins from wheat and rye without overestimation and the proposed sensitivity ranges between 5 and 80 mg/kg [194].

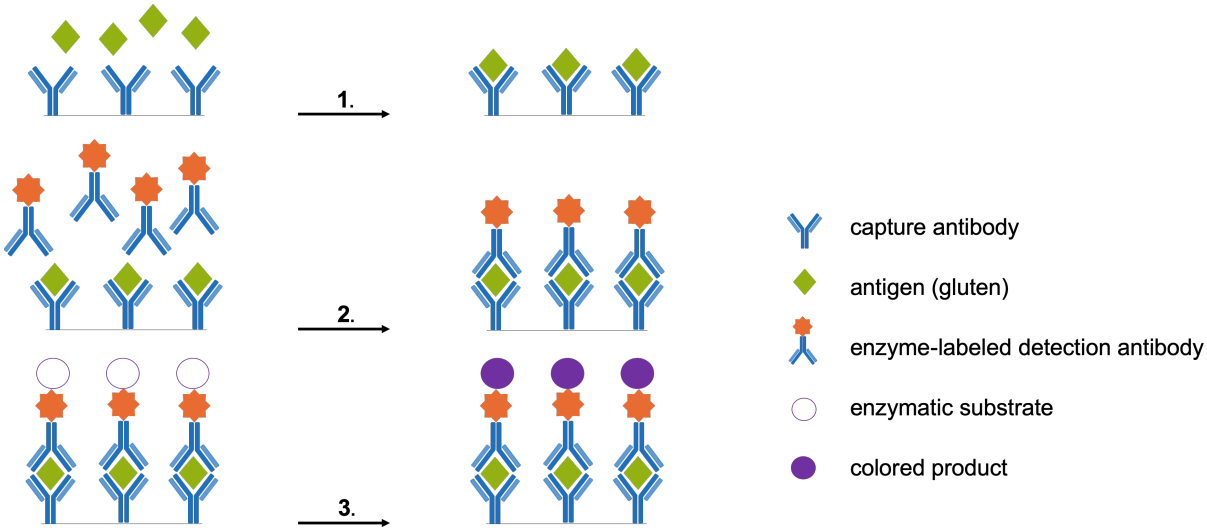

Figure 4: Sandwich ELISA (enzyme-linked immunosorbent assay) approach. For steps 1) to 3), see text.

1.5.2 Chromatographic and mass spectrometry-based methods

Mass Spectrometry (MS)

The most commonly used analytical methods for qualitative and quantitative determination of gluten proteins are reversed phase (RP)- and gel permeation (GP)-high-performance liquid chromatography (HPLC). A major drawback of these techniques is the limitation to the polarity and approximate molecular weight, respectively. In contrast, mass spectrometry allows an advanced characterization of proteins. Generally, in gluten analyses, peptides are identified by means of untargeted LC-MS/MS [195], whereas targeted LC-MS/MS analysis in the multiple reaction monitoring mode (MRM) is used for quantification of selected peptides [115]. The quantitation can be carried out either by means of an external calibration [114] or addition of an isotope-labelled internal standard during sample preparation, including a

preparation of a calibration curve [115]. The isotope-labelled internal standard is usually labelled with ^{2}H or ^{13}C and ^{15}N and therefore has a different weight compared to the quantified analyte but ideally the same chemical and physical properties. Because the concentration of the isotope-labelled internal standard is known, the concentration of the analyte can be quantified precisely. The principle workflow for quantitation of gluten peptides by LC-MS/MS involves the extraction of proteins from the sample (see 1.3), eventually the reduction of disufide bonds and the alkylation of cysteine residues, followed by enzymatic digestion of gluten proteins. Usually, chymotrypsin, pepsin, trypsin or a mixture of these enzymes is used for gluten hydrolysis in order to generate gluten peptides. Chymotrypsin is able to cleave the peptide bonds on the C-terminal side of phenylalanine, tyrosine and tryptophan, which occur frequently in gluten [196]. The degradation with chymotrypsin consequently leads to a variety of peptides. In contrast, trypsin is only able to cleave at the carboxylic side of the amino acid lysine or arginine, which are not present in high amounts in gluten [197]. Together with the enzymes, the labelled internal standard with a known concentration is added to compensate losses during the workflow. The standard can also be added prior to alkylation, if it contains cysteine residues. The quantitation of the peptides is enabled based on the comparison of the peak areas of internal standard with known concentration and analyte and also based on the calibration with a reference material [198]. The targeted LC-MS/MS approach in the MRM mode and the application of an internal standard is presented in Figure 5. After completing the workflow, the peptides are separated by means of RP-columns, which are connected in line to the electrospray ionization (ESI) source of a triple quadrupole mass spectrometer. The first quadruple (Q1) is applied as the mass filter for the precursor ion of the targeted peptide. Usually, peptides are detected in different charge states in Q1. The precursor ions of selected mass-to-charge ratios (m/z) are transferred to the collision cell (Q2). In Q2 the fragmentation of the precursor ions by collision with gas atoms (N_2, He, or Ar) and a collision energy between 10 and 40 eV takes place. In the third quadrupole (Q3), specific product ions are selected and a detection of the MRM transition (from precursor to the specific product ion) occurs. For a reliable identification of the targeted peptide, two to three peptide-specific product ions (MRM transitions) are analyzed. Finally, a detector visualizes the ions as MS spectra with respective retention times and intensities. Based on the mass difference in the MS spectra, the internal standard and the analyte can be distinguished. For quantification the most abundant transition

is used (quantifier), whereas less abundant transitions are used for qualification (qualifier) [199].

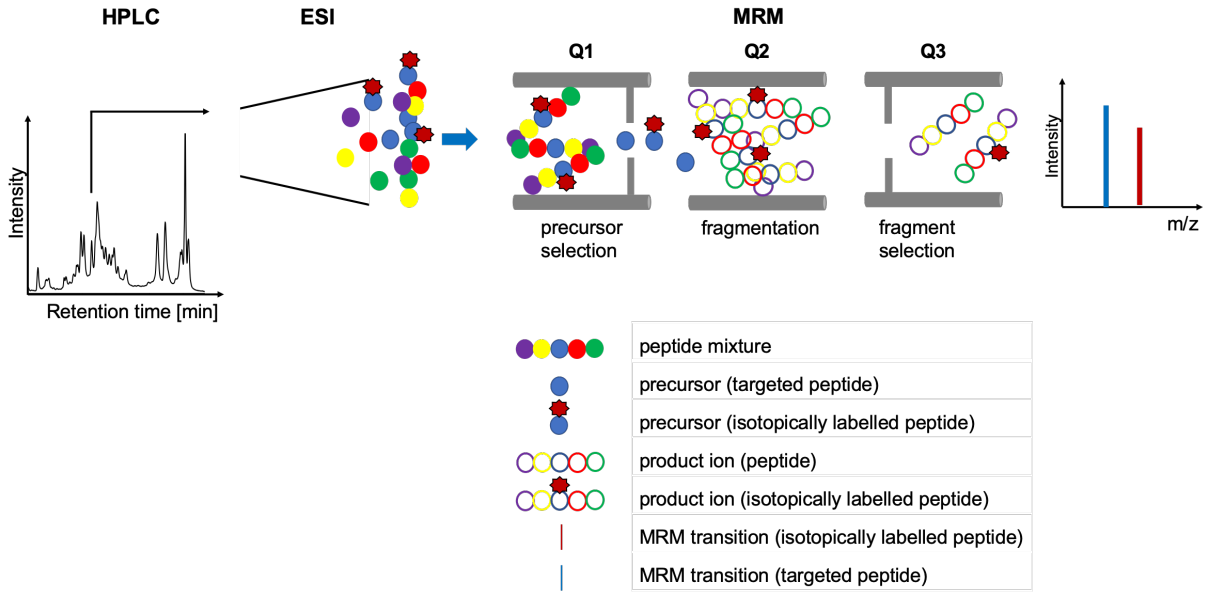

Figure 5: Principle of targeted LC-MS/MS in the multiple reaction monitoring (MRM) mode, including the application of an isotope-labelled peptide as internal standard [modified according to Gillette and Carr, (2013) [199]].

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2 Aim of the work

Wheat and wheat products have been staple foods since the beginning of cereal cultivation about 10000 years ago. However, certain wheat proteins are held to be responsible for triggering inflammatory disorders in the human body. The scientific literature comprises evidence for an increased prevalence of patients who suffer from celiac disease (CD). The consumption of wheat can also cause a reaction in which neither allergic nor autoimmune mechanisms are involved. This entity is called non-celiac gluten sensitivity (NCGS) and is characterized by intestinal and/or extraintestinal symptoms and has an estimated prevalence of 1-6%. While CD is a well-studied disorder, the causative factors of NCGS are not yet elucidated in detail. The critical point is the diagnosis and the differentiation of NCGS from CD, wheat allergy (WA), other food intolerances and irritable bowel syndrome (IBS), so that the diagnosis is a process of elimination. Moreover, the pathogenesis is poorly recognized so far and the triggering factors are unclear. Fermentable oligo-, di-, and monosaccharides and polyols (FODMAPs) and wheat constituents, such as α-amylase-trypsin-inhibitors (ATIs) and gluten proteins have been suggested as potentially harmful. Gluten is considered as triggering factor because the symptoms disappear on a gluten-free diet (GFD). Besides growing attention and improved diagnostic techniques, changes in the protein content and composition over the last 100 years due to breeding are considered to be one reason for the increasing number of patients who suffer from NCGS. Wheat breeding may have as a consequence that modern cultivars (registered after 1950) have a higher immunostimulatory potential than older cultivars (registered before 1950).

The aim of this study was to investigate 60 German winter wheat cultivars, which were registered in the period of 1891 to 2010, for their qualitative and quantitative protein composition and distribution of CD-active peptides. In order to take environmental effects into account, samples from three different harvest years (2015-2017) were analyzed.

To achieve this, the first part of the work involved a fractionation of proteins according to their solubility (modified Osborne fractionation) and their characterization by means of a reversed-phase high performance liquid chromatography (RP-HPLC).

The second part of this work focused on achieving in-depth insights into the variation of the protein distribution of wheat cultivars, their specific protein fingerprints and

further a better differentiation between wheat cultivars and species. This is why a new peak integration approach of the RP-HPLC patterns was developed, in order to gain insights into the changes of the protein distribution of wheat cultivars over the past 100 years due to breeding.

The third part of this work was to address the immunoreactive potential of the wheat cultivars and how potential changes in the antigenicity can be traced back to the identified qualitative changes in the protein composition. For this purpose, the contents of CD-active immunoactive peptides the 60 German hexaploid wheat cultivars were investigated by means of a targeted LC-MS/MS method. Moreover, 16 selected and isolated gliadin fractions and types were investigated regarding their influence on the ELISA responses, using the R5 and G12 monoclonal antibodies (mAbs).

3 Results

3.1 Wheat (*Triticum aestivum* L.) breeding from 1891 to 2010 contributed to increasing yield and glutenin contents but decreasing protein and gliadin contents

A sample selection of 60 hexaploid German winter wheat cultivars common in the period of 1891 to 2010, was grown under the same conditions in Gatersleben, Germany, in the years 2015, 2016 and 2017. All samples were milled into wholegrain flours and the flours were analyzed for their protein content and distribution. The crude protein contents were determined by the Dumas method, followed by the investigation of the protein fractions albumins/globulins, gliadins and glutenins. Therefore, the fractions were extracted based on their solubility according to the modified Osborne fractionation procedure and subsequently characterized by means of RP-HPLC. Darina Pronin analyzed all data generated by the RP-HPLC analyses and determined the contents of the fractions and of gliadin types and glutenin subunits. Further, she compared the agronomic characteristics of all wheat cultivars with the identified changes in the protein composition. Moreover, she determined the influence of the wheat genotypes and the harvest year on the protein distribution. Darina Pronin used statistical tools, such as analysis of variance, correlation analysis and box plot analysis in order to evaluate and to visualize the data set. Darina Pronin designed all figures, wrote the manuscript and revised it according to the reviewer comments.

This work revealed changes in the protein content and distribution in wheat cultivars over the past 120 years. The results of the agronomic factors, such as plant height and yield, showed a clear reduction of the height, whereas the yield increased continuously from old to modern cultivars. Further, the protein content slightly declined over time and the contents of gliadins, including α-and γ-gliadins also decreased. On the other hand, the glutenin contents increased. Given these trends, no changes in the content of gluten and albumins/globulins were identified. Based on a unique sample set cultivated in the same area over three years, it was possible to identify that the differences were rather affected by environmental conditions than by the wheat cultivars.

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Wheat (*Triticum aestivum* L.) Breeding from 1891 to 2010 Contributed to Increasing Yield and Glutenin Contents but Decreasing Protein and Gliadin Contents

Darina Pronin, Andreas Börner, Hans Weber, and Katharina Anne Scherf*

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ABSTRACT: Epidemiologic studies suggest an increasing prevalence of celiac disease and non-celiac gluten/wheat sensitivity. With wheat proteins being the main triggers, changes in wheat protein composition are discussed as a potential cause. The goals of breeding toward increased yield and resistance might have inadvertently contributed to a higher immunostimulatory potential of modern wheat cultivars compared to old wheat cultivars. Therefore, agronomic characteristics, protein content, and gluten composition of 60 German winter wheat cultivars first registered between 1891 and 2010 grown in 3 years were analyzed. While plant height and spike density decreased over time, yield and harvest index increased. The protein and gliadin contents showed a decreasing trend, whereas glutenin contents increased, but there were no changes in albumin/globulin and gluten contents. Overall, the harvest year had a more significant effect on protein composition than the cultivar. At the protein level, we found no evidence to support an increased immunostimulatory potential of modern winter wheat.

KEYWORDS: *albumins/globulins, breeding, celiac disease, gliadins, gluten, glutenins, non-celiac gluten sensitivity (NCGS), protein, wheat*

INTRODUCTION

Wheat (*Triticum aestivum* L. ssp. *aestivum*) is one of the most important cereals in terms of production and utilization for human consumption. According to the Food and Agriculture Organization of the United Nations, about 734 million tons are produced worldwide annually (data from 2018), up from 222 million tons in 1961 (earliest entry in the database).[1] Wheat proteins are traditionally classified into so-called Osborne fractions according to their solubility. Albumins and globulins are soluble in water and salt solution, respectively, whereas gliadins are soluble in aqueous alcohols (e.g., 60−70% ethanol). Glutenins are only partly soluble in diluted acids and bases or completely in alcoholic solvents containing disaggregating and reducing agents.[2] The albumin/globulin fraction accounts for 20−25% of wheat proteins and mainly includes protective and metabolic proteins, such as enzymes and enzyme inhibitors. Gliadins and glutenins are storage proteins and account for 75−80% of wheat proteins and form the viscoelastic mass known as gluten. The gliadin fraction mainly contains monomeric proteins with relative molecular masses (M_r) from 28 000 to 55 000 that contribute viscosity to wheat flour dough and are subdivided into ω5-, ω1,2-, α-, and γ-gliadins.[3] The glutenin fraction is polymerized by intermolecular disulfide bonds, which are responsible for dough elasticity and are subdivided into high-molecular-weight glutenin subunits (HMW-GS, M_r of 67 000−88 000) and low-molecular-weight glutenin subunits (LMW-GS, M_r of 28 000−35 000).[3,4]

Wheat proteins are known to trigger adverse reactions, such as celiac disease (CD)[5] and wheat allergy,[6] that affect about 1 and 0.5% of the adult population worldwide, respectively. More recently, a third entity, called non-celiac gluten sensitivity (NCGS), involving neither autoimmunogenic nor allergic mechanisms, has been receiving more and more attention.[7−10] With an estimated prevalence of up to 6% of the population in Western countries, NCGS is characterized by intestinal (e.g., bloating, abdominal pain, diarrhea, and epigastric pain) and/or extra-intestinal symptoms (e.g., lack of wellbeing, tiredness, headache, anxiety, foggy mind, and joint/muscle pain).[9] While gluten proteins are known to trigger CD and a variety of wheat proteins, including gluten, are known to cause wheat allergy,[11] the situation is less clear in the case of NCGS. Gluten proteins have been considered as causative factors for NCGS, because symptoms disappear on a gluten-free diet, which is the main treatment thus far.[12] Recent findings suggest α-amylase/trypsin inhibitors (ATIs) as main activators of NCGS by stimulating the innate immune response via toll-like receptor 4 (TLR4).[13] Besides ATIs, fermentable oligo-, di-, and monosaccharides and polyols (FODMAPs) might contribute to NCGS symptoms.[14,15]

There is more and more evidence that the prevalence of CD has risen over the last 50 years[16−18] as well as that of NCGS,[10] but the underlying reasons remain unclear. Apart from growing awareness and better diagnostic tools, changes on the side of the human immune system might have contributed to increasing the susceptibility to develop adverse reactions to

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wheat.[19] On the side of cereals, modifications in processing, such as short dough fermentation times,[20] the use of gluten as a technofunctional ingredient in many foods,[21] and changes in protein composition as a result of agronomic practices, including nitrogen fertilization,[22] are possible causes. Additionally, it has been suggested that wheat breeding may have resulted in a higher immunostimulatory potential of modern wheat cultivars (varieties cultivated after 1950) compared to old wheat cultivars (landraces cultivated prior to 1950).[23] While increasing yield is the main goal of wheat breeding, secondary goals include improvement of adaptation, resistance to biotic and abiotic stress factors, and baking quality.[24] The influence of breeding during the last century on wheat protein contents is inconclusive, with one study from Canada reporting an increase,[25] two studies from the U.S. essentially reporting no change,[21,26] and three others from the U.K., Germany and the U.S. reporting a decrease.[27−29]

With regard to protein composition, there are only a few studies comparing the gliadin and glutenin composition of old and modern wheats.[30−32]

Therefore, the aim of our study was to investigate changes in agronomic characteristics, protein content, and composition of German winter wheat cultivars first registered from 1891 to 2010. The five most important cultivars per decade were selected and grown in 3 years (2015, 2016, and 2017) to account for the effects of genetics, the environment, and their interaction.

MATERIALS AND METHODS

Wheat Samples. A selection of hexaploid wheat (*T. aestivum* L.) cultivars was sampled from the German Federal *ex situ* Genebank of crops at the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK, Gatersleben, Germany). For each of the 12 decades between 1891 and 2010, the five cultivars most widely grown in Germany in that decade were selected among the cultivars first registered in that decade (Table S1 of the Supporting Information). The cultivars were grown together using an incomplete block design (α lattice) with three biological replications at Gatersleben [latitude, 51° 49′ 19.74″ N; longitude, 11° 17′ 11.80″ E; 110.5 m above sea level (masl); black soil of clayey loamy type].[33] The recorded mean temperature for the harvest years 2015, 2016, and 2017 was 10.2, 10.0, and 10.1 °C, respectively. With regard to the mean precipitation, annual values were 533, 366, and 557 mm, respectively.[34]

The experimental plot was 1.5 × 2.5 m in size with a sowing density of 60 g of grains per plot. Soil nutrient composition of IPK experimental fields underlying a 5 year rotation is analyzed each season, with sugar beet as a forecrop. Standard agronomic management according to good agricultural practice, including fungicide and insecticide treatments, was applied. Because a low to medium nutrient input is strived for, additional fertilizers were not applied. The soil nitrogen content was determined in early spring at a depth of 0−60 cm. Between 50 and 70 kg/ha nitrogen was available to plants (NO_3 N + NH_4 N). The whole experiment was repeated 3 times, and the samples were harvested in 2015, 2016, and 2017.

Agronomic Characteristics. The days from sowing until flowering were registered (mean of n = 1 per plot), as well as plant height (main tillers, mean of n = 3 per plot), grain weight and straw weight per 20 tillers (sum of the 20 values per plot), spike density (mean of n = 20 per plot, respectively, evaluated on a scale ranging from 5, very dense, to 4, dense, 3, intermediate, 2, loose and 1, very loose) and yield per plot (n = 3). The harvest index was calculated from the mean values for grain weight and straw weight using the formula: grain weight/(grain weight + straw weight). The minimum/maximum flowering times were 214/255 days, 212/221 days and 211/223 days for the years 2015, 2016 and 2017, respectively.

Milling and Flour Characterization. Grains from the three biological replications were pooled, and 20 g per cultivar was milled into wholemeal flours using a laboratory grinder (Bosch, Stuttgart, Germany) and sieved to a particle size of 0.2 mm. The nitrogen content of the flours was measured in triplicate by the Dumas combustion method using a TruSpec nitrogen analyzer (Leco, Moenchengladbach, Germany) according to ICC Standard 167. Ethylenediaminetetraacetic acid was used for calibration, and the nitrogen content was multiplied with a factor of 5.7 to calculate the crude protein content.[35]

Extraction of Albumins/Globulins, Gliadins, and Glutenins. Wheat flours (100 mg) were extracted twice with 1.0 mL of salt solution (0.067 mol/L Na_2HPO_4/KH_2PO_4 buffer and 0.4 mol/L NaCl at pH 7.6) to obtain albumins/globulins. The residues were extracted 3 times with 0.5 mL of 60% (v/v) ethanol to obtain gliadins. For the extraction of glutenins, the residues were extracted twice with 1.0 mL of glutenin extraction solution [50% (v/v) 1-propanol, 0.1 mol/L Tris−HCl at pH 7.5, and 0.06 mol/L (w/v) dithiothreitol] at 60 °C under nitrogen.[2]

Each extraction step consisted of vortex mixing for 2 min at 22 °C, followed by magnetic stirring for 10 min. The suspensions were centrifuged (3550g, 25 min, and 22 °C), and the respective supernatants were combined and filled up to 2.0 mL with the respective extraction solution. All supernatants were filtered (Whatman, Spartan 13/0.45 RC, GE Healthcare, Freiburg, Germany) prior to analysis by reversed-phase high-performance liquid chromatography (RP-HPLC). The extractions for each sample were performed in triplicate.

RP-HPLC Analysis. The following system was used: Jasco XLC (Jasco, Gross-Umstadt, Germany) with Chrom Pass software; column, Acclaim 300 C_{18} (particle size, 3 μm; pore size, 30 nm; 2.1 × 150 mm; Thermo Fisher Scientific, Braunschweig, Germany); elution solvents, trifluoroacetic acid (TFA) (0.1%, v/v) in water (A) and TFA (0.1%, v/v) in acetonitrile (B); gradient for albumins/globulins: 0 min, 0% B; 0.5 min, 20% B; 7 min, 60% B; 7.1−11 min, 90% B; and 11.1−17 min, 0% B; linear gradient for gliadins and glutenins: 0 min, 0% B; 0.5 min, 24% B; 20 min, 56% B; 20.1−24.1 min, 90% B; and 24.2−30 min, 0% B; flow rate, 0.2 mL/min; temperature, 60 °C; injection volume, 10 μL for gliadins and 20 μL for albumins/globulins and glutenins; and detection, ultraviolet (UV) absorbance at 210 nm.[35] Prolamin Working Group (PWG)-gliadin (2.5 mg/mL in 60% ethanol) was used as a calibration reference for the calculation of protein contents.[36]

Statistical Analysis. Mean values (n = 3), absolute standard deviation (SD), and relative standard deviation (RSD) were calculated for all quantitative values of each wheat cultivar. One-way analysis of variance (ANOVA) with Tukey's test ($p < 0.05$) was carried out to identify significant differences between decades within each harvest year and for the average of 3 harvest years. Two-way ANOVA with Tukey's test ($p < 0.05$) was performed using the harvest year and wheat cultivar as factors. ANOVA was carried out with Sigma Plot 12.0 (Systat Software, San Jose, CA, U.S.A.). Pearson's product moment correlations ($p < 0.05$) were calculated between the agronomic characteristics (plant height, yield, harvest index, and spike density) and the protein fractions (albumins/globulins, ω5-, ω1,2-, α-, γ-gliadins, and ωb-gliadins, HMW-GS, and LMW-GS). The correlation coefficients were defined as $r \leq \pm 0.54$, no correlation; $\pm 0.54 < r \leq \pm 0.67$, weak correlation; $\pm 0.67 < r \leq \pm 0.78$, medium correlation; and $r > \pm 0.78$, strong correlation.[37]

RESULTS

A total of 60 hexaploid wheat samples of 3 harvest years (2015, 2016, and 2017) were examined for their protein content and distribution. The contents of albumins/globulins, gliadins, glutenins, and crude protein (mg/g of flour) are displayed in Tables S2, S4, and S6 of the Supporting Information, and the contents of ω5-, ω1,2-, α-, γ-, and ωb-gliadins, HMW-GS, and LMW-GS (mg/g of flour) are displayed in Tables S3, S5, and S7 of the Supporting Information for each sample, respectively.

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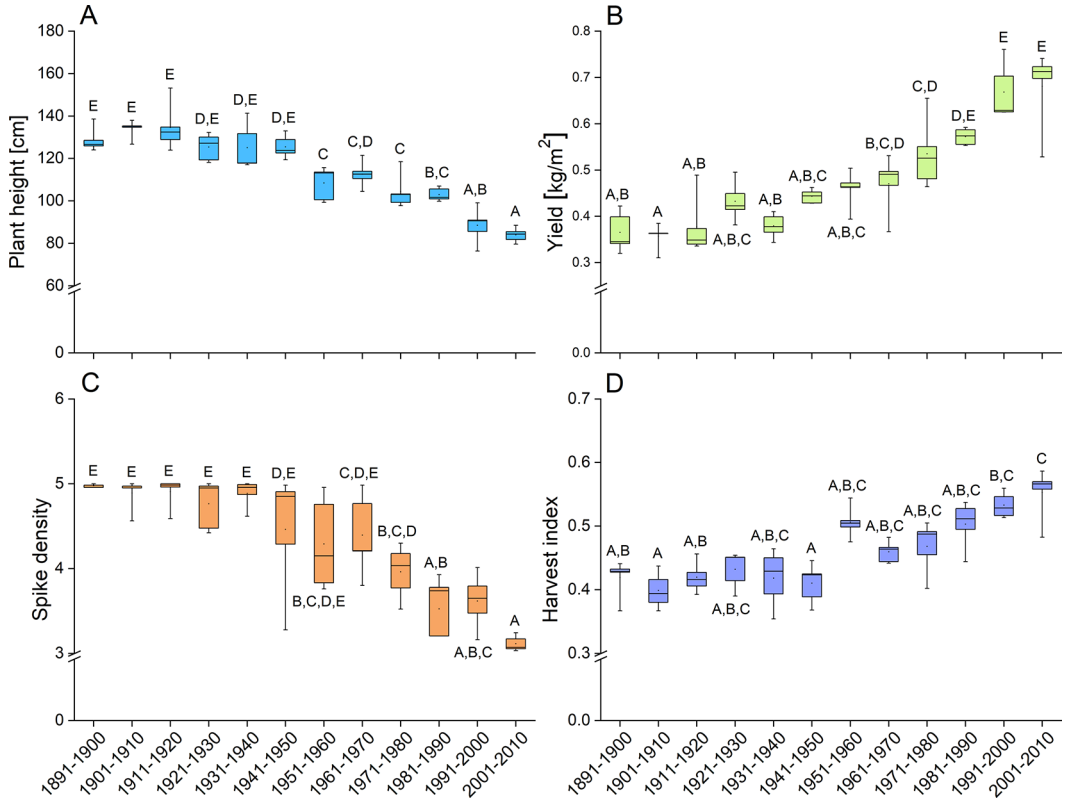

Figure 1. Agronomic characteristics, i.e., (A) plant height, (B) yield, (C) spike density, and (D) harvest index, in five cultivars per decade averaged over 3 harvest years 2015, 2016, and 2017. Boxes represent the interquartile range with the median (line in the box) and mean (point in the box), and whiskers represent the minima and maxima. Different capital letters designate significant differences between the decades (one-way ANOVA and Tukey's test; $p < 0.05$).

In the following, wheat cultivars are referred to as samples 1−60, starting with the oldest cultivars from the decade 1891−1900 (cultivars 1−5) to the most recent cultivars from the decade 2001−2010 (cultivars 56−60) (Table S1 of the Supporting Information). Modern cultivars are defined as cultivars that were first registered in Germany after 1950 because dwarfing genes were introduced in the 1950s.[38]

Agronomic Characteristics. The agronomic characteristics, i.e., plant height, yield, spike density, and harvest index, are presented as the median of the five cultivars per decade averaged over the 3 harvest years (Figure 1). The plant height showed a significant reduction from old to modern cultivars. The height of old cultivars ranged between 117 and 153 cm, whereas the height of the most modern cultivars (1991−2010) was 76−99 cm. This corresponds to a decrease of about 40% from old to modern cultivars regarding plant height. In contrast, the yield increased continuously and significantly over time. While the yield of the oldest cultivars ranged between 31 and 49 dt/ha, the yield for the modern cultivars was up to 76 dt/ha. The spike density changed significantly from a very dense spike toward a more loose spike. The old cultivars had a value of 5 on the scale (very dense), whereas modern cultivars showed values of about 3 (intermediate). In terms of the harvest index, we observed a significant increase over the decades. During the period from 1891 to 1950, the index remained largely constant, with a value around 0.40, but from 1951, the index increased to up to 0.58. Considering the results for plant height and yield, the changes in harvest index are consistent.

Crude Protein Contents. The crude protein contents of the flours of all 3 harvest years are presented in Figure 2. The contents varied between 11.7% (sample 30) and 7.1% (sample 52) for the average over 3 years. With regard to the particular harvest years, the values ranged between 14.0% (sample 8) and 7.4% (sample 60) for the harvest year 2015, between 10.6% (sample 38) and 6.0% (sample 33) for the harvest year 2016, and between 13.1% (sample 30) and 6.0% (sample 43) for the harvest year 2017. Overall, about 90% of the samples had less than 10% protein as a result of the fact that they were not fertilized. The median values averaged over 3 harvest years showed high variation within the five cultivars per decade and a slight decreasing trend over the last 120 years, even if it was not significant. The results from the year 2015 showed a clear decreasing trend from 1891 to 2010, but the results of the other 2 years 2016 and 2017 were not as unambiguous, partly because of high variability, especially in the samples from 1941 to 1960 in the year 2017. Because wheat contains many different protein fractions and types with various immunoreactivities, the protein composition was determined in more detail in the following.

Percentages of Albumins/Globulins, Gliadins, Glutenins, and Gluten. The proportions of the so-called Osborne fractions albumins/globulins, gliadins, glutenins, and gluten (as the sum of gliadins and glutenins) of all 60 wheat cultivars were calculated relative to the sum of all extractable proteins to see changes in protein composition without the confounding factor of changes in the overall protein content. The mean recovery relative to the total protein content was 80.2% for the sum of the protein fractions. The values were

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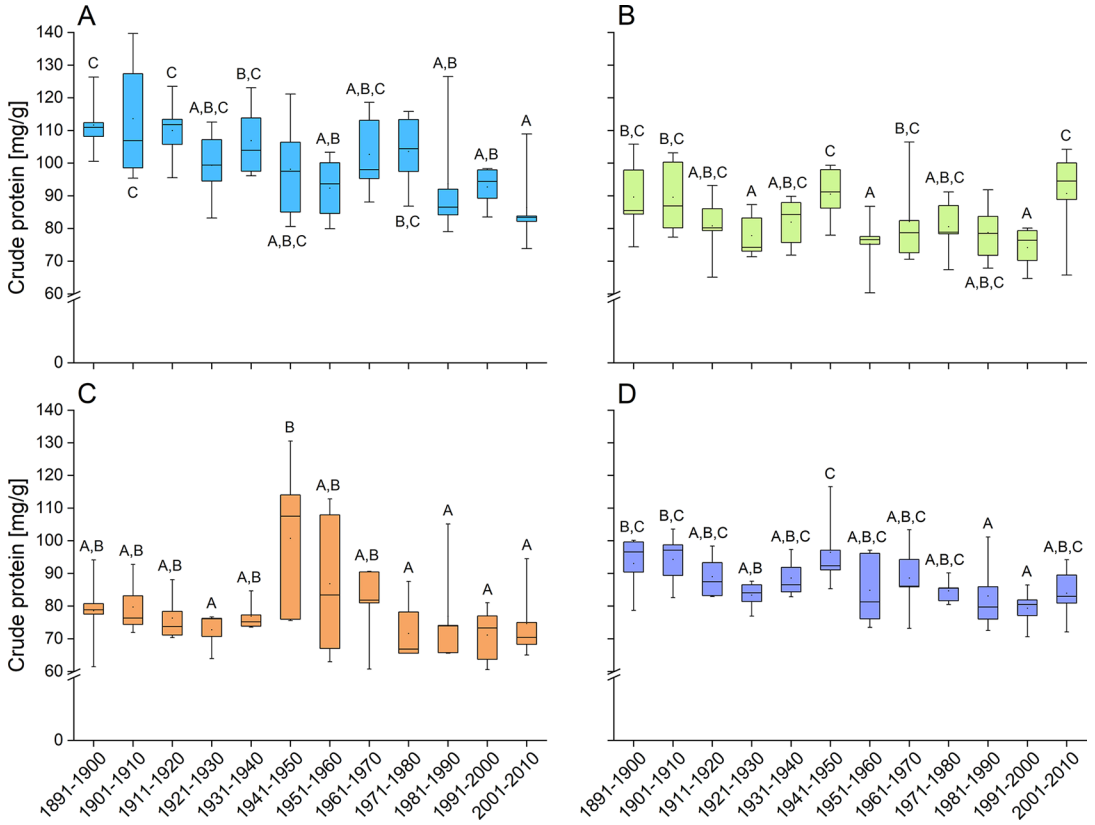

Figure 2. Crude protein contents in five cultivars per decade of the harvest years (A) 2015, (B) 2016, and (C) 2017 and (D) averaged over 3 harvest years 2015, 2016, and 2017. Boxes represent the interquartile range with the median (line in the box) and mean (point in the box), and whiskers represent the minima and maxima. Different capital letters designate significant differences between the decades (one-way ANOVA and Tukey's test; $p < 0.05$).

further averaged over the 3 harvest years to identify trends independent of environmental conditions. The percentages of the albumin/globulin fraction ranged between 25.5% (sample 12) and 16.9% (sample 30), and there was no clear increase or decrease apparent (Figure 3A). The proportions of gliadins decreased significantly over time and ranged between 62.0% (sample 30) and 45.7% (sample 57) (Figure 3B). In contrast, glutenins increased significantly with proportions between 33.1% (sample 56) and 17.1% (sample 44) (Figure 3C). In this context, the minimum of the glutenin proportions was obviously a special outlier in the decade 1971−1980. This is why the second lowest percentage should be considered as well. In line with the increasing trend for the glutenin fraction, an old cultivar (sample 3) contained the second lowest proportion, with 17.7%. As a result of the decrease of gliadins and simultaneous increase of glutenins, the gliadin/glutenin ratio (Figure S5 of the Supporting Information) decreased significantly. The values for the highest and second highest ratios were 3.6 (sample 44) and 3.5 (sample 3), respectively, and 1.4 (sample 56) for the lowest ratio. The gluten proportion and content did not show any significant changes over time, because its components gliadins and glutenins decreased and increased, respectively, thus largely canceling each other out (Figure 3D).

The absolute values for each harvest year are additionally presented in Figures S1−S4 of the Supporting Information. Although we have to consider the different absolute protein contents of the samples, we still see largely the same trends regarding the contents of albumins/globulins, gliadins, glutenins, and gluten. Taken together, the protein composition has in fact changed over the last 120 years as a result of breeding.

Percentages of Gluten Protein Types. The gliadin fraction was additionally subdivided into its protein types ω5-, ω1,2-, α-, and γ-gliadins, and the glutenin fraction was additionally subdivided into HMW-GS and LMW-GS, all quantitated by RP-HPLC, as described above. Within gliadin types, ω5-gliadins had the lowest proportions overall, with values between 5.2% (sample 31) and 1.0% (sample 24), followed by ω1,2-gliadins, with values from 6.4% (sample 59) to 3.1% (sample 25) (panels A and B of Figure 4). Neither ω5- nor ω1,2-gliadins showed a specific trend from 1891 to 2010. However, α- and γ-gliadins revealed a decreasing trend with proportions between 30.3% (sample 17) and 19.7% (sample 57) and between 28.3% (sample 15) and 17.5% (sample 56), respectively. Both HMW-GS and LMW-GS showed the highest proportions for sample 56 (7.8 and 24.2%, respectively) and the lowest proportions for sample 44 (3.8 and 12.7%, respectively) (panels C and D of Figure 4). The second lowest percentages of HMW-GS and LMW-GS were observed for sample 3 with 3.9 and 13.1%, respectively, and this agrees with the results for total glutenins. In accordance with the results for the whole glutenin fraction, the proportions of HMW-GS and LMW-GS both increased significantly (panels A and B of Figure 5). The absolute contents of the protein types for each harvest year are additionally presented in Figures S6−S11 of the Supporting Information. As already

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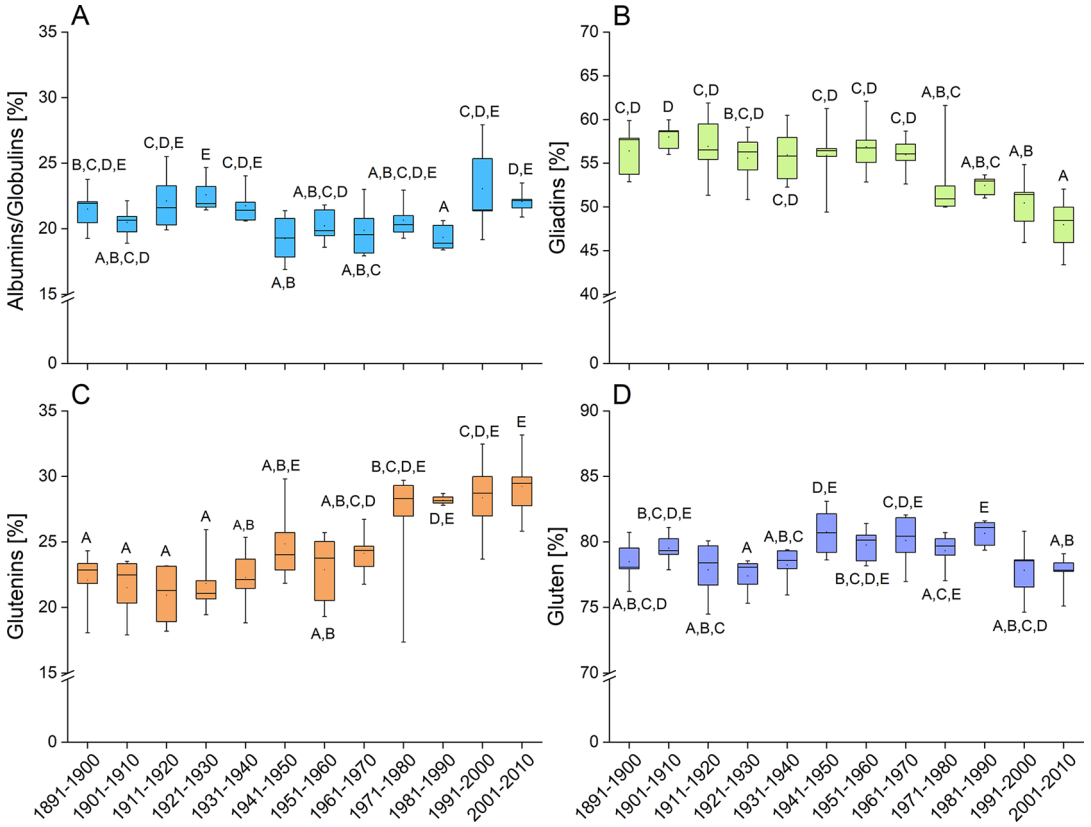

Figure 3. Proportions (%) of (A) albumins/globulins, (B) gliadins, (C) glutenins, and (D) gluten in five cultivars per decade in the total RP-HPLC content averaged over 3 harvest years 2015, 2016, and 2017. Boxes represent the interquartile range with the median (line in the box) and mean (point in the box), and whiskers represent the minima and maxima. Different capital letters designate significant differences between the decades (one-way ANOVA and Tukey's test; $p < 0.05$).

discussed for the gliadin and glutenin fractions, the relative and absolute values essentially showed similar trends.

Correlations between Agronomic and Proteomic Characteristics. On the basis of agronomic and proteomic findings, a correlation matrix was calculated to clarify relationships between the different factors (Figure S12 of the Supporting Information). We observed strong negative correlations between the plant height and yield as well as the plant height and harvest index (r = −0.87 and −0.90, respectively). Further, the yield showed a strong negative correlation with the spike density (r = −0.79) and a strong positive correlation with the harvest index (r = 0.82). With regard to the proportions of the protein fractions, albumins/globulins showed no correlation with either of the other parameters. Gliadins showed weak positive correlations with the plant height (r = 0.60) and spike density (r = 0.64) and a weak negative correlation with the yield (r = −0.58). Gliadin proportions further showed strong negative correlations with glutenins (r = −0.89) and LMW-GS (r = −0.90) and a medium negative correlation with HMW-GS (r = −0.73). Interestingly, the total gliadin proportions did not show any correlations with ω5- and ω1,2-gliadins but medium positive correlations with α- and γ-gliadins (r = 0.77 and 0.73, respectively). In addition, the proportions of α- and γ-gliadins showed medium negative correlations with the glutenin proportions (r = −0.68 and −0.74). Concerning glutenins, medium negative correlations were observed for the plant height and spike density (r = −0.65 and −0.73, respectively). The glutenin subunits (HWM-GS and LMW-GS) supported the findings for the glutenins.

Influence of Genotype and Harvest Year on the Protein Distribution. Two-way ANOVA was performed on the entire sample set of 60 wheat cultivars to examine the effect of different cultivars (G variability) and harvest years (E variability) as well as their interaction on the contents of crude protein and proportions of the protein fractions and types (Table 1). We observed a significant $G \times E$ interaction for all parameters investigated, so that G and E variabilities cannot be clearly distinguished from one another. The harvest year had a greater influence on crude protein contents than the cultivar. The major determinant factor for the albumin/globulin and glutenin proportions was the harvest year. However, it was noticeable that the gliadin proportions were more strongly influenced by the cultivar and not by the harvest year. These findings are supported by the fact that, although the minima and maxima of the proportions of the protein fractions were almost similar, different cultivars had the highest/lowest proportions for each harvest year. With regard to the albumin/globulin fraction, the minima (16.3, 16.3, and 13.9%) were found for the samples 37, 27, and 48, whereas the maxima were observed for the samples 60 (27.4%), 52 (27.8%), and 18 (26.3%) for the harvest years 2015, 2016, and 2017, respectively. As for the proportions of the gliadin fraction, the lowest values were found for the samples 52 (42.3%), 57 (44.0%), and 27 (42.0%) and the highest values were found for samples 4 (67.2%), 23 (60.8%), and 14 (64.7%) for the harvest years 2015, 2016, and 2017,

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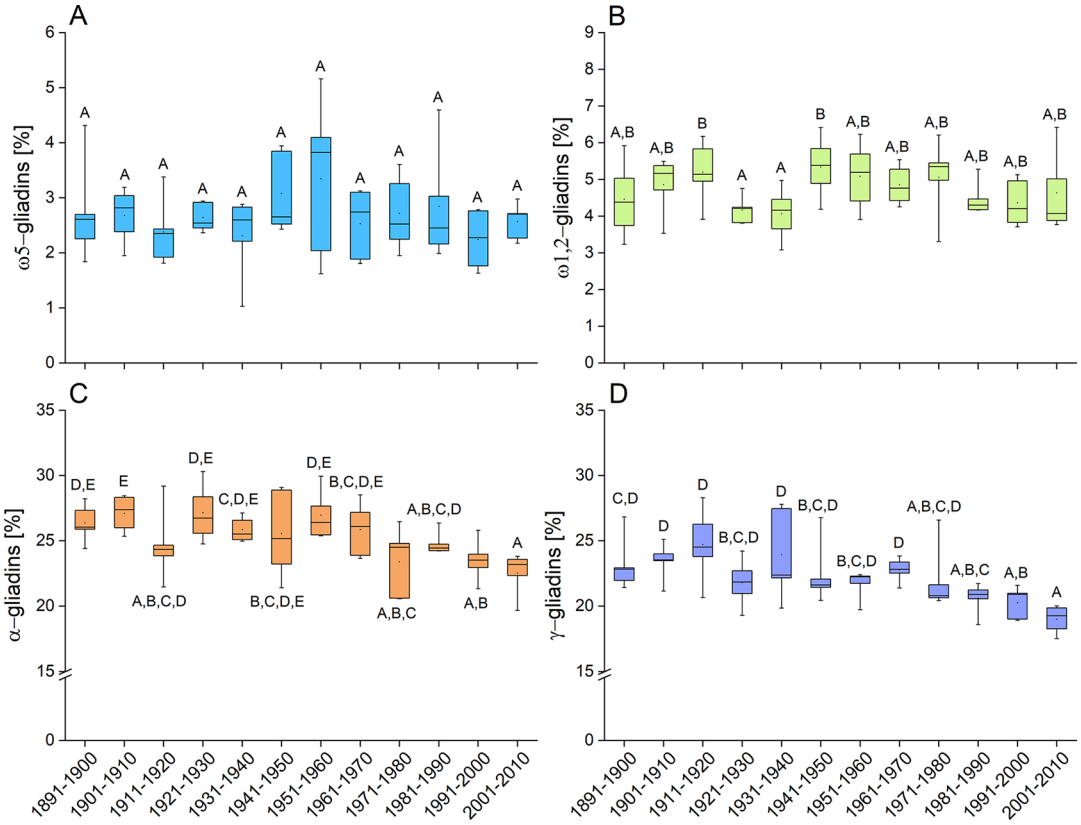

Figure 4. Proportions (%) of (A) ω5-gliadins, (B) ω1,2-gliadins, (C) α-gliadins, and (D) γ-gliadins in five cultivars per decade in the total RP-HPLC content averaged over 3 harvest years 2015, 2016, and 2017. Boxes represent the interquartile range with the median (line in the box) and mean (point in the box), and whiskers represent the minima and maxima. Different capital letters designate significant differences between the decades (one-way ANOVA and Tukey's test; $p < 0.05$).

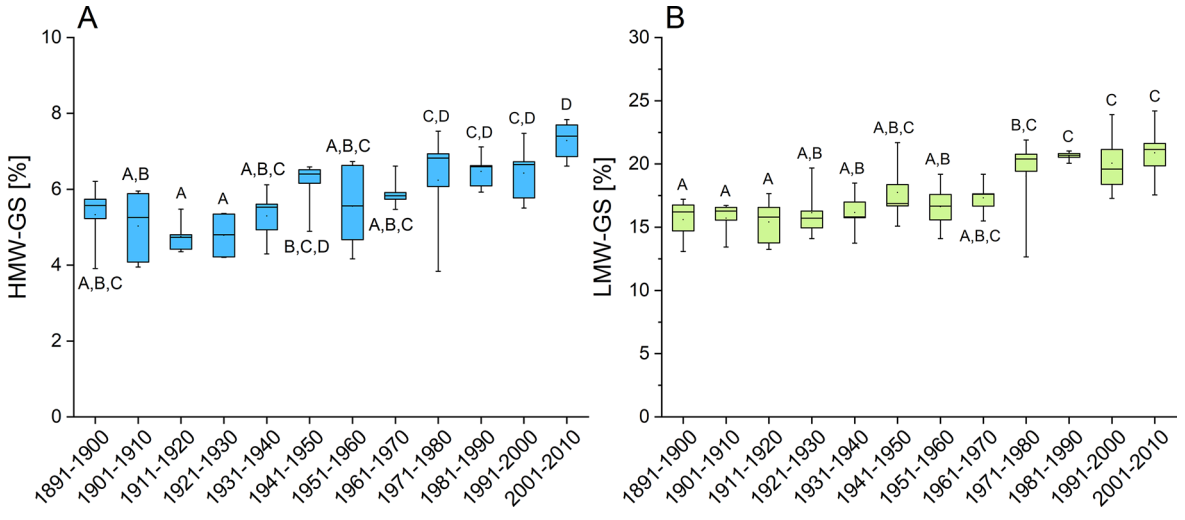

Figure 5. Proportions (%) of (A) HMW-GS and (B) LMW-GS in five cultivars per decade in the total RP-HPLC content averaged over 3 harvest years 2015, 2016, and 2017. Boxes represent the interquartile range with the median (line in the box) and mean (point in the box), and whiskers represent the minima and maxima. Different capital letters designate significant differences between the decades (one-way ANOVA and Tukey's test; $p < 0.05$).

respectively. It was noticeable that old cultivars that had the highest proportions for each harvest year were displayed, thus reinforcing the decrease of gliadins. In contrast, the highest proportions for the glutenin fraction were represented by modern cultivars 53 (35.8%), again 53 (33.8%), and 56 (34.7%) for the harvest years 2015, 2016, and 2017, respectively, whereas samples 3 (12.8%), 44 (18.1%), and 9 (15.9%) had the lowest proportions, with the old sample 20 having the second lowest proportion in 2016 (18.4%). The second and third lowest or highest proportions of each protein fraction were randomly distributed over old and modern cultivars. Concerning the protein types, we observed a high influence of the cultivar on the proportions of ω5-gliadins and

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Table 1. Two-Way ANOVA Testing Wheat Cultivar (Factor *a*), Harvest Year (Factor *b*), and Cultivar by Harvest Year Interactions for Total Protein Content and the Proportions of Each Protein Fraction and Type[a]

parameter	wheat cultivar (factor *a*)	harvest year (factor *b*)	*a* × *b* interaction
	F value	*F* value	*F* value
crude protein (mg/g)	400.3	13778.0	280.3
Protein Fractions			
albumins/globulins (%)	26.5	85.4	13.3
gliadins (%)	34.1	4.8	13.0
glutenins (%)	84.2	137.0	13.0
Gluten Protein Types			
ω5-gliadins (%)	321.6	62.5	51.8
ω1,2-gliadins (%)	154.1	1482.1	29.2
α-gliadins (%)	49.9	219.8	8.2
γ-gliadins (%)	63.3	717.9	7.4
HMW-GS (%)	118.6	1080.2	23.9
LMW-GS (%)	78.6	28.6	11.4

[a]All *p* values were below 0.001, except for gliadins, factor *b*, with $p = 0.009$.

LMW-GS. The proportions of ω1,2-, α-, and γ-gliadins and HMW-GS were strongly influenced by the harvest year.

DISCUSSION

In this study, 60 German wheat cultivars, first registered in the period of 1891−2010 were grown under the same conditions in 3 harvest years (2015, 2016, and 2017) and analyzed for agronomic and proteomic characteristics. The plant height decreased by up to 40% over the past 120 years, and reduced height is associated with a lower susceptibility toward diseases and stress.[24] These observations are in accordance with Liatukas and Ruzgas,[39] who reported that European wheat breeding efforts resulted in an optimal plant height of 80−100 cm. The main agronomic and quality traits reported by Longin et al.[40] also showed equivalent results for the plant height of winter wheat cultivars. The significant increase in wheat yield over the past 120 years agrees well with observations from the U.K. in the time period between 1950 and 1980[41] and also with German official trial data from 1983 to 2014.[28] The crude protein contents were highly variable in the 60 cultivars and also within the five cultivars per decade. About 90% of the samples had less than 100 mg/g of protein, because we decided not to fertilize the samples. It is well-known that the amount and type of fertilization has a significant effect on the grain protein content and composition.[21,22,42] However, we explicitly wanted to focus on the differences between the cultivars in this study and not encounter potential confounding effects resulting from different plant responses to fertilization.

The overall crude protein content tended to decrease from 1891 to 2010, which is in agreement with previous studies from the U.K.,[43] Germany,[28] and the U.S.[29] In contrast to our findings, a Canadian study reported an increase of protein contents in 36 red spring wheat cultivars first registered between 1860 and 2000.[25] Interestingly, two U.S. studies, one of which carried out a survey of data from the 20th and 21st centuries for the U.S.[21] and the other one that analyzed 30 red spring wheat cultivars from 1910 to 2013,[26] did not find changes in the protein contents. Because wheat protein composition is complex, we further analyzed the distribution of protein fractions and types. We observed an increase for glutenins and a decrease for gliadins and gliadin/glutenin ratios over the past 100 years but essentially no change for albumins/globulins and gluten. These results are in accordance with De Santis et al.[32] and also Ozuna and Barro,[30] who demonstrated that breeding contributed to a decrease of gliadin contents and the gliadin/glutenin ratio. That study also reported that the gluten content decreased, but this is not supported by our findings. The evidence that we found points to elevated glutenin contents, because glutenins are related to improved bread-making properties and have always been a target for breeders.[44] Concerning the gluten contents, our results support the findings by Shewry et al.,[27] who noted that the gluten content did not increase and suggested that the protein composition may have changed as a result of breeding practices targeting wheat with elastic dough properties.

Concerning gluten protein types, there was no definite trend for ω5- nor ω1,2-gliadins but α- and γ-gliadins showed a significant decrease. In contrast, De Santis et al.[32] suggested that the ω-gliadins, mainly ω5-gliadins, decreased significantly in Italian durum wheat cultivars from the 20th century, while no trend was determined for α- and γ-gliadins. Specific CD immunogenic epitopes are present in all gliadin types,[45] with ω5-gliadins being additionally known for triggering wheat-dependent exercise-induced anaphylaxis (WDEIA) in sensitized individuals.[46] We can confirm the decreasing trend of α- and γ-gliadins reported by Ozuna and Barro[30] but not the decreasing trend of ω-gliadins. Given the fact that α-gliadins are frequently considered to be the most immunogenic gluten protein type,[11] the decrease suggests no association to the rising prevalence of CD and NCGS. Still, further data are required to determine how the total α-gliadin content affects the amounts of immunogenic peptides, because correlations between the two have been reported[47] or not.[48,49] Our findings on HMW-GS and LMW-GS are partly consistent with Gulati et al.,[29] who reported that HMW-GS increased while LMW-GS decreased between 1870 and 2013. Ozuna and Barro[30] reported increasing trends, for both HMW-GS and LMW-GS. Especially the increase in HMW-GS can be explained as a result of their importance for dough elasticity and bread-making performance.[3]

Several studies have focused on the impact of breeding on the potential immunoreactivity of modern and old wheat cultivars but with partially conflicting results. A survey of data from the U.S.[21] showed that wheat breeding did not contribute to higher gluten contents and, consequently, to the increase in CD and NCGS prevalence. Using R5 ELISA, modern wheat cultivars had less immunogenic epitopes compared to wheat landraces.[31] In line with this, modern cultivars had lower amounts of immunogenic epitopes compared to old cultivars.[47,48] However, another study did find more epitopes in modern cultivars compared to old cultivars.[23]

The influence of the growing conditions on the protein composition was confirmed by previous studies,[50,51] but sometimes the effect of the genotype was higher[52] than reported here.

Our findings indicated that the variability in the protein composition is attributable to different cultivars but even more to the harvest year. Merely, the gliadin fraction, ω5-gliadins, and LMW-GS were predominantly affected by the genotype. Because the annual mean temperature was very similar across the harvest years, this factor did not seem to influence the protein content and distribution. In contrast, we observed a relation between absolute contents of the protein fractions and

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annual precipitation. The contents of crude protein, gliadins, and glutenins were lowest in the harvest year 2016 that had the least amount of precipitation. The albumin/globulin contents did not seem to be affected by precipitation. However, precipitation had no influence on the relative proportions of the gluten protein fractions, only the overall protein content. Because we only included German winter wheat cultivars and grown at one location in Germany, albeit in 3 consecutive years, the results of this study are not intended for generalization. However, this study based on our well-defined sample set is one of the first to answer questions related to the effect of wheat breeding since 1891 on the protein composition.

Taken together, we have characterized the protein content, fractions, and types demonstrating that the composition of wheat proteins has changed over the last 100 years as a result of breeding. Nonetheless, the picture is still incomplete, and a new approach for a better distinction of different wheat cultivars is already in progress. Moreover, further work will focus on elucidating the immunoreactive potential of the wheat cultivars and how it can be traced back to the identified changes in protein composition.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.0c02815.

Overview of the 60 German winter wheat cultivars analyzed (Table S1), contents of crude protein, albumins/globulins, gliadins, glutenins, and gluten as well as gliadin/glutenin ratios of the 60 wheat cultivars harvested in 2015 (Table S2), 2016 (Table S4), and 2017 (Table S6), contents of ω5-, ω1,2-, α-, and γ-gliadins, HMW-GS, and LMW-GS of the 60 wheat cultivars harvested in 2015 (Table S3), 2016 (Table S5), and 2017 (Table S7), albumin/globulin contents (Figure S1), gliadin contents (Figure S2), glutenin contents (Figure S3), gluten contents (Figure S4), gliadin/glutenin ratios (Figure S5), ω5-gliadin contents (Figure S6), ω1,2-gliadin contents (Figure S7), α-gliadin contents (Figure S8), γ-gliadin contents (Figure S9), HMW-GS contents (Figure S10), LMW-GS contents (Figure S11), and correlations between wheat agronomic and proteomic parameters (Figure S12) (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Katharina Anne Scherf − *Leibniz-Institute for Food Systems Biology at the Technical University of Munich, 85354 Freising, Germany; Department of Bioactive and Functional Food Chemistry, Institute of Applied Biosciences, Karlsruhe Institute of Technology (KIT), 76131 Karlsruhe, Germany;* orcid.org/0000-0001-8315-5400; Phone: +49-721-608-44176; Email: katharina.scherf@kit.edu; Fax: +49-721-608-44241

Authors

Darina Pronin − *Leibniz-Institute for Food Systems Biology at the Technical University of Munich, 85354 Freising, Germany*

Andreas Börner − *Genebank Department, Leibniz Institute of Plant Genetics and Crop Plant Research, 06466 Seeland/OT Gatersleben, Germany*

Hans Weber − *Department of Molecular Genetics, Leibniz Institute of Plant Genetics and Crop Plant Research, 06466 Seeland/OT Gatersleben, Germany*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.jafc.0c02815

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Notes

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■ ABBREVIATIONS USED

ANOVA, analysis of variance; CD, celiac disease; ELISA, enzyme-linked immunosorbent assay; HMW-GS, high-molecular-weight glutenin subunits; ICC, International Association for Cereal Science and Technology; LMW-GS, low-molecular-weight glutenin subunits; NCGS, non-celiac gluten sensitivity; RP-HPLC, reversed-phase high-performance liquid chromatography

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3.2 Fingerprinting of wheat protein profiles for improved distinction between wheat cultivars and species

The characterization of 60 hexaploid wheat cultivars by means of RP-HPLC revealed significant changes in the qualitative RP-HPLC profiles of the albumin/globulin, gliadin and glutenin fractions. Clear alterations in the peak patterns between cultivars were observed but these changes were not reflected in the contents of the protein fractions and types. To trace the changes in the patterns and to extract more information, a novel approach of peak integration was developed. All RP-HPLC chromatograms were integrated in an automated way every 20 seconds with regard to the retention time. In this manner, 22 integration events were performed for albumins/globulins, 42 for gliadins and 46 for glutenins. Thus, it was possible to achieve a better recognition of the protein fingerprints and a comprehensive discriminability of different wheat cultivars over the past 120 years due to the fact that almost every peak was detected and determined individually. By this means an improved differentiation between old and modern wheat cultivars was achieved, compared to the common integration method. Second, unique genotypes with specific protein fingerprints were determined, which should be examined in further research.
The application of new method was also extended to the five further wheat species common wheat, spelt, durum wheat, emmer and einkorn. The results substantiated the applicability of the method and allowed a distinction of einkorn from common wheat, however similarities regarding the overlap of spelt, durum wheat and emmer were observed.

Darina Pronin used statistical tools, such as analysis of variance, correlation analysis, principle component analysis (PCA) and hierarchical cluster analysis (HCA) in order to evaluate and visualize the data set. Darina Pronin performed all evaluation and statistical analyses, designed all figures, wrote the manuscript and revised the manuscript according to the reviewer comments.

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ORIGINAL RESEARCH ARTICLE

Fingerprinting of wheat protein profiles for improved distinction between wheat cultivars and species

Darina Pronin[1] | **Sabrina Geisslitz**[1,2] | **Andreas Börner**[3] | **Katharina A. Scherf**[1,2]

[1]Leibniz-Institute for Food Systems Biology at the Technical University of Munich, Freising, Germany

[2]Department of Bioactive and Functional Food Chemistry, Institute of Applied Biosciences, Karlsruhe Institute of Technology (KIT), Karlsruhe, Germany

[3]Genebank Department, Leibniz Institute of Plant Genetics and Crop Plant Research, Sealand, Gatersleben, Germany

Correspondence
Katharina A. Scherf, Department of Bioactive and Functional Food Chemistry, Karlsruhe Institute of Technology (KIT), Karlsruhe, Germany.
Email: katharina.scherf@kit.edu

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Abstract

Background and objectives: Wheat protein composition is commonly characterized by reversed-phase (RP)-HPLC-UV after extraction of albumins/globulins, gliadins (ω5-, ω1,2-, α-, and γ-gliadins), and glutenins (high- and low-molecular-weight glutenin subunits). However, this traditional classification does not consider the individual distribution of peaks, resulting in loss of information on protein fingerprints. We developed a new approach to peak integration and evaluated its suitability to differentiate between wheat cultivars and species.

Findings: Integration events were performed every 20 s, and the relative proportions of the peaks were calculated. We compared the traditional and new integration methods on two sample sets, the first comprising 60 common wheat cultivars from 1891 to 2010 and the second comprising 40 common wheat, spelt, durum wheat, emmer, and einkorn cultivars. The new integration method performed better in differentiating old and modern common wheat cultivars and was also applicable to different wheat species.

Conclusions: Unique cultivars were identified that stood out because of their protein composition. Four samples warrant further research to identify the specific proteins that are responsible for the differences.

Significance and novelty: The new integration allowed us to map the cultivar- and species-specific fingerprints, identify cultivars with exceptional protein composition, and group similar cultivars.

KEYWORDS

breeding, gliadins, glutenins, principal component analysis (PCA), reversed-phase (RP)-HPLC, wheat

1 | INTRODUCTION

Wheat belongs to the *Triticeae* of the *Poaceae* grass family and is subdivided into different species according to ploidy levels. Common wheat, also called bread wheat (*Triticum aestivum L.*), and spelt (*Triticum spelta* L.) are hexaploid species (genome AABBDD). Durum wheat (*Triticum durum* Desf.) and emmer (*Triticum dicoccon* (Schrank) Schübler) are tetraploid species (genome AABB), and einkorn (*Triticum monococcum* L.) is diploid (genome AA) (Delcour et al., 2012). Spelt,

emmer, and einkorn are hulled and often referred to as ancient wheats, whereas common wheat and durum wheat are free-threshing and designated as modern wheats (Dubcovsky & Dvorak, 2007; Kasarda, 2013). The gluten proteins of common wheat and durum wheat are responsible for the suitability of processing the grains into bread or pasta, respectively. The grains contain 9%–20% of protein, of which about 80% are storage proteins (gluten proteins) and about 20% are metabolic, protective, and structural proteins (Davis, Cain, Peters, Le Tourneau, & McGinnis, 1981; Wieser, Antes, & Seilmeier, 1998). Wheat proteins are typically divided into three fractions: albumins/globulins, gliadins, and glutenins. Albumins and globulins are water- and salt-soluble. Gliadins are predominantly monomeric proteins soluble in aqueous alcohols, whereas glutenins are polymerized by interchain disulfide bonds and only partly soluble in diluted acids and bases or solvents containing detergents and reducing agents (Scherf, Koehler, & Wieser, 2016). Gliadins and glutenins are further subdivided into protein types, and there are three different ways to classify them. The first is based on the amino acid sequence and classification into sulfur (S)-rich, S-poor, and high-molecular-weight (HMW) prolamins (Shewry, Miflin, & Kasarda, 1984). The second classification is based on relative molecular masses (M_r): (a) HMW group ($M_r \approx 67{,}000–88{,}000$), (b) medium-molecular-weight (MMW) group ($M_r \approx 40{,}000–55{,}000$), and (c) low-molecular-weight (LMW) group ($M_r \approx 30{,}000–42{,}000$). The third classification into gluten protein types is the separation of the gliadin fraction into ω5-, ω1,2-, α-, and γ-gliadins and of the glutenin fraction into HMW-glutenin subunits (GS) and LMW-GS based on electrophoretic mobility (Anjum et al., 2007). Each gluten protein type contains different numbers of single proteins, for example, 7 ω-gliadins, 23 α-gliadins, 13 γ-gliadins, 5 HMW-GS, and 22 LMW-GS as identified in the wheat cultivar Butte 86 (Dupont, Vensel, Tanaka, Hurkman, & Altenbach, 2011). However, these numbers and the protein quantities vary depending on the genotype (G), the environment (E), and the G × E interaction (Geisslitz, Longin, Scherf, & Koehler, 2019; Shewry et al., 2010; Ward et al., 2008). Usually, the classification into albumins/globulins and gluten protein types is applied for the qualitative and quantitative characterization of wheat proteins by means of reversed-phase (RP)-HPLC, that can be used to elucidate relationships between protein structure, functionality (e.g., baking performance), and bioactivity (e.g., potential to elicit immune reactions) (Schalk, Lexhaller, Koehler, & Scherf, 2017). However, one disadvantage is the lack of insight into the distribution of individual peaks for particular protein fractions and types. There are only very few reports in the literature that deal with more in-depth characterizations of protein or peptide profiles. One example is the extensive comparison of gliadin peaks by RP-HPLC to identify the effects of temperature and fertilization on the gliadin composition of a winter wheat cultivar (Daniel & Triboi, 2000), or isolation of different gliadins by preparative HPLC (Bin & Peterson, 2016). Other methods to compare differences in wheat genotypes and species include SDS-PAGE (Lagrain, Rombouts, Wieser, Delcour, & Koehler, 2012), a combination of RP-HPLC and SDS-PAGE (DuPont, Vensel, Encarnacao, Chan, & Kasarda, 2004), a combination of RP-HPLC and MS (DuPont, Vensel, Chan, & Kasarda, 2000), and LC-MS/MS (Bromilow et al., 2017; Colgrave et al., 2015; Geisslitz, America, & Scherf, 2020). Due to the complexity of the gluten protein types, it is even more important to provide accurate information about their qualitative and quantitative composition. These insights are crucial to determine changes in protein distribution, distinguish between cultivars and species, and identify protein fingerprints using RP-HPLC patterns.

Therefore, the aim of this study was to develop a new peak integration approach for the RP-HPLC patterns, in order to detect specific protein fingerprints and to allow a better differentiation between wheat cultivars within one species and between species. In that way, it was possible to achieve comprehensive insights into the changes of the protein distribution of wheat cultivars over the past 120 years due to breeding.

2 | MATERIALS AND METHODS

2.1 | Grain samples

Grains of 60 hexaploid German winter wheat cultivars from 1891 to 2010 were analyzed. For each decade, the five most common cultivars for the respective decade were selected (Table S1) and cultivated in a randomized field order without fertilization and harvested in 2015, 2016, and 2017 at the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) in Gatersleben, Germany (Pronin, Börner, Weber, & Scherf, 2020). Wholemeal flours were obtained by milling the grains with a laboratory grinder (Bosch, Stuttgart, Germany) and sieving to a particle size of 0.2 mm. Cultivars first registered prior to 1950 were designated as "old" (samples 1–30) and those registered after 1950 as "modern" (samples 31–60).

In addition, a second sample set containing different wheat species, that is, spelt, durum wheat, emmer, einkorn, and also common wheat, from a different region were analyzed (Table S1). Eight cultivars per wheat species were harvested in 2013 by the State Plant Breeding Institute (SPBI), University of Hohenheim (Stuttgart, Germany) at Seligenstadt, Germany. The cultivars were fertilized according to the demand of the particular wheat species. Common wheat, spelt, and durum wheat were fertilized with 95 kg N/ha and emmer and einkorn with 75 kg N/ha. In case of spelt,

emmer, and einkorn, the grains were dehulled. The grains were milled into wholemeal flour using a cross-beater mill (Perten Instruments, Hamburg, Germany) (Geisslitz, Wieser, Scherf, & Koehler, 2018; Longin et al., 2016).

2.2 | Characterization of the protein composition by RP-HPLC

The extraction of the wheat flours was performed stepwise based on solubility of the wheat protein fractions. First, the albumin/globulin fraction was obtained by extracting the wheat flours (100 mg) twice with 1.0 ml buffered salt solution (0.067 mol/L K_2HPO_4/KH_2PO_4-buffer, 0.4 mol/L NaCl, pH = 7.6). Next, the gliadin fraction was extracted three times with 0.5 ml 60% (v/v) aqueous ethanol from the residues. In the final step, the residues were extracted twice with 1.0 ml glutenin extraction solution (50% (v/v) 1-propanol, 0.1 mol/L Tris-HCl, pH 7.5, 0.06 mol/L (w/v) dithiothreitol) at 60°C under nitrogen, in order to obtain the glutenin fraction. The extraction conditions for each fraction were vortex mixing for 2 min at 22°C, followed by magnetic stirring for 10 min or 30 min (glutenins). After centrifugation of the suspensions (4,600 × *g*, 25 min, 22°C), the supernatants were combined, diluted to 2.0 ml with the respective extraction solvent, and filtered (Whatman™, Spartan 13/0.45 RC, GE Healthcare, Freiburg, Germany). The extractions for each sample were done in triplicate and analyzed by means of RP-HPLC according to Schalk et al., 2017.

For analysis, the following system was used: Jasco XLC system (Jasco, Gross-Umstadt, Germany) with ChromPass software (Jasco); column, Acclaim™ 300 C_{18} (particle size 3 µm, pore size 30 nm, 2.1 × 150 mm, Thermo Fisher Scientific, Braunschweig, Germany); temperature, 60°C; injection volume, 10 µl for gliadins and 20 µl for albumins/globulins and glutenins; elution solvents, trifluoroacetic acid (TFA) (0.1%, v/v) in water (A) and TFA (0.1%, v/v) in acetonitrile (B); linear gradient for albumins/globulins: 0 min 0% B, 0.5 min 20% B, 7 min 60% B, 7.1–11 min 90% B, 11.1–17 min 0% B; linear gradient for gliadins and glutenins: 0 min 0% B, 0.5 min 24% B, 20 min 56% B, 20.1–24.1 min 90% B, 24.2–30 min 0% B; flow rate 0.2 ml/min; and detection, UV absorbance at 210 nm. For external calibration, Prolamin Working Group (PWG)-gliadin (2.5 mg/ml in 60% ethanol) was used (van Eckert et al., 2006).

2.3 | Integration methods

Peak area integration for each protein fraction was performed by the ChromPass software. Following the traditional approach, the albumin/globulin fraction is integrated as one, starting at the retention time of 5.6 min and terminating at 12.9 min. The integration of the gliadin fraction is divided into ω5-gliadins at 7.8–10.0 min, ω1,2-gliadins at 10.0–13.4 min, α-gliadins at 13.4–18.0 min, and γ-gliadins at 18.0–21.7 min. Concerning the glutenin fraction, the integration starts with ωb-gliadins at 7.5–10.0 min, followed by HMW-GS at 10.0–14.5 min and LMW-GS at 14.5–22.7 min.

In case of the new integration approach, integration events were carried out every 20 s for all protein fractions. For albumins/globulins, 22 integration events were performed, from 5.6 min to 12.9 min. For gliadins, 42 integration events were performed from 7.8 min to 21.7 min and the integration for glutenins started at 7.5 min and ended at 22.7 min, which makes 46 integration events in total. For the determination of the area proportions (%) for each integration event, the ratio of the area beneath the peak relative to the area of the total chromatogram was calculated.

2.4 | Statistical analysis

Principal component analysis (PCA) was used to differentiate between old and modern wheat cultivars. For the traditional integration strategy, the percentages of albumins/globulins, gliadins (ω5-, ω1,2-, α-, γ-gliadins) and glutenins (ωb-gliadins, HMW-GS, LMW-GS) in the total RP-HPLC protein content for each of the 60 wheat cultivars were correlated with each other per harvest year. Additionally, the percentages were averaged over three harvest years and also correlated with each other. Using the new integration approach, the percentages of all integration events in the total RP-HPLC protein content for each of the 60 wheat cultivars were correlated with each other, both per each harvest year and averaged over three harvest years. PCA was carried out using Excel with the XLSTAT statistical software (Addinsoft, New York, NY, USA). Hierarchical cluster analysis was performed using the Origin 2018b software (OriginLab, Northampton, Massachusetts, USA), and the corresponding data were normalized (0;1) before distance calculation for clustering observations. The correlation coefficients (r) were defined as $r \leq \pm.54$ no correlation, $\pm.54 < r \leq \pm.67$ weak correlation, $\pm.67 < r \leq \pm.78$ medium correlation, and $r > \pm.78$ strong correlation (Thanhaeuser, Wieser, & Koehler, 2014).

3 | RESULTS AND DISCUSSION

3.1 | Distinction of wheat cultivars using the traditional integration method

The traditional way of peak integration for cereal proteins as described in Wieser et al. (1998) is as follows: The albumin/globulin fraction is not subdivided further, while the gliadin and glutenin fractions are subdivided into ω5-, ω1,2-, α-, and

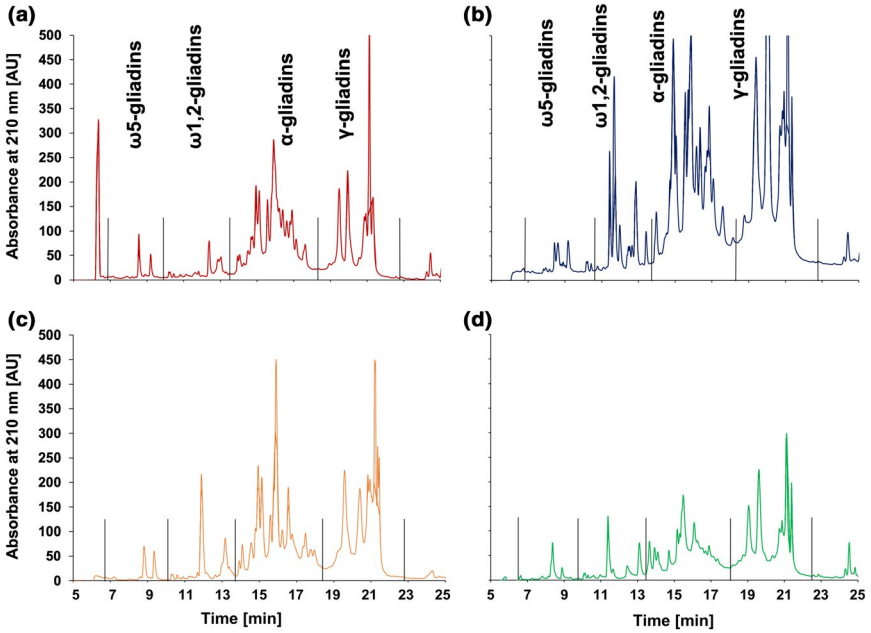

FIGURE 1 Reversed-phase (RP)-HPLC chromatograms of the gliadin fractions of the samples 1 (a), 24 (b), 35 (c) 58 (d) from the harvest year 2015

γ-gliadins as well as ωb-gliadins, HMW-GS, and LMW-GS, respectively (Figure 1). A PCA was performed in order to reduce the dimensionality of the data set and to see possible changes between old and modern wheat cultivars. The percentages of albumins/globulins, ω5-, ωb-, ω1,2-, α-, and γ-gliadins, HMW-GS, and LMW-GS relative to the sum of extractable proteins for each of the 60 common wheat samples of each harvest year (Figure S1) and of the average over three years were correlated with each other (Figure 2a). The principal components (PC)1 and PC2 accounted for 57.84% and 17.61% of the observed variability, respectively. PC1 was positively correlated with γ-gliadins ($r = .775$) and with gliadins and gliadin/glutenin ratios ($r \geq .978$), but negatively correlated with HMW-GS ($r = -.849$), glutenins, and LMW-GS ($r \geq -.971$). In contrast, PC2 was only negatively correlated with albumins/globulins ($r = -.868$). A negative correlation ($r = -.885$) was observed between gliadins and glutenins, but there was no correlation between ω5-, ω1,2-gliadins and gliadins or glutenins. Further, there was no correlation between albumins/globulins and gliadins or glutenins. The majority of the old wheat cultivars was placed on the right, because of their high gliadin and comparatively low glutenin proportions, as also reflected in the gliadin/glutenin ratios. Vice versa, the majority of the modern wheat cultivars was placed on the left, because of their high glutenin and low gliadin proportions. Neither old nor modern wheat cultivars were related to albumin/globulin proportions. Both old and modern cultivars showed a cluster formation, but with clear overlap in the middle of the biplot and several exceptions. The PCA revealed that the old cultivars 20, 22, and 26 were located among modern cultivars because of their exceptionally high glutenin proportions. Moreover, the modern wheat cultivars 31 and 44 were always located among old cultivars, because of their relatively high gliadin proportions. Essentially, the PCA of each of the three harvest years showed comparable correlations regarding wheat cultivars and corresponding proportions of the protein fractions. A further difference between old and modern cultivars was that the respective old cultivars of one decade were distributed unevenly, whereas the majority of the modern wheat cultivars showed cluster formation. Thus, it appears that the distinction between old and modern cultivars is limited using the traditional integration method, because information on individual peaks is lost.

3.2 | Qualitative characterization of the protein composition by RP-HPLC

By comparing all chromatograms of the 60 samples, significant changes in the peak patterns were determined over the period of 120 years. We identified cultivars with distinct patterns both for old and modern cultivars, which were 1, 24 and 35 and 58. Figure 1 shows RP-HPLC profiles of the corresponding gliadin fractions (harvest year 2015, overlay in Figure S2) and these agreed well with previous reports (Eggert, Wieser, & Pawelzik, 2010; Geisslitz et al., 2018; Schalk et al., 2017).

Due to comparatively clear baseline separation and presence of few peaks, the most noticeable differences were observed for the ω5- and ω1,2-gliadins. Compared to sample 1 (Figure 1a), the RP-HPLC profile of sample 24 (Figure 1b) revealed one additional peak for the ω5-gliadins and two additional high intensity peaks for ω1,2-gliadins at retention times of 8.5–9.0 min and 11.0–12.5 min, respectively. Sample 35 (Figure 1c) contained two sharp and baseline separated peaks for the ω5-gliadins at the retention times 8.5–10

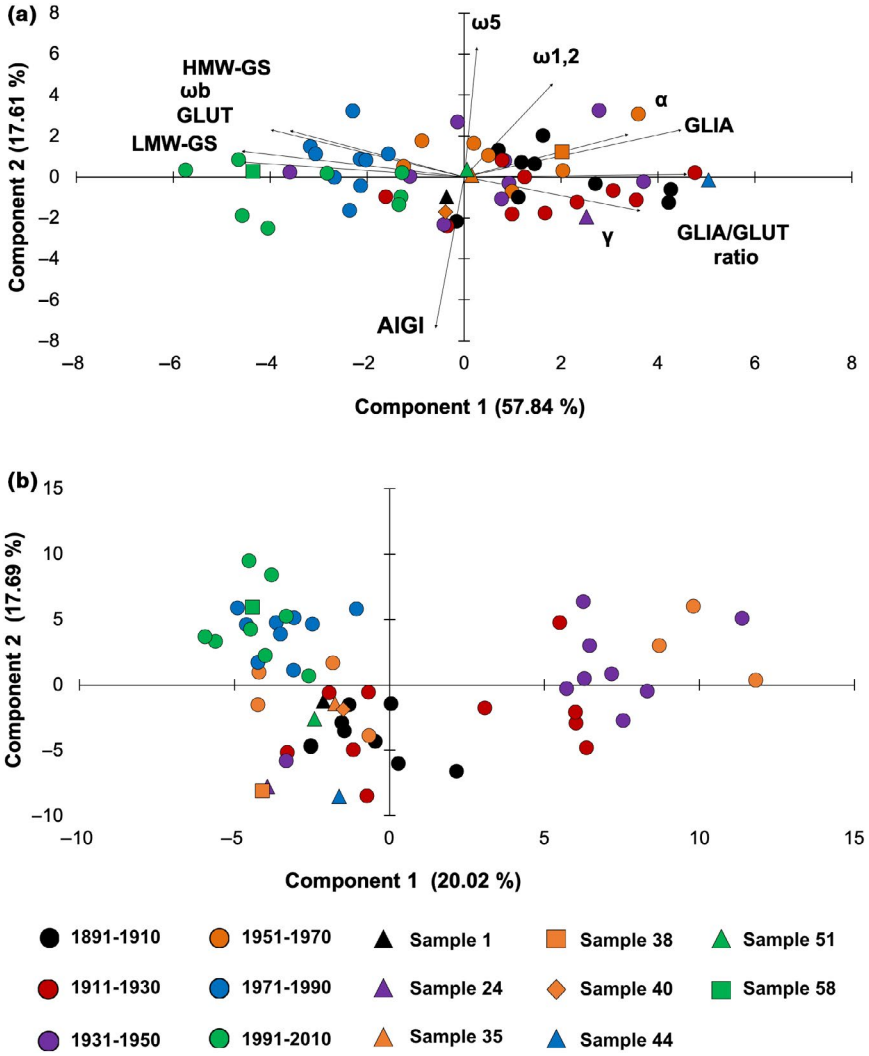

FIGURE 2 PCA biplot of proportions of albumins/globulins, ω5-, ω1,2-, α-, γ-gliadins, ωb-gliadins, HMW-GS, and LMW-GS relative to the sum of extractable proteins (a) and PCA biplot of proportions of each integration event of albumins/globulins, gliadins, and glutenins relative to the sum of extractable proteins (b). The data are displayed as average of the three harvest years 2015, 2016, and 2017. Distinct samples 1, 24, 35, 38, 40, 44, 51, and 58 were marked with different symbols. PCA, principal component analysis

min and one sharp and one shoulder peak for ω1,2-gliadins at 11.0–13.8 min, respectively. In contrast, sample 58 (Figure 1d) showed an additional peak for the ω1,2-gliadins between 11.0 and 13.8 min. Further differences in the peak distribution were observed for the patterns of α-gliadins. Especially for the samples 24 and 35, it was clearly visible that certain peaks were more pronounced than comparable ones at the same retention time. The patterns of γ-gliadins mostly remained unchanged, except for one peak at the retention time of 20 min, which changed its intensity from sample to sample. However, the detailed characterization of the patterns of α- and γ-gliadins was limited due to their accumulation and complexity of peaks and the absence of a baseline separation. Nonetheless, it was striking that the RP-HPLC profiles have significantly changed from sample 1 to 60 in terms of peak variability and distribution. Our findings did not support the results of Malalgoda, Ohm, Meinhardt, and Simsek (2017), who reported no changes in the profiles of hard red spring wheats between 1910 and 2013. In fact, we found that there seem to be differences between the profiles of old and modern cultivars. Interestingly, the profile changes were not reflected in the contents of the protein fractions and types (Pronin et al., 2020). This is why a new approach of peak integration was required to allow a better distinction of different wheat cultivars and an advanced detection of the protein fingerprints.

3.3 | Distinction of wheat cultivars using the new integration method

The new method involves continuous integration every 20 s, regardless of the protein fraction. In this way, nearly every peak was recognized individually (Figure S3). Compared to the traditional classification, peaks 1–7 belong to ω5-gliadins, 8–18 to ω1,2-gliadins, 19–33 to α-gliadins, and 34–42 to γ-gliadins. Concerning the glutenin fraction, peaks 1–9 belong to ωb-gliadins, 10–22 to HMW-GS, and 23–46 to LMW-GS.

Principal component analysis biplots were created by correlating all proportions of each integration event of albumins/globulins, gliadins, and glutenins relative to the sum of

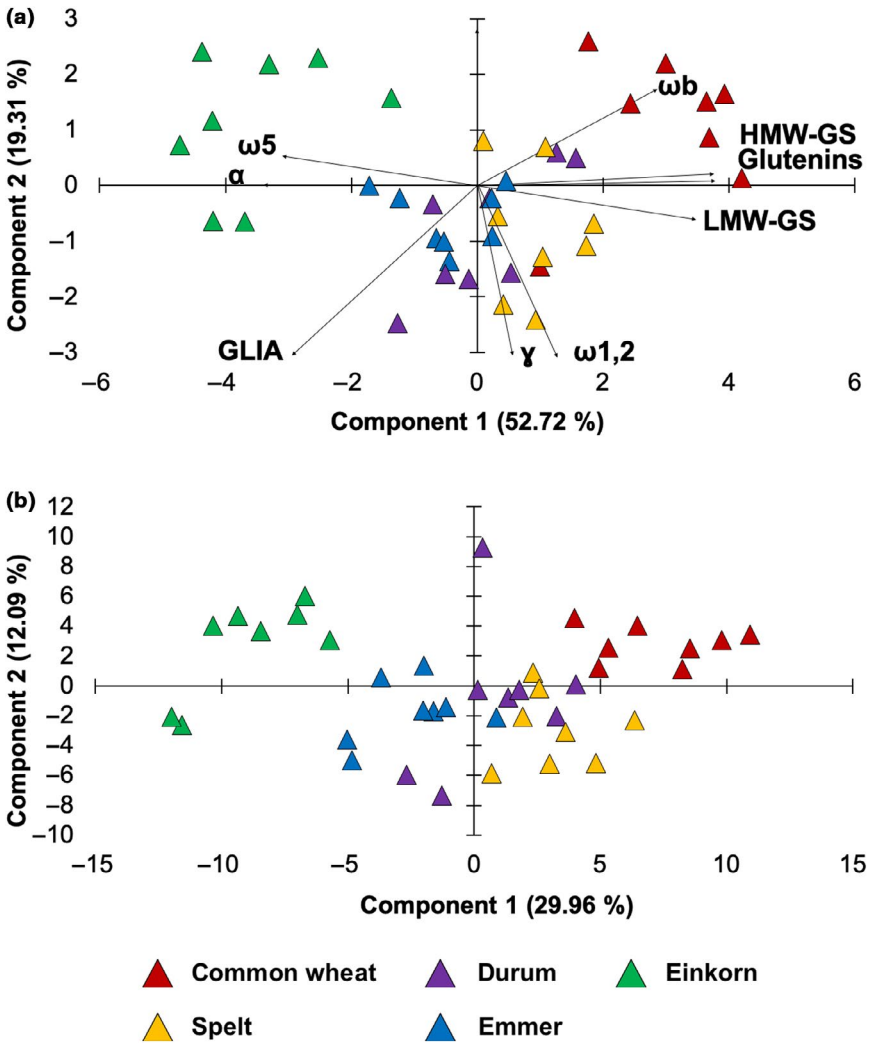

FIGURE 3 PCA biplot of proportions of albumins/globulins, ω5-, ω1,2-, α-, γ-gliadins, ωb-gliadins, HMW-GS, and LMW-GS relative to the sum of extractable proteins (a) and PCA biplot of proportions of each integration event of albumins/globulins, gliadins, and glutenins in the sum of extractable proteins from common wheat, spelt, durum, emmer, and einkorn (b). PCA, principal component analysis

extractable proteins. At first glance, it becomes obvious that the harvest year had a strong influence on the cluster formation and distribution of the cultivars (Figure S4), which is in accordance with our previous study (Pronin et al., 2020) and also evident using the traditional integration method. One reason for the stronger clustering of the wheat cultivars might be that this year had the least amount of annual mean precipitation (366 mm, as opposed to 533 mm and 557 mm in 2015 and 2017, respectively), but further investigations would be necessary to establish a clear cause–effect relation here. However, to even out the environmental effects, the results of the average over three harvest years are discussed in the following (Figure 2b). PC1 and PC2 accounted for 20.02% and 17.69% of the observed variability, respectively. PC1 was positively correlated with gliadin peaks 17–19 ($r \geq .81$) and with glutenin peaks 9 and 10 ($r = .83$ and .89). On the contrary, PC2 was only positively correlated with glutenin peaks 4 and 20–22 ($r \geq .72$), but negatively with total gliadin proportions ($r = -.76$) and gliadin peaks 24, 30–41 ($r \geq -.72$). Furthermore, we observed a cluster formation for modern cultivars (cluster I), which were located in the upper left corner, whereas old cultivars were grouped into two clusters. One cluster was placed on the right side and mainly contained samples, which encompassed the transition from old to modern cultivars including modern samples 31, 32, and 34 (cluster II). The other cluster also included both old and a few modern samples (35, 38, 40, 44, 51) and was placed in the bottom left corner (cluster III). Moreover, we observed a cluster formation for five cultivars of each decade, except the cultivars listed above. Considering the correlation between the three clusters and protein peaks, we observed clear determinants for each cluster, which were also true over all three harvest years. First, modern cultivars (cluster I) were correlated with peaks for albumins/globulins (2–5 and 16–22), gliadins (3, 35, and 37), and the majority of glutenins (4–7, 15–20, and 32–43). Next, old cultivars (cluster II) were correlated with peaks for albumins/globulins (7, 8, 10, 11), gliadins (1, 2, 5–10 15–23), and glutenins (2, 3, 8–11 13, 14, 23–27, 29, 30). This cluster was barely correlated with the fractions, in particular only with peaks of albumins/globulins (12–15), gliadins (4, 13, 14, 25–29, and 36–42), and only with a few of glutenins (37, 44, 46). Thus, old cultivars were

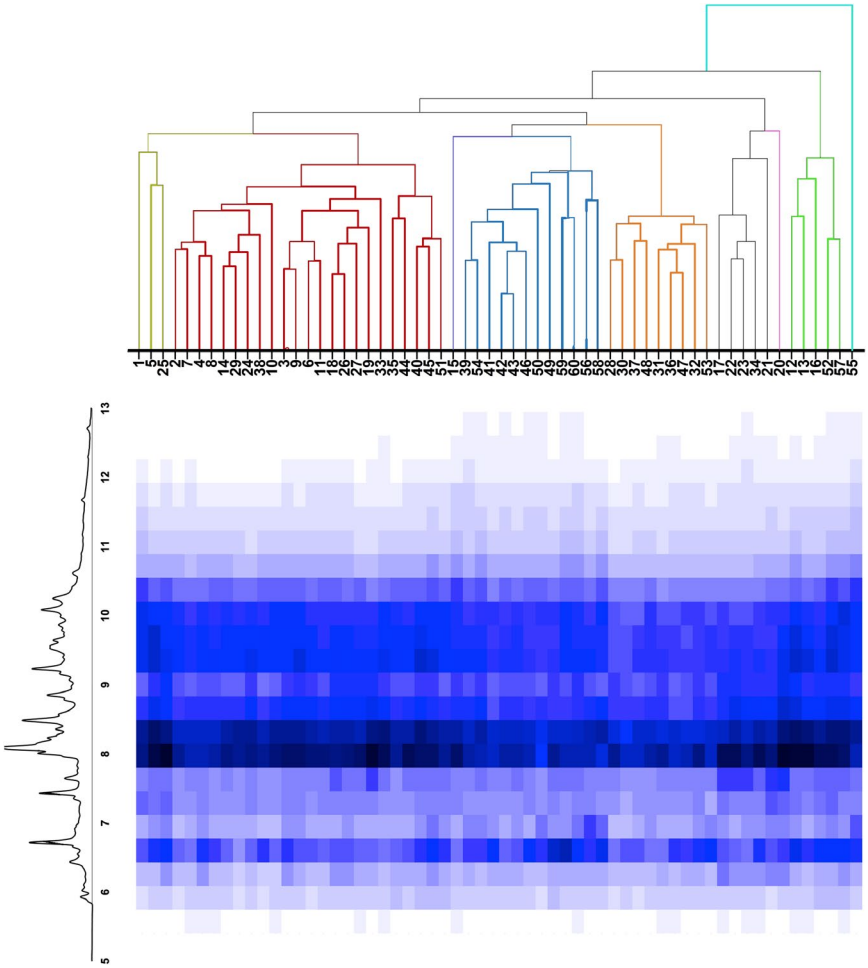

FIGURE 4 Dendrogram and heatmap based on RP-HPLC data for albumins/globulins using the new integration approach. The dendrogram on top shows the 60 analyzed wheat cultivars, and the qualitative profile on the side shows the pattern of the corresponding protein fraction. The data are displayed as average of the three harvest years 2015, 2016, and 2017. RP, reversed-phase

mostly associated with the middle part of albumins/globulins, the front parts of ω5- and α-gliadins, and the back part of ω1,2-gliadins. Further, old cultivars were associated with ωb-gliadins, the front and middle parts of HMW-GS, and the front part of LMW-GS. On the other hand, modern cultivars were mostly related to glutenins, especially to the back parts of ω5- and ωb-gliadins, HMW-GS and LMW-GS, to the front part of albumins/globulins and the front part of γ-gliadins. Cluster III was mostly influenced by the back parts of albumins/globulins, ω5-gliadins and α-gliadins and by the total of ω1,2- and γ-gliadins. The observations made for the average over three harvest years were influenced by the harvest year 2016 (Figure S4b), since the clusters for the harvest years 2015 and 2017 were mostly overlapping and therefore difficult to distinguish. These observations are consistent with the fact the harvest year had a significant effect on the protein distribution of the 60 wheat cultivars (Pronin et al., 2020). Effectively, there is a lack of studies performing a comprehensive comparison of wheat protein patterns by means of RP-HPLC, except for the study by Daniel and Triboi (2000) that revealed significant differences between the gliadin chromatograms of a winter wheat cultivar influenced by the effects of temperature and fertilization.

3.4 | Distinction of wheat species using the new integration method

To test the applicability of the new integration method, a second sample set including the five wheat species common wheat, spelt, durum wheat, emmer, and einkorn was analyzed as described above (Table S1). We observed a distinct cluster formation for the cultivars of common wheat and einkorn, but clear overlap between spelt, durum wheat, and emmer with both integration methods (Figure 3). The new integration performed better in terms of distinguishing emmer from spelt, but the durum wheat samples still lay in between, because they showed the greatest within-species variability. As expected, common wheat was associated with glutenins (Geisslitz et al., 2018), whereas ω5- and α-gliadins mostly determined the placement of einkorn cultivars. The results are in agreement with the PCA reported by Geisslitz et al. (2019) that also showed an association between glutenin contents and the location of common wheat cultivars, as well as between gliadin contents and einkorn. Our new approach allowed a better differentiation of wheat species than before, but there were also similarities regarding the overlap of spelt, durum wheat, and emmer.

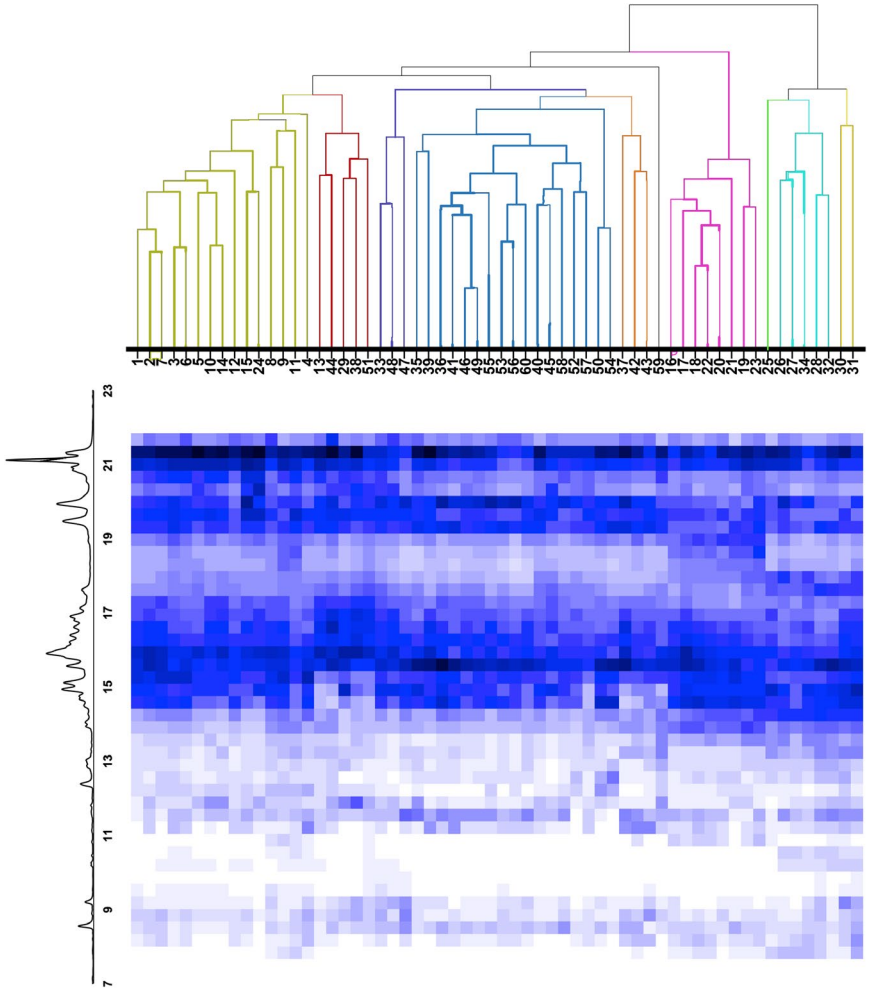

FIGURE 5 Dendrogram and heatmap based on RP-HPLC data for gliadins using the new integration approach. The dendrogram on top shows the 60 analyzed wheat cultivars, and the qualitative profile on the side shows the pattern of the corresponding protein fraction. The data are displayed as average of the three harvest years 2015, 2016, and 2017. RP, reversed-phase

3.5 | Hierarchical cluster analysis

To determine the differences and similarities for the cultivars in more detail, the data for each protein fraction were clustered by similarity using hierarchical clustering analysis (Figures 4–6). The following characterization of the cultivars refers to the average of the three harvest years. First, the dendrogram of the albumins/globulins shows that the 60 cultivars can be divided into nine major groups according to their distribution (Figure 4). The most modern samples (56–60) showed a strong correlation with fairly modern samples (41–49) and were also correlated with samples of the transition from old to modern (28, 30, 31). Further, the samples from each decade were often distributed unevenly over the 9 groups, but modern samples showed more similarities than old cultivars. We further observed individual cultivars, that did not appear jointly with the cultivars from the same decade, but were found in different groups and showed greater distances to the cultivars from the same decade, for example, samples 18, 19, 24, 25, 29, and 38. Interestingly, these cultivars showed rather short distances among each other. In detail, we found samples which appeared to be different within one group, because they belonged to other decades, for example, the second group contained modern samples 33, 35, 40, 44, 45, and 51, which were similar to each other based on their distance, but were located among old cultivars within the group. Interestingly, these cultivars were always correlated irrespective of the harvest year and were not among the other cultivars from the same decade. What is more, the modern sample 38 was found in group 2, among old cultivars and with no correlation to the samples from the corresponding decade. Further outstanding samples, which showed disparities within the group on the one hand and between the group on the other, were 15 (group 3); 47, 48, 53 (group 5); 34 (group 6); 20 (group 7); 52, 57 (group 8) and 55 (group 9).

Next, the cultivars of the gliadin fraction were categorized into 10 groups (Figure 5). It was noticeable that old cultivars of the particular decade from sample 1 to 25, except for sample 24, were mostly located in the same group, while cultivars from the 1940s and 1950s were rather evenly distributed. Especially, the samples 29, 33, and 35 showed greater distances to the cultivars from the same period. Modern samples were widely distributed, but remained in the same or in an adjoining section (groups 3 and 4). Exceptions with wider distances to the modern cultivars from the same decade were observed for samples 38, 44, 51,

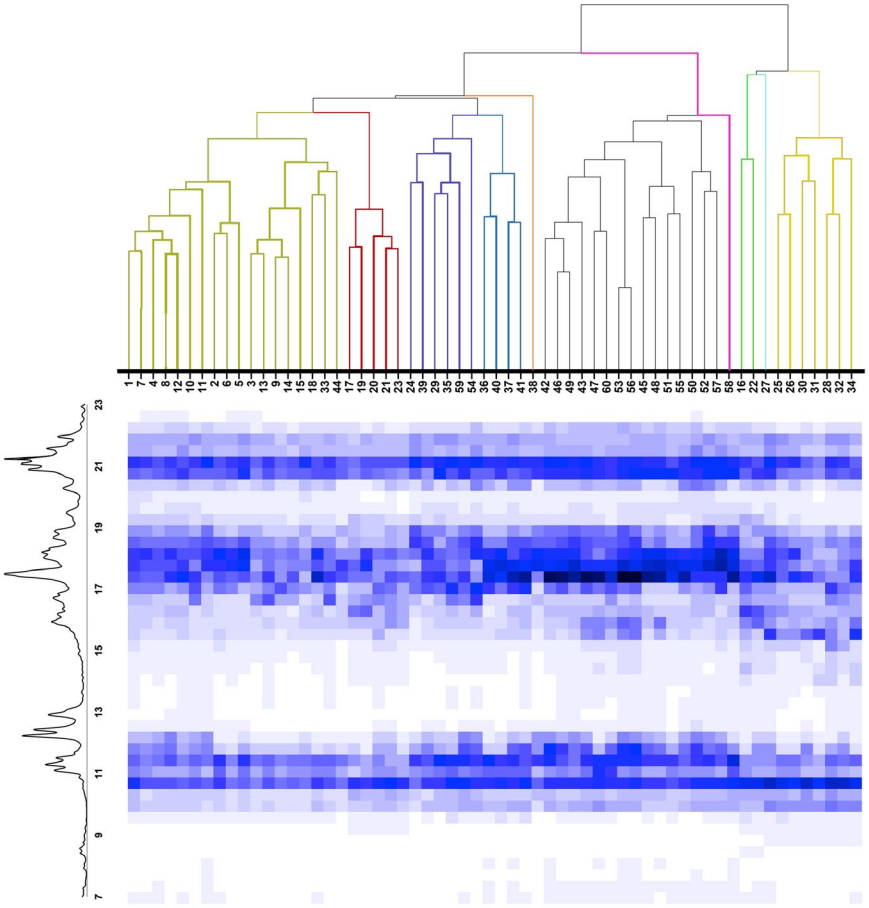

FIGURE 6 Dendrogram and heatmap based on RP-HPLC data for glutenins using the new integration approach. The dendrogram on top shows the 60 analyzed wheat cultivars, and the qualitative profile on the side shows the pattern of the corresponding protein fraction. The data are displayed as average of the three harvest years 2015, 2016, and 2017. RP, reversed-phase

and 59. However, we found that the oldest samples (1–15) were most likely correlated with modern samples (36–60) and not with cultivars from the 1940s and 1950s (26–35). Noticeable samples were also found for the gliadin fraction, in particular 24 (group 1); 29, 38, 44, 51 (group 2); 33 (group 3); 59 (group 6) and 25 (group 8).

Last, the cultivars of the glutenin fraction were categorized into 10 groups (Figure 6). Old and modern samples, respectively, were mostly correlated among themselves. It was striking that the samples of the same decade were found in the same or adjacent group, but with a few exceptions. These exceptions were 33, 44 (group 1); 59 (group 3). Effectively, samples 38 (group 5); 58 (group 7) and 27 (group 8) showed great distances and were not correlated to other groups.

The genealogical relations between the cultivars were obtained from the Genetic Resources Information System (GRIS) database, but some cultivars were not included (e.g., 9, 12, 23, 30) and some are directly derived from German landraces (e.g., 8, 10, 14 and 20). Some cultivars included in the sample set did have relations, for example, 59 has 31 and 43 in its close ancestry, but these were only partially reflected. While 43 was next to 59 in the gliadin dendrogram, 31 was not; and in the glutenin dendrogram these three were not any closer to one another than to totally unrelated cultivars. It seems that the resolution of the RP-HPLC chromatograms is not sufficiently detailed enough to map genealogical relations or individual protein isoforms.

Overall, it can be concluded that the observations above revealed associations among cultivars and emphasized the uniqueness of particular cultivars. We were able to find one cultivar, which was outstanding with respect to its unique albumin/globulin and gliadin pattern and moreover three further cultivars which had unique patterns for all protein fractions. These samples were 59 and 33, 38, and 44, respectively. In a recent study, 30 hard red spring wheat cultivars from a period of 100 years were analyzed to elucidate the clustering patterns of gliadins by means of RP-HPLC (Malalgoda et al., 2017). The data were produced by determining the presence and absence time of a particular peak for each cultivar and indicated that the cultivars have in fact changed over the last 100 years in terms of quality characteristics. However, no clusters according to the release year of the cultivars were observed. In the view of the above, the insights given by the new method of peak integration are crucial for an in-depth analysis of wheat proteins. The major advantage of this method is that the distribution of individual peaks in the complex gluten chromatograms is now captured more comprehensively, which leads to better results. For future research, these insights can be used to isolate specific

peaks from the outstanding cultivars for in-depth MS and immunological studies.

4 | CONCLUSION

The new integration approach for wheat protein fingerprints analyzed by means of RP-HPLC was suitable to trace changes in peak variability depending on the wheat cultivar or wheat species. The method was also applicable to spelt, emmer, durum wheat, and einkorn compared to common wheat, although the traditional method of peak integration performed almost equally well in this case. Within the set of 60 common wheat cultivars, we were able to differentiate between old and modern wheat cultivars and additionally identify unique cultivars that stood out because of their protein composition. Four samples were identified that warrant further research to identify the specific proteins that are responsible for the differences.

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CONFLICT OF INTEREST

The authors declare no competing financial interest.

ORCID

Sabrina Geisslitz https://orcid.org/0000-0002-6811-4069
Andreas Börner https://orcid.org/0000-0003-3301-9026
Katharina A. Scherf https://orcid.org/0000-0001-8315-5400

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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3.3 Elucidation of the immunoreactivity of old and modern common wheat cultivars caused by breeding.

The investigation of 60 wheat cultivars, which were common in the time between 1891 and 2010 showed a trend towards lower gliadin contents, including α- and ɣ-gliadins. To trace the immunoreactive potential of the wheat cultivars to the identified changes in the protein composition and distribution, the cultivars were analyzed by means of LC-MS/MS. In this way, the question of whether breeding practices have led to a higher antigenicity of modern cultivars was addressed. Therefore, two different methods were applied in this study based on the sample set of 60 wheat cultivars. Therefore, Darina Pronin carried out a stable isotope dilution assay (SIDA) paired with targeted LC-MS/MS, in order to allow the elucidation of the antigenicity of all cultivars. The investigated epitopes were LQLQPFPQPQLPYPQPQPF (P1), RPQQPYPQPQPQY (P2), LQLQPFPQPQLPYPQPQLPYPQPQPLPYPQPQPF (33-mer) from α-gliadins and LQPQQPQQSFPQQQQPL (P3) from γ-gliadins. These peptides were chosen based on their known immunogenic activity. Especially the immunodominant 33-mer plays a crucial role in the initiation of CD, because it contains six partly overlapping epitopes. With one exception, all cultivars contained each analyzed peptide. Both, the 33-mer an P1 proportions in α-gliadin revealed a tendency to increase from old to modern cultivars. However only the values relative to the α-gliadins and not the absolute contents showed a slightly increasing trend. In contrast, neither absolute contents nor the proportions of P2 and P3 peptides in α- and ɣ-gliadins, respectively, showed a clear trend. Further, a high variability of the proportions and contents of CD-active peptides over 60 different cultivars and within the five cultivars per decade was observed. Darina Pronin evaluated the statistical analysis of the data, which suggested that old and modern cultivars might have changed over the past decades, however primarily affected by the harvest year and less by the cultivar.

The second part of the study comprised the extraction of gliadin fractions and types out of 16 selected cultivars and their investigation by means of two different sandwich ELISA kits, namely R5 and G12. Given the fact that the antibodies used in these kits are specific for certain CD-active peptide motifs, the corresponding gliadin types showed a strong correlation with the R5-ELISA results. On the contrary, the G12 ELISA resulted in a weak correlation with the 33-mer contents, although the G12 mAb was raised against the 33-mer peptide. Darina Pronin carried out all experiments,

performed the statistical analysis, wrote the manuscript and revised it according to the comments of the coauthors.

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Old and modern wheat (*Triticum aestivum* L.) cultivars and their potential to elicit celiac disease

Darina Pronin[a], Andreas Börner[b], Katharina Anne Scherf[a,c,*]

[a] Leibniz-Institute for Food Systems Biology at the Technical University of Munich, Lise-Meitner-Str. 34, 85354 Freising, Germany
[b] Genebank Department, Leibniz Institute of Plant Genetics and Crop Plant Research, Corrensstr. 3, 06466 Seeland, OT Gatersleben, Germany
[c] Department of Bioactive and Functional Food Chemistry, Institute of Applied Biosciences, Karlsruhe Institute of Technology (KIT), Adenauerring 20a, 76131 Karlsruhe, Germany

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ABSTRACT

One potential explanation for the increasing prevalence of celiac disease (CD) over the past decades is that breeding may have inadvertently changed the immunoreactive potential of wheat. To test this hypothesis, we quantitated four CD-active peptides, namely the 33-mer and peptides containing the DQ2.5-glia-α1a/DQ2.5-glia-α2 (P1), DQ2.5-glia-α3 (P2) and DQ2.5-glia-γ1 (P3) epitopes, in a set of 60 German hexaploid winter wheat cultivars from 1891 to 2010 and grown in three consecutive years. The contents of CD-active peptides were affected more by the harvest year than by the cultivar. The 33-mer and P1 peptides showed no tendency regarding their absolute contents in the flour, but they tended to increase slightly over time when calculated relative to the α-gliadins. No trends in relative or absolute values were observed for the P2 and P3 peptides derived from α- and γ-gliadins. Therefore, the immunoreactive potential of old and modern wheat cultivars appears to be similar.

1. Introduction

Wheat is a staple food of mankind, but its consumption is also linked to wheat-related disorders, such as celiac disease (CD), wheat allergy or non-celiac gluten sensitivity. CD affects about 1% of the population worldwide and is characterized by chronic inflammation of the small intestine, which results in destruction of the epithelial cells and consequently in nutrient malabsorption (Caio et al., 2019). In genetically predisposed individuals, CD can be initiated by ingestion of the storage proteins of wheat (gliadins, glutenins), barley (hordeins) and rye (secalins) (Abadie, Sollid, Barreiro & Jabri, 2011). Wheat storage proteins comprise gliadins and glutenins and are usually classified into gliadin types (ω5-, ω1,2-, α-, and γ-gliadins) and high- (HMW-GS) and low-molecular-weight glutenin subunits (LMW-GS) (Scherf, Koehler & Wieser, 2016). As their name suggests, prolamins are rich in the amino acids proline and glutamine (Arentz-Hansen et al., 2002; Shan et al., 2005). The disability of human gastrointestinal enzymes to cleave proteins before or after proline and glutamine results in the persistence of large CD-active peptides with nine or more amino acid residues, which are absorbed into the lamina propria and initiate the inflammatory reaction in CD patients (Plugis & Khosla, 2015).

All gluten proteins contain CD-active epitopes with distinct levels of stimulatory potential (Tye-Din et al., 2010), but gliadin peptides are usually considered to be most toxic, *inter alia*, because the incomplete digestion of gliadins results in a 33-mer peptide (LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF) (Qiao et al., 2004). The 33-mer is derived from α2-gliadin of hexaploid wheats, but not present in tetraploid emmer and durum wheat and diploid einkorn, because the D-genome, which encodes α2-gliadin, is absent (Ozuna, Lehisa, Gimenez, Alvarez, Sousa & Barro, 2015). The 33-mer is considered to be the most immunodominant peptide (Qiao, Bergseng, Molberg, Jung, Fleckenstein & Sollid, 2005; Shan et al., 2005), because it contains six partly overlapping epitopes, in particular PFPQPQLPY (DQ2.5-glia-α1a, one repetitive unit), PYPQPQLPY (DQ2.5-glia-α1b, two repetitive units) and PQPQLPYPQ (DQ2.5-glia-α2, three repetitive units) (Arentz-Hansen et al., 2000; Sollid, Tye-Din, Qiao, Anderson, Gianfrani & Koning, 2020). Two monoclonal antibodies (mAbs, A1 and G12) have been developed against the 33-mer for use in enzyme-linked immunosorbent assays (ELISA) for gluten detection (Morón, Bethune et al., 2008; Morón, Cebolla et al., 2008). The two most commonly used sandwich ELISA test kits are based on the R5 (Valdés, García, Llorente & Méndez, 2003) and G12 mAbs (Morón, Bethune et al., 2008; Morón, Cebolla

* Corresponding author at: Department of Bioactive and Functional Food Chemistry, Institute of Applied Biosciences, Karlsruhe Institute of Technology (KIT), Adenauerring 20a, 76131 Karlsruhe, Germany.
E-mail addresses: d.pronin.leibniz-lsb@tum.de (D. Pronin), boerner@ipk-gatersleben.de (A. Börner), katharina.scherf@kit.edu (K.A. Scherf).

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Table 1
LC-MS/MS parameters for the quantitative analysis of CD-active peptides. Multiple reaction monitoring (MRM) parameters for the 33-mer, P1 and P2 derived from α-gliadins, P3 derived from γ-gliadins and the stable isotope labelled peptide standards.

Peptide	Amino acid sequence	Precursor ion [*m/z*] (charge state)	Product ion[1] [*m/z*]	Collision energy [V]
33-mer	LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF	979.0 (4+)[2]	263.3 (y2)[2]	14
		1305.2 (3+)[3]	973.5 (y8)[3]	12
*33-mer	LQLQP*FPQPQLPYPQPQLPYPQPQLPYPQ*PQ*P*F	987.0 (4+)[2]	279.0 (y2)[2]	14
		1316.0 (3+)[3]	996.0 (y8)[3]	12
P1	LQLQPFPQPQLPYPQPQPF	755.20 (3+)	262.96(y2)[2]	18
			973.64 (y8)[3]	20
*P1	LQLQPFPQPQLPYPQPQ*P*F	760.50 (3+)	278.96 (y2)[2]	14
			989.64 (y8)[3]	10
P2	RPQQPYPQPQPQY	814.24 (2+)	407.12 (y3)[2]	14
			770.48 (b6)[3]	10
P3	LQPQQPQQSFPQQQQPL	1011.42 (2+)	839.02 (y7)[2]	20
			228.96 (y2)[3]	18

*Labelled with *P: L-[$^{13}C_5$][^{15}N]-proline and *F: L-[$^{13}C_9$][^{15}N]-phenylalanine, [1]charge state: 1+, [2]precursor to product ion transition was used as quantifier, [3]precursor to product ion transition was used as qualifier.

et al., 2008).

One hypothesis to explain the rising prevalence of CD in the population (Gatti et al., 2020; Singh et al., 2018) is that wheat breeding may have contributed to increasing the immunostimulatory potential of modern wheats (cultivars developed after 1950) compared to old wheats and landraces (van den Broeck et al., 2010). Several studies have already set out to verify or falsify this hypothesis, but with partly contradictory results. A recently developed method based on a stable isotope dilution assay (SIDA) paired with liquid chromatography tandem mass spectrometry (LC-MS/MS) was used to quantitate the 33-mer in 15 old and 23 modern common wheat cultivars, but no trends towards increased contents of the 33-mer in modern cultivars were identified (Schalk, Lang, Wieser, Koehler & Scherf, 2017; Schalk, Koehler & Scherf, 2018). Malalgoda, Meinhardt & Simsek (2018) used SIDA LC-MS/MS to quantitate two CD-active epitopes, namely DQ2.5-glia-α1 (PFPQPQLPY) and DQ2.5-glia-α3 (FRPQQPYPQ), present in α-gliadins of 30 different spring wheat cultivars from North Dakota of the past 100 years. This study suggested a random variation of the epitope contents over all cultivars and no relation to the cultivar release year. Boukid et al. (2017) determined the antigenicity of 100 Tunisian durum wheat cultivars of the 20th century and the results indicated a high variability in the contents of immunogenic peptides resulting from different environmental conditions, but again not related to the release year of the cultivars. A comparable study found that the DQ2.5-glia-α1a peptide was present in all durum wheat cultivars studied and that the amount was affected more by the cultivar than by the environmental conditions (Prandi, Mantovani, Galaverna & Sforza, 2014). The same group determined ten immunogenic peptides in old and modern Italian common wheat cultivars and showed that the old cultivars had higher contents of immunogenic peptides than modern ones (Prandi, Tedeschi, Folloni, Galaverna & Sforza, 2017). Ribeiro, Rodriguez-Quijano, Nunes, Carrillo, Branlard & Igrejas (2016) analyzed 53 modern wheat varieties and 19 old landraces using the R5 competitve ELISA and found that the reactivity did not increase over time, but that landraces even had higher reactivity. Another study by Escarnot, Gofflot, Sinnaeve, Dubois, Bertin, & Mingeot (2018) used A1 and G12 mAbs to analyze 195 wheat accessions and determined no significant differences in reactivity of landraces, old, mid and modern wheat varieties. In contrast, van den Broeck, Cordewener, Nessen, America & van der Meer (2015) performed a label-free determination of the immunogenic epitopes DQ2.5-glia-α1a, DQ2.5-glia-α2 and DQ2.5-glia-α3 in three wheat cultivars, demonstrating that one modern wheat cultivar contained a higher amount of the epitopes. In a previous study, van den Broeck et al. (2010) analyzed 36 modern European wheat cultivars and 50 landraces using ELISA. The results indicated that higher amounts of DQ2.5-glia-α1a were present in modern wheat cultivars than in landraces, with the exception of a few modern cultivars.

Taken together, the evidence whether old or modern wheat cultivars have higher or lower contents of CD-active peptides is inconclusive so far, because only small sample sets were studied or samples that were not grown under the same conditions to ensure comparable environmental conditions. Therefore, the aim of our study was to investigate the influence of breeding on the contents of four major CD-active peptides by LC-MS/MS in a very well-characterized sample set of 60 German hexaploid wheat cultivars from 1891 to 2010 grown in three consecutive harvest years. All cultivars and selected gliadin fractions and types were also investigated regarding their influence on the ELISA responses, using the R5 and G12 mAbs to get further insights into their immunoreactive potential.

2. Materials and methods

2.1. Materials

Analytical grade quality or higher was used for all chemicals and reagents, unless stated otherwise. The reagents were purchased from VWR Merck (Darmstadt, Germany), Serva (Heidelberg, Germany), LECO (Kirchheim, Germany) and Sigma-Aldrich (Steinheim, Germany). α-Chymotrypsin (from bovine pancreas, TLCK-treated, 40 U/mg protein) was used for gluten digestion. Water for reversed-phase high-performance liquid chromatography (RP-HPLC) was deionized by an Arium 611VF water purification system (Sartorius, Goettingen, Germany). Two commercially available sandwich ELISA test kits for gluten detection in foods were used: RIDASCREEN® Gliadin (R-Biopharm, Darmstadt, Germany) (R5) and AgraQuant® ELISA Gluten G12 (Romer Labs, Tulln, Austria) (G12). The peptides LQLQPFPQPQLPYPQPQLPYPQPQPLPYPQPQPF (33-mer), LQLQPFPQPQLPYPQPQPF (P1), RPQQPYPQPQPQY (P2), LQPQQPQQSFPQQQQPL (P3) and the stable isotope labelled peptides LQLQP*FPQPQLPYPQPQLPYPQPQLPYPQ*PQ*P*F (*33-mer) and LQLQPFPQPQLPYPQPQ*P*F (*P1) with *P: L-[$^{13}C_5$][^{15}N]-proline and *F: L-[$^{13}C_9$] [^{15}N]-phenylalanine, were purchased from Genscript (Hongkong, PR China) with a purity of > 90% (Table 1).

2.2. Grain samples

In this study 60 different hexaploid wheat cultivars (*Triticum aestivum* L.), common between 1891 and 2010 in Germany, were analyzed. The five most widely grown cultivars for each of the twelve decades were selected (Supplementary Table S1) and provided by the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) in Gatersleben, Germany as 20 g of cleaned grains. All cultivars were harvested in three different years, namely 2015, 2016 and 2017, and cultivated in a randomized field order without fertilization (Pronin, Börner, Weber & Scherf, 2020). The grains were milled into wholemeal flour using a

laboratory grinder (Bosch, Stuttgart, Germany) and sieved to a particle size of 0.2 mm.

On the basis of clear differences between RP-HPLC chromatograms as described in Pronin, Geisslitz, Börner & Scherf (2020), 16 particular cultivars were selected (samples 5, 10, 14, 19, 24, 25, 29, 35, 38, 40, 44, 45, 48, 51, 58 and 59) and propagated in the years 2016 and 2017 for further analysis. The grains were milled into flour using a Quadrumat Junior mill (Brabender, Duisburg, Germany), sieved to a particle size of 0.2 mm and left to rest for two weeks.

2.3. Quantitation of CD-active peptides by LC-MS/MS

2.3.1. Sample preparation

The quantitative analysis of CD-active peptides was performed according to Schalk, et al. (2017). Wheat flours (150–200 mg) were defatted twice with pentane/ethanol (95/5, v/v, 2.0 mL). Then, the albumin/globulin fraction was extracted from the flour with 2 × 1.0 mL of buffered salt solution (0.067 mol/L K_2HPO_4/KH_2PO_4-buffer, 0.4 mol/L NaCl, pH = 7.6). In the next step, the residues were extracted three times with 0.5 mL 60% (v/v) ethanol to obtain the gliadin fraction. For each extraction step the suspensions were vortex mixed for 2 min at 22 °C followed by magnetic stirring for 10 min and centrifugation (4600*g*, 25 min, 22 °C). The supernatants of the albumin/globulin fraction were discarded, while the supernatants of the gliadin fraction were combined and dried by centrifuging under reduced pressure (40 °C, 6 h, 800 Pa). After re-suspension in a TRIS-HCl-buffer (2.0 mL, 0.1 mol/L TRIS-HCl, pH 7.8, urea 120 mg/mL) the labeled *P1 and *33-mer peptides were added (300 µL; 10 µg/mL). The mixture was hydrolyzed with α-chymotrypsin (enzyme-to-protein (E:P) ratio of 1:200) for 24 h at 37 °C. The digestion was stopped with trifluoracetic acid (TFA) (5 µL) and dried by centrifuging under reduced pressure (40 °C, 6 h, 800 Pa). The residue was re-dissolved in formic acid (FA) (0.1%, v/v, 500 µL), filtered (0.45 µm) and analyzed by targeted LC-MS/MS (Table 1). α-Chymotrypsin was used for enzymatic hydrolysis instead of trypsin, because α-chymotrypsin has been shown to release the 33-mer from gluten proteins in its intact form (Schalk et al., 2017).

The determination of 33-mer and P1 was performed for each harvest year (2015, 2016, 2017), whereas P2 and P3 were only determined as an average over the three harvest years in order to focus on the genetic variability rather than the environmental effect. Therefore, 300 mg of flour from each wheat cultivar per harvest year were combined to a pooled sample and subsequently prepared as described above. All determinations were performed in triplicate.

2.3.2. Calibration

First the 33-mer, P1, P2 and P3 and the labelled *33-mer and *P1 standards were each dissolved in FA (0.1%, v/v, 10 µg/mL). For calibration, the stock solutions of P1 and 33-mer were mixed with *P1 and *33-mer in molar ratios *n*(peptide)/*n*(*standard peptide) between 1.0 and 9.1 (1 + 1, 1 + 4, 1 + 9, 4 + 1, 9 + 1), respectively. The calibration for P2 and P3 was performed based on *P1 as reported previously by Schalk et al. (2018), since no labelled standards were available. Therefore, different amounts of P2 and P3 stock solution (0 µL, 50 µL, 100 µL, 250 µL, 500 µL, 1000 µL) were each added to a mixture of wheat samples 24, 38 and 51, which were pooled from three different harvest years. The obtained mixture was prepared with addition of *P1 as described above to create a response line based on standard addition. The quantitation was performed as matrix-matched calibration relative to the labeled *P1 standard peptide.

2.3.3. Targeted LC-MS/MS

The following system was used: UltiMate 3000 HPLC system (Dionex, Idstein, Germany) coupled to a triple-stage quadrupole mass spectrometer (TSQ Vantage, Thermo Fisher Scientific, Dreieich, Germany). A XBridge Peptide BEH-C_{18} column (1.0 × 150 mm, 3.5 µm, 13 nm; Waters, Eschborn, Germany) was used for peptide separation with the following conditions: solvent A, FA (0.1%, v/v) in water, solvent B, FA (0.1%, v/v) in acetonitrile; gradient 0–5 min isocratic 5% B, 5–22 min linear 5–55% B, 25–30 min isocratic 90% B; 30–35 min linear 90–5% B, 35–45 min isocratic 5% B; flow rate, 0.1 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. A declustering voltage of −10 V was set for all transitions.

2.4. Quantitation of the gliadin/gluten content with ELISA

All ELISA measurements were carried out in a separate, closed room where the surfaces and equipment had been cleaned with 60% (v/v) ethanol to prevent gluten contamination. The gliadin/gluten contents of the 60 wheat cultivars were measured by two commercially available ELISA test kits: R5 RIDASCREEN® Gliadin (R-Biopharm, Darmstadt, Germany) and G12 AgraQuant® Gluten (Romer Labs, Tulln, Austria). Wheat flours (0.025 g) were incubated with 2.5 mL Cocktail solution (R5) or 2.5 mL extraction solution (G12) for 40 min at 50 °C, followed by shaking with 7.5 mL ethanol (80%, v/v in water) for 1 h at 20 °C. The samples were diluted 1:500 (R5) and 1:1000 (G12) with ethanol (60%, v/v in water) and the last dilution step was performed with the sample dilution buffer provided by the manufacturers (1:12.5 for R5, 1:10 for G12). All extractions were carried out in triplicate. The following ELISA procedures were performed according to the manufacturers' protocols. The absorbance was measured at 450 nm with a microplate reader (Expert 96 microplate reader, Asys Hitech, Eugendorf, Austria). Calibration curves with the respective standard provided in each test kit were created using the cubic spline function of the Rida® Soft Win Software (R-Biopharm).

2.5. Characterization of the protein fractions and types with ELISA

2.5.1. Sample preparation

To obtain protein fractions, the 16 propagated wheat samples were used. Each wheat flour (120 g) was defatted twice with 250 mL pentane/ethanol (95/5, v/v) for 30 min, followed by stirring once with 250 mL pentane. The suspensions were centrifuged (4600*g*, 20 min, 22 °C) and the solvent was discarded. After vacuum-drying overnight, the defatted flours (100 g) were extracted four times each with 200 mL buffered salt solution (0.067 mol/L K_2HPO_4/KH_2PO_4-buffer, 0.4 mol/L NaCl, pH = 7.6) by homogenizing with an Ultra Turrax blender (16 000 rpm, IKA-Werke, Staufen, Germany) for 5 min at 22 °C and centrifuged (4600*g*, 20 min, 22 °C). The supernatants of the obtained albumin/globulin fraction were discarded. The flour residues were extracted three times each with 200 mL 60% (v/v) ethanol as described for the albumin/globulin fraction to obtain the gliadin fraction. Then, the residues were extracted three times each with 200 mL of the glutenin extraction solution (50% (v/v) 1-propanol, 0.1 mol/L TRIS-HCl, pH 7.5, 0.06 mol/l (w/v) dithiothreitol) by homogenizing with an Ultra Turrax blender for 5 min under nitrogen and stirring for 30 min at 60 °C, cooling and centrifugation (4600*g*, 20 min, 22 °C). The supernatants of the gliadin and glutenin fractions were each concentrated under reduced pressure, dialyzed (molecular weight cut-off: 12,000–14,000, Medicell Membranes, London, UK) and lyophilized.

To obtain gliadin and glutenin types, preparative RP-HPLC was used according to Schalk, Lexhaller, Koehler & Scherf (2017). The lyophilized protein fractions and types were dissolved in the glutenin extraction solution (1 mg/mL), filtered (0.45 µm) and used for the ELISA experiments. They were also simultaneously analyzed by analytical RP-HPLC to determine the exact protein concentration, as reported by Lexhaller, Tompos & Scherf (2017). The stock solutions were diluted with 60% (v/v) ethanol to yield five serial dilutions each, so that the absorbance lay within the respective calibration ranges of the two

ELISA test kits (5–80 ng/ml for R5, 10–500 ng/ml for G12). The last dilution step was carried out with the sample dilution buffer provided in each test kit (1:12.5 for R5, 1:10 for G12). Each dilution was applied to two cavities of the 96-well plate (n = 4). The following ELISA procedures were performed as described above.

2.5.2. Analytical RP-HPLC

The following system was used: Jasco XLC system (Jasco, Gross-Umstadt, Germany) with Chrom Pass software; column, Acclaim™ 300 C_{18} (particle size 3 µm, pore size 30 nm, 2.1 × 150 mm, Thermo Fisher Scientific, Braunschweig, Germany); elution solvents, TFA (0.1%, v/v) in water (A) and TFA (0.1%, v/v) in acetonitrile (B); linear gradient: 0 min 0% B, 0.5 min 24% B, 20 min 56% B, 20.1–24.1 min 90% B, 24.2–30 min 0% B; flow rate 0.2 mL/min; temperature, 60 °C; injection volume, 40 µL for gliadins and 40 µL for glutenins, 40 µL for ω5- and ω1,2-gliadins, 10 µL for α- and γ-gliadins; detection, UV absorbance at 210 nm. Prolamin Working Group (PWG)-gliadin (2.5 mg/mL in 60% ethanol) was used as a calibration reference for the calculation of the protein contents (van Eckert et al., 2006).

2.6. Statistical analysis

One-way analysis of variance (ANOVA) with Tukey's test at a significance level of $p < 0.05$ using SigmaPlot 12.0 (Systat Software, San José, CA, USA) was used to determine statistically significant differences between the contents of 33-mer, P1, P2 and P3 of the five wheat cultivars per decade. The significant differences between the contents of P1 and 33-mer in all cultivars and the harvest years (2015, 2016, 2017) were analyzed by two-way ANOVA with harvest year and cultivar as factors. Principle component analysis (PCA) was performed with XLSTAT 2016 (Addinsoft, New York, NY, USA) to analyze whether the contents of the CD-active peptides, α-gliadins, γ-gliadins and gliadins can be used to differentiate between old and modern wheat cultivars. Pearson's product moment correlations were calculated between RP-HPLC contents of gliadins and the corresponding contents analyzed by R5 and G12 ELISA. Since the interpretation of correlation coefficients (r) varies significantly among research areas and there are no absolute rules to interpret their strength (Akoglu, 2018), we defined them according to Thanhaeuser, Wieser & Koehler (2014) ($r > 0.78$, strong correlation; 0.67–0.78, medium correlation; 0.54–0.66, weak correlation; $r < 0.54$, no correlation). The mean concentrations determined by RP-HPLC (n = 3) plotted against the corresponding mean ELISA absorbances (n = 4), while taking the five serial dilutions into account, were applied to create quadratic fits ($f = y0 + ax + bx^2$).

Next, the 16 curves of the gliadin fraction and also gliadin types were taken together for dynamic fitting to obtain the overall best fit and also 95% confidence bands and 95% prediction bands, as in Schopf & Scherf (2018).

3. Results and discussion

The contents of the CD-active 33-mer, P1, P2 and P3 peptides in the 60 wheat cultivars from 1891 to 2010 from three different harvest years (2015–2017) are presented in Supplementary Tables S2 and S3. The contents of crude protein and gluten proteins (ω5-, ω1,2-, α- and γ-gliadins, HMW-GS and LMW-GS) were already reported for the whole sample set (Pronin et al., 2020).

3.1. Determination of the 33-mer peptide in the 60 wheat cultivars

The proportions of 33-mer were calculated based on the contents of α-gliadins for each particular cultivar (Fig. 1), but also as absolute values and proportions based on the contents of crude protein (Supplementary Figs. S1 and S2). The 33-mer was present in all cultivars and showed a range from 2.7% (sample 50) to 0.6% (sample 10) considering the average over all three harvest years (Fig. 1D). Concerning each harvest year, the values ranged between 2.7% (sample 50) and 0.6% (sample 31) in 2015 (Fig. 1A), between 3.4% (sample 50) and 0.5% (sample 10) in 2016 (Fig. 1B) and between 2.3% (sample 60) and 0.5% (sample 10) in 2017 (Fig. 1C). The five cultivars per decade showed comparatively low variability in some decades, e.g., the 1920s and 1960s, but also higher variability, e.g., in the 1940s and 1950s. Considering the absolute values based on flour weight and the proportions relative to crude protein contents for the 33-mer averaged over three years, we observed no clear change. When looking at the 33-mer proportions based on α-gliadin contents, there was no clear trend in 2015, but a slight increasing trend in the year 2016, because there was a significant increase between the old (1901–1930) and modern cultivars (1981–1990, 2001–2010), but also already to samples from the 1930s. In 2017, there was a significant difference between old (1891–1930) and the most modern cultivars (2001–2010). The average values of all three years showed significantly lower 33-mer proportions in the old cultivars (1901–1930) compared to the most modern ones (2001–2010). However, due to large variability within the five samples per decade, there was no significant difference between the oldest (1891–1900) and the most modern cultivars (2001–2010). This result can be explained by the large influence of environmental factors, e.g., the harvest year, on 33-mer contents, as has been reported earlier (Schalk et al., 2017). We also observed no correlation between the contents of 33-mer and α-gliadins ($r = 0.41$, $p < 0.001$).

3.2. Determination of CD-active peptides P1, P2 and P3 in the 60 wheat cultivars

The results for P1 based on α-gliadin contents for each harvest year, the absolute contents and the proportions based on crude protein contents are presented in Supplementary Figs. S3–S5. The absolute values and the proportions based on the crude protein contents for P2 and P3 are available in Supplementary Figs. S6 and S7. P1 was present in 59 out of 60 cultivars, with the exception of sample 12 (Fig. 2A). Apart from this sample, the proportions of P1 based on α-gliadins averaged over all three harvest years were between 0.6% (sample 52) and 0.3% (sample 31), with a comparatively narrow range. The proportions of P1 based on α-gliadins showed an increasing trend for the average over the three harvest years with significant differences between the samples from 1891 to 1940 and also from 1951 to 1970 to those from 1971 to 2010. Concerning the absolute contents of P1, no apparent trend was identified, because the overall protein contents decreased from 1891 to 2010. As already observed for the 33-mer, the contents of P1 were not correlated to the α-gliadin contents ($r = 0.45$, $p < 0.001$).

No significant trends were detected for the CD-active peptides P2 and P3 over the past 120 years (Fig. 2B, C). Compared to P1, the variability of the proportions of P2 and P3 was higher within the five cultivars per decade, even if all cultivars contained both P2 and P3. In contrast to the 33-mer and P1, correlations were observed between the contents of P3 and γ-gliadins ($r = 0.79$, $p < 0.001$) and between the contents of P2 and α-gliadins ($r = 0.75$, $p < 0.001$).

All investigated common wheat cultivars contained all four CD-active peptides investigated, except sample 12, that did not contain P1. The contents of the peptides varied across the 120 years of breeding and also within the five cultivars per decade, regardless of the harvest year. Further, we showed that two CD-active peptides (33-mer and P1) did not change over the past 120 years in terms of their absolute contents in the flours, but their proportions in α-gliadins increased. The increasing trend of the proportions in α-gliadins is in agreement with van den Broeck et al. (2010) and van den Broeck et al. (2015), who reported that modern cultivars tend to contain more CD-active peptides compared to old cultivars. However, these studies were limited either to the use of ELISA or to only three wheat cultivars, respectively. Malalgoda et al. (2018) reported a high variability of CD-active peptides in wheat cultivars without a clear trend from old to modern cultivars, which is in

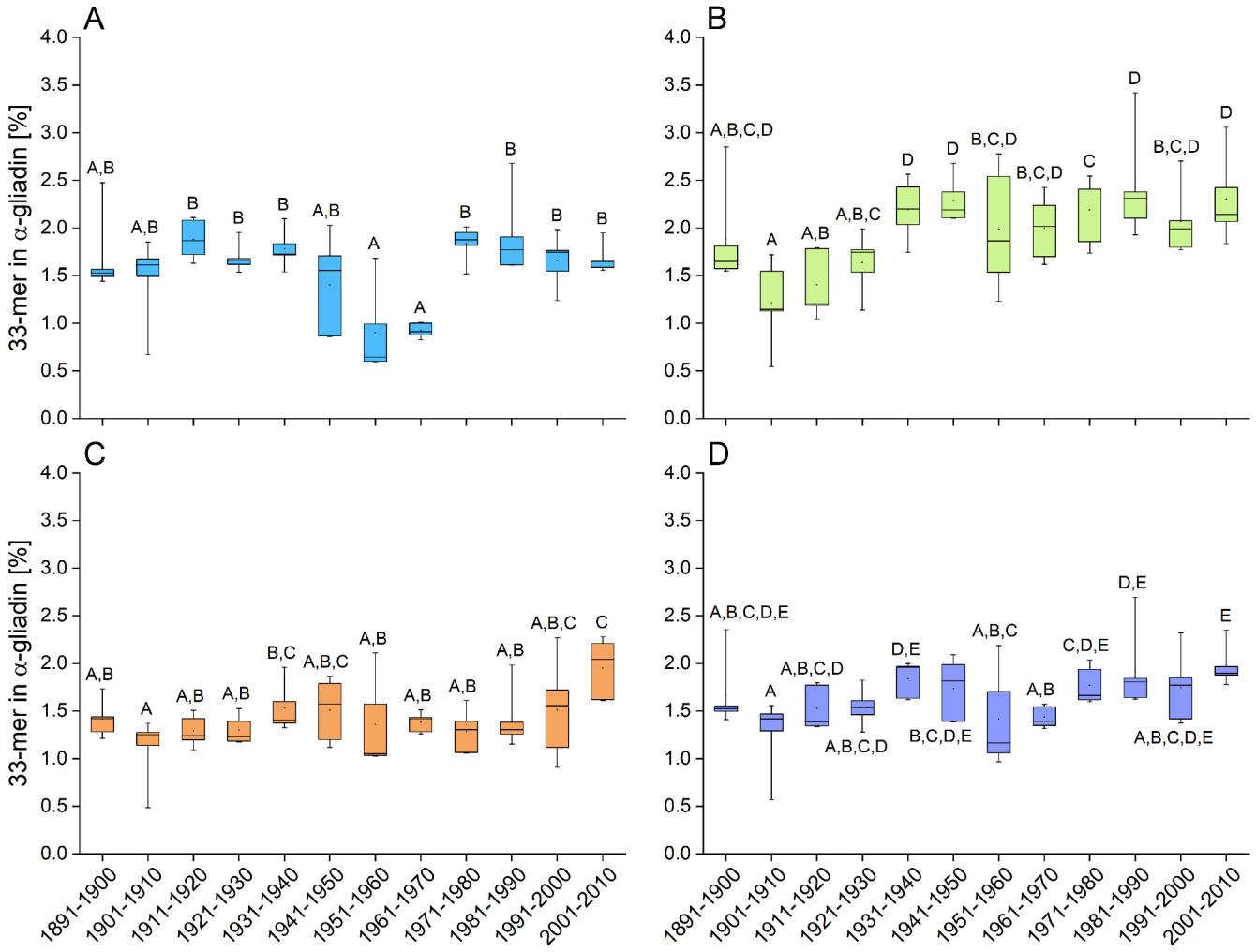

Fig. 1. Proportions [%] of 33-mer based on α-gliadin contents in five cultivars per decade of the harvest years 2015 (A), 2016 (B), 2017 (C), and averaged over three harvest years 2015–2017 (D). Boxes represent the interquartile range with the median (line in the box) and mean (point in the box) and whiskers represent the minima and maxima. Different capital letters designate significant differences between the decades (one-way ANOVA, Tukey's test, $p < 0.05$).

agreement with our findings for P2 and P3. Given the fact that α-gliadin is considered the most immunoreactive fraction, the results of our study suggest that α-gliadin may not be associated with the amount of CD-active peptides. Another study by Boukid et al. (2017) on 100 Tunisian durum wheat cultivars of the 20th century found significant differences between the amounts of immunogenic peptides between abandoned and modern cultivars but did not find increased amounts in modern durum lines. In contrast to our findings, Prandi et al. (2017) reported that old Italian common cultivars had higher contents of CD-active peptides compared to modern ones.

3.3. Influence of cultivar and harvest year on the contents of 33-mer and P1

Two-way ANOVA to determine the effect of different cultivars (G variability) and different harvest years (E variability) on the contents of the 33-mer and P1 revealed a statistically significant interaction between the effects of cultivar and harvest year (G × E, (F [118, 360] = 108.2 and 7.6, respectively, $p < 0.001$)). Additionally, the effect of the harvest year for P1 was greater for old cultivars (1–30) (F [2, 180] = 3678.3, $p < 0.001$), which implies that modern cultivars (31–60) (F [2, 180] = 829.8, $p < 0.001$) are less sensitive to environmental changes. No substantial difference was observed for the 33-mer, thus old and modern cultivars were affected equally (F [2, 180] = 1258.9 and 1688.8, for old and modern cultivars respectively, $p < 0.001$).

Due to the significantly higher variance between the harvest years (F [2, 360] = 1959 and 3661.5, for 33-mer and P1 respectively, $p < 0.001$) compared to the variance within one particular harvest year, the harvest year had the greatest influence on the peptide contents. The effect of the cultivar on the peptide contents was smaller compared to that of the harvest year (F [59, 360] = 190 and 154.5, for 33-mer and P1 respectively, $p < 0.001$).

These findings are further corroborated by the fact that different cultivars had the highest/lowest contents for each harvest year. Regarding the 33-mer, the minima (86.0 μg/g, 117.6 μg/g, 79.6 μg/g) were observed for samples 34, 10 and 10 for the harvest years 2015, 2016 and 2017, respectively, whereas the maxima were found for samples 8 (541.9 μg/g), 50 (634.6 μg/g) and 54 (445.3 μg/g). Interestingly, the maxima of P1 were detected for the same samples as for the 33-mer. We observed maximal values of 235.4 μg/g (sample 8), 197.9 μg/g (sample 50) and 188.7 μg/g (sample 54) and minimal values for sample 34 (84.8 μg/g), sample 57 (71.1 μg/g) and sample 51 (55.4 μg/g) for the harvest years 2015, 2016 and 2017, respectively.

Our results are in agreement with Schalk et al. (2017), who demonstrated a significant influence of the harvest year on the contents of the 33-mer, based on the analysis of four wheat cultivars from three different harvest years. Prandi et al. (2014) on the other hand, found that the genotype affected the contents of the DQ2.5-glia-α1a peptide in durum wheat species more than environmental factors. Boukid et al. (2017) showed that modern genotypes appear to be less affected by

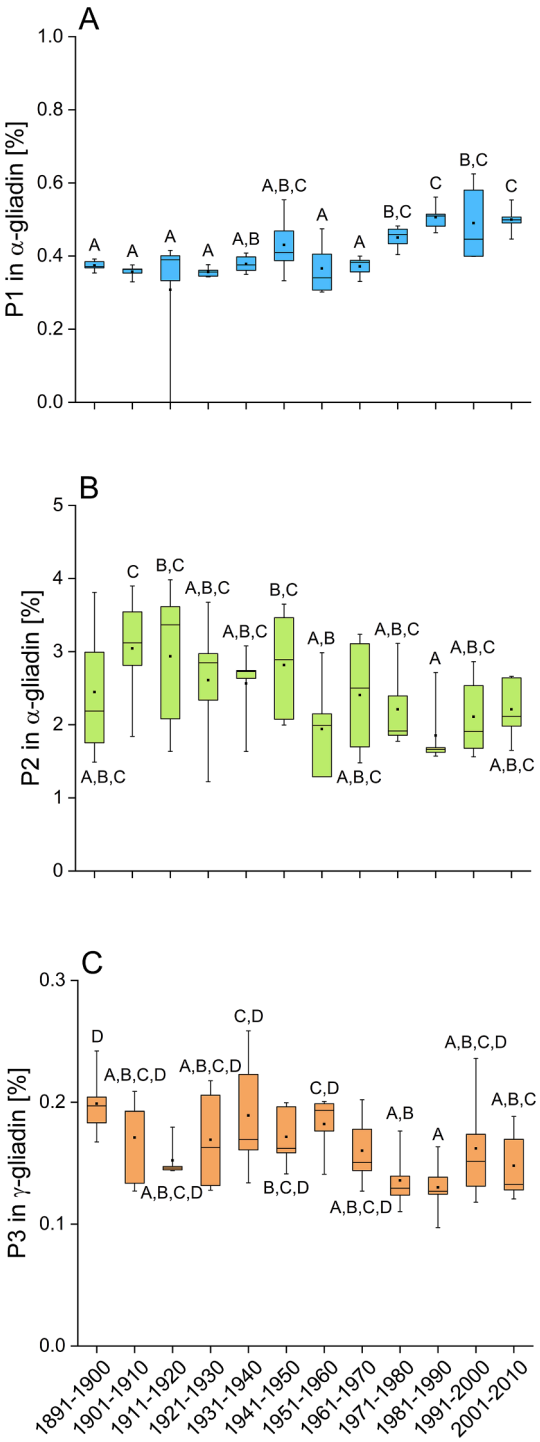

Fig. 2. Proportions [%] of P1 based on α-gliadins (A), of *P*2 based on α-gliadins (B) and of *P*3 based on γ-gliadins in five cultivars per decade (C) averaged over three harvest years 2015–2017. Boxes represent the interquartile range with the median (line in the box) and mean (point in the box) and whiskers represent the minima and maxima. Different capital letters designate significant differences between the decades (one-way ANOVA, Tukey's test, $p < 0.05$).

environmental changes and this is in line with our results for P1 in the present study. The same research group further identified a greater impact of the cultivar on the content of CD-active peptides in Tunisian durum wheat cultivars, which is in contrast to our results. However, these discrepancies in the findings may be caused by different environmental conditions and the fact that we studied common wheat, but no durum wheat cultivars.

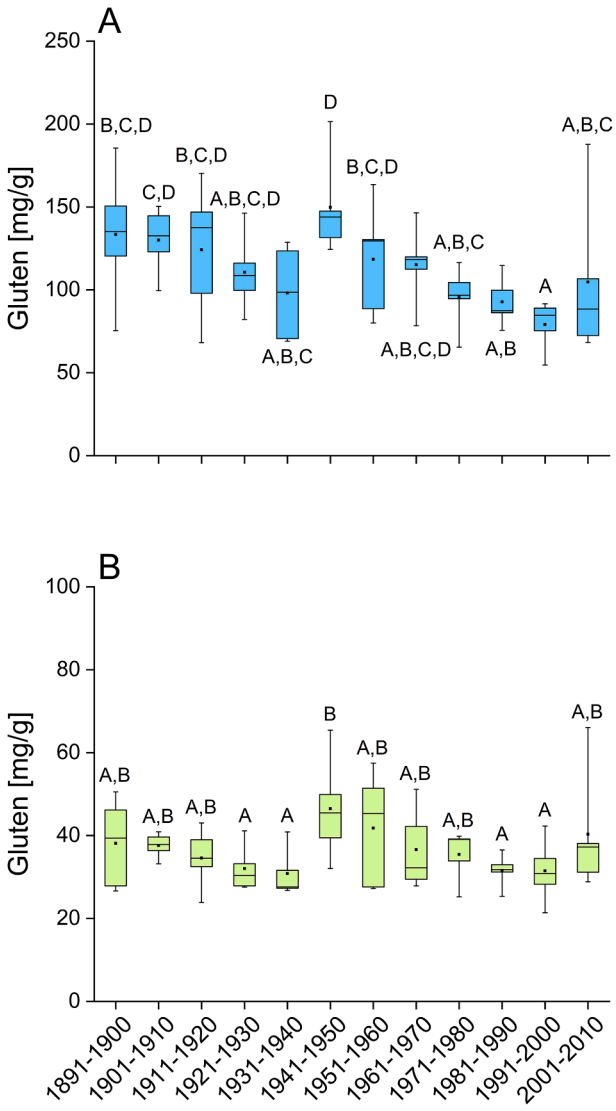

Fig. 3. Gluten contents determined by R5 (A) and G12 (B) ELISA test kits in five cultivars per decade as an average over three harvest years 2015–2017. Boxes represent the interquartile range with the median (line in the box) and mean (point in the box) and whiskers represent the minima and maxima. Different capital letters designate significant differences between the decades (one-way ANOVA, Tukey's test, $p < 0.05$).

3.4. Reactivity of common wheat cultivars with ELISA test kits

The gliadin and gluten contents of the 60 common wheat flours from three different harvest years were analyzed with two different sandwich ELISA test kits, namely R5 and G12 (Supplementary Table S4). The antibodies used in these test kits are specific for certain CD-active peptide motifs. The R5 mAb recognizes the pentapeptides QQPFP and QLPFP present in ω1,2-, γ- and α-gliadins, whereas the G12 test kit recognizes QPQLPY and QPQLPF sequences, that occur in α-gliadins (Morón, Bethune et al., 2008; Morón, Cebolla et al., 2008). Usually, the R5 test kit reports the results as gliadin concentrations and the G12 test kit as gluten concentrations. In order to allow a comparison between both test kits, a conversion factor of 2 (2 × gliadins = gluten) is used, even though this procedure has been questioned (Scherf, 2016; Wieser & Koehler, 2009). This is why we compared results by multiplying the R5 gliadin concentrations with the factor 2 and also with the actual gliadin-to-glutenin ratios (Pronin et al., 2020) and correlation of either value with the gluten contents determined by RP-HPLC. We observed medium correlations for both scenarios ($r = 0.745$ and $r = 0.685$,

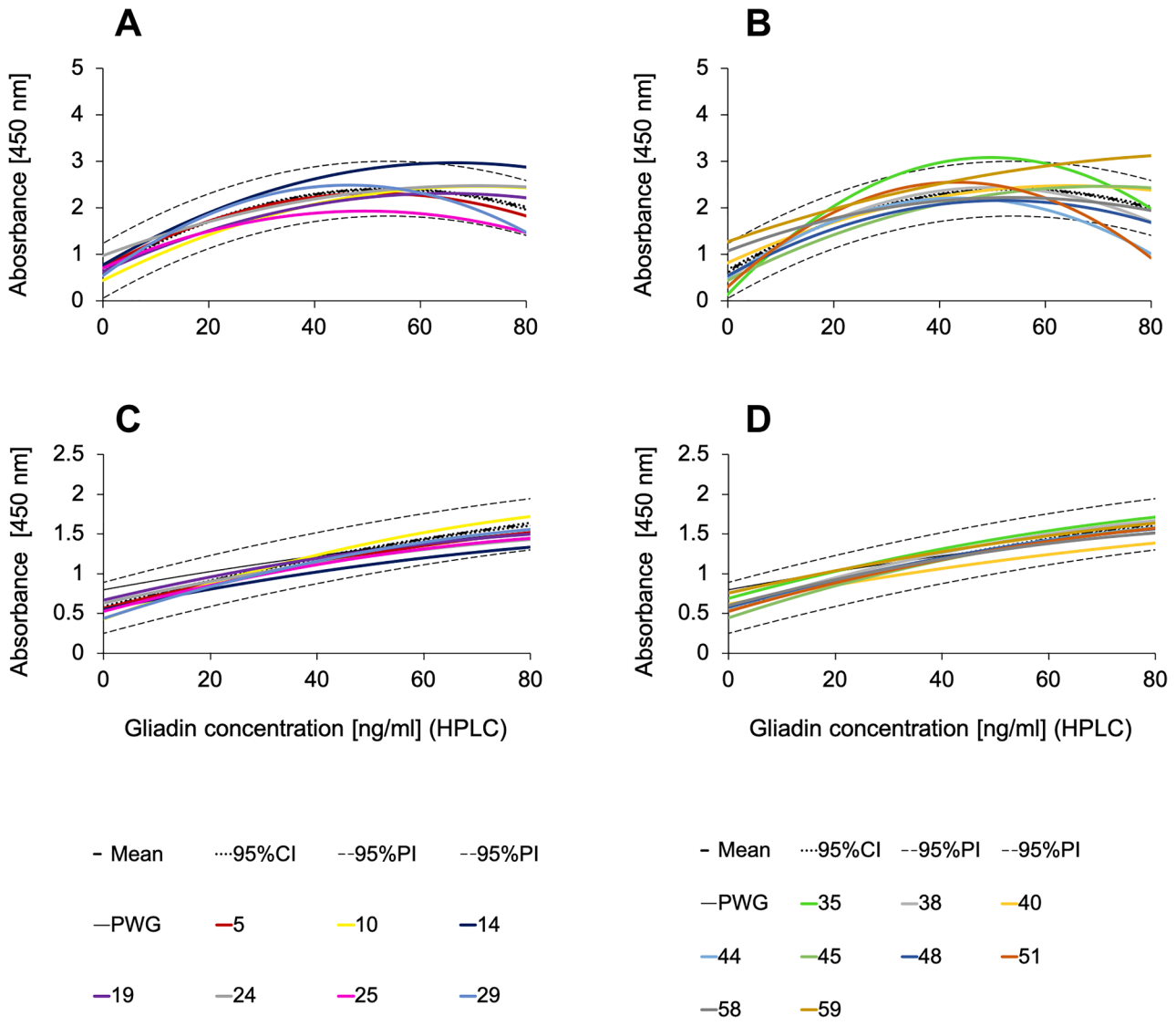

Fig. 4. Array of quadratic fits for ELISA absorbances (λ = 450 nm) as a function of the concentrations quantitated by RP-HPLC including overall mean, 95% confidence bands (CI) and 95% prediction bands (PI) as well as the PWG-gliadin reference material. Gliadin fractions from old wheat cultivars 5, 10, 14, 19, 24, 25 and 29 by R5 ELISA (A), from modern wheat cultivars 35, 38, 40, 44, 45, 48, 51, 58 and 59 by R5 ELISA (B), from the same old wheat cultivars by G12 ELISA (C), and from the same modern wheat cultivars by G12 ELISA (D).

respectively, $p < 0.001$), but the conversion factor 2 had a higher correlation coefficient (Supplementary Fig. S8).

Correlation analyses revealed no correlation between gluten contents determined by R5 ELISA and ω5-gliadin contents ($r = 0.47$, $p < 0.001$), but a strong correlation with ω1,2-gliadins ($r = 0.824$, $p < 0.001$) and medium correlations with α- and γ-gliadins ($r = 0.667$ and $r = 0.685$, respectively, $p < 0.001$). Concerning the G12 ELISA, the gluten contents were strongly correlated with the ω1,2-gliadins ($r = 0.80$, $p < 0.001$), weakly correlated with α-gliadins ($r = 0.660$ and $r = 0.548$, respectively, $p < 0.001$), but not with the ω5- and γ-gliadins ($r = 0.48$ and $r = 0.44$, respectively, $p < 0.001$). Interestingly, no correlation was observed between the gluten contents determined by G12 ELISA and the contents of 33-mer, although this mAb was raised against the 33-mer peptide ($r = 0.47$, $p < 0.001$)

In contrast to the R5 test kit, the G12 test kit showed significantly lower gluten contents, because of different gluten protein extraction procedures and characteristics towards detectable peptide motifs. As expected, both test kits showed an overestimation of the gluten contents (Hajas et al., 2017). The absolute values for both test kits are presented in Fig. 3 as an average over three harvest years and additionally for each harvest year in Supplementary Figs. S9 and S10. For both test kits, the median values were highly variable over the 120 years and also within the decades. Considering the average of the three harvest years, we found no clear trend in gluten contents irrespective of the test kit used and this is in agreement with our previous findings using RP-HPLC (Pronin et al., 2020).

3.5. Reactivity of gliadin protein fractions and protein types between ELISA test kits

To investigate the differences between gliadin protein fractions of 16 selected wheat cultivars, the total gliadin concentrations determined by RP-HPLC were plotted against the ELISA absorbances of the five serial dilutions (Fig. 4). In the specified range, the R5 test kit showed higher absorption values overall compared to the G12 test kit. One modern cultivar (sample 59) showed the highest response to the R5 mAb due to a linear increase after a strong response at low concentrations. The old (sample 14) and the modern (sample 35) cultivars showed weak R5 ELISA responses for small concentrations, but had significantly higher absorptions at their maxima, especially compared

to the old cultivar (sample 25). However, the differences in the overall absorption values between old and modern cultivars were not significant and subsequently no clear distinction could be made.

The G12 test kit did not allow any distinctions between the old and modern gliadin fractions isolated from 16 common wheat cultivars, because the absorption values for all cultivars were nearly equal. These results are in accordance with previous studies performed by Schopf et al. (2018), who did not find any tendencies for R5 or G12. We performed the same experiments for isolated ω5-, ω1,2-, α- and γ-gliadins from one old (sample 10) and one modern cultivar (sample 51), but essentially found no differences between the old and modern cultivars when looking at the same gliadin type (data not shown). Further work could focus on the relations between the presence of CD epitope sequences in the genetic information, their expression levels based on quantitative PCR analysis of cDNA and the final protein/peptide levels (Dubois, Bertin, Hautier, Muhovski, Escarnot & Mingeot, 2018).

4. Conclusions

To test the hypothesis that wheat breeding may have contributed to increasing the immunostimulatory potential of modern wheats compared to old wheats and landraces, we quantitated four CD-active peptides by LC-MS/MS and gluten contents by ELISA in a well-characterized set of 60 German common winter wheat cultivars from 1891 to 2010. We found no clear trend for gluten contents over the 120-year period studied. Regarding the CD-active peptides, we demonstrated a high variability of the contents of immunogenic peptides, both over 60 different cultivars and within the five cultivars per decade, primarily affected by the harvest year and less by the cultivar. The 33-mer and P1 peptides showed a tendency to increase over breeding, but only when looking at the proportions of peptides relative to the respective protein type, the α-gliadins, not when looking at the absolute contents. No trends in relative or absolute values were observed for the P2 and P3 peptides present in α- and γ-gliadins. Taken together, our results suggest that the contents of some CD-active peptides may have changed over the course of breeding, but that the effect of different harvest years is much more important compared to the genetic effect, because we identified both old and modern cultivars containing high and low contents of CD-active peptides, respectively.

CRediT authorship contribution statement

Darina Pronin: Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing - original draft. **Andreas Börner:** Conceptualization, Investigation, Project administration, Resources, Writing - review & editing. **Katharina Anne Scherf:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.127952.

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4 General discussion

In this study the sample set consisted of the five most widely grown German hexaploid winter wheat cultivars per decade from 1891 to 2010. The cultivars were selected and grown in Gatersleben, Germany, in three different harvest years: 2015, 2016 and 2017. Altogether, a total of 180 samples (60 cultivars * 3 harvest years) were available. The growing of the cultivars under the same agronomic conditions and at the same location, had the advantage of a consistent comparability between the cultivars based on their genetic background and also provided information about the influence of genotypes and environmental conditions on the protein content and composition. The environmental factors had the highest impact (75%) on the variation of the protein content and composition, followed by genetic factors (25%) and the interaction between the two factors [1]. Based on the large data set generated by the wide range of samples, the requirements for the differentiation of genotypes were accomplished [2].
The goals of this work were divided into three parts, which were achieved stepwise:
1) the extraction of the protein fractions albumins/globulins, gliadins and glutenins from 60 common wheat cultivars according to the modified Osborne fractionation and their characterization by means of RP-HPLC; 2) the development of a novel integration approach of RP-HPLC patterns, in order to allow a better characterization of the cultivar-specific fingerprints; 3) the quantitative determination of four CD-active peptides, including the 33-mer peptide, in all wheat flours by SIDA based on targeted LC-MS/MS.

Table 4.1: Overview of the 60 German winter wheat cultivars analyzed

Sample	Decade	Wheat cultivar
1	1891-1900	Rimpaus Früher Bastard
2	1891-1900	Rimpaus Dickkopf
3	1891-1900	Strubes Dickkopf
4	1891-1900	Cimbals Großherzog von Sachsen
5	1891-1900	Steigers Leutewitzer Dickkopf
6	1901-1910	Breustedts Extra Dickkopf
7	1901-1910	Janetzkis Früher Kreuzung
8	1901-1910	Kraffts Siegerländer
9	1901-1910	Ruppiner Brauner Landweizen
10	1901-1910	Ackermanns Brauner Dickkopf
11	1911-1920	Lembkes Obotriten Weizen
12	1911-1920	Heinrichs von Hindenburg
13	1911-1920	Friedrichswerther Berggoldweizen
14	1911-1920	Stadlers Brauner Dickkopf
15	1911-1920	Strubes General von Stöcken

Sample	Decade	Wheat cultivar
16	1921-1930	Mettes Quedlinburger Schloss
17	1921-1930	Passendorfer Goldweizen
18	1921-1930	Heges Basalt
19	1921-1930	Salzmünder Ella
20	1921-1930	Ackermanns Jubel
21	1931-1940	Heines II
22	1931-1940	Poernbacher Graf Toerring
23	1931-1940	Grundmanns Wotan
24	1931-1940	Nordost Sandomir
25	1931-1940	Rimpaus Bastard II
26	1941-1950	Dippes Strum Weizen
27	1941-1950	Firlbeck I
28	1941-1950	Strengs Marschall
29	1941-1950	Erbachshofer Braun
30	1941-1950	Walz Oberrheinperle
31	1951-1960	Heines VII
32	1951-1960	Carstacht
33	1951-1960	Fanal
34	1951-1960	Merlin
35	1951-1960	Hadmerslebener Qualitas
36	1961-1970	Jubilar
37	1961-1970	Format
38	1961-1970	Konsul
39	1961-1970	Poros
40	1961-1970	Pilot
41	1971-1980	Caribo
42	1971-1980	Diplomat
43	1971-1980	Kormoran
44	1971-1980	Alcedo
45	1971-1980	Vuka
46	1981-1990	Kanzler
47	1981-1990	Ares
48	1981-1990	Rektor
49	1981-1990	Okapi
50	1981-1990	Miras
51	1991-2000	Ritmo
52	1991-2000	Zentos
53	1991-2000	Astron
54	1991-2000	Borenos
55	1991-2000	Orestis
56	2001-2010	Akteur
57	2001-2010	Cubus
58	2001-2010	Dekan
59	2001-2010	Drifter
60	2001-2010	Tommi

4.1 Wheat (Triticum aestivum L.) breeding from 1891 to 2010 contributed to increasing yield and glutenin contents but decreasing protein and gliadin contents

4.1.1 Agronomic traits

The grain yield is the main breeding goal, which increased with the introduction of new cultivars with higher resistance towards environmental stress factors and possible diseases, mostly due to their decreased height. The grain yield and the plant height of the 60 wheat cultivars were analyzed for three harvest years. A decline of about 40% of the plant height from old to modern cultivars was determined, since old cultivars

showed heights between 117 cm and 153 cm, while modern cultivars were significantly shorter, with heights between 76 cm and 99 cm. Subsequently, this trend resulted in a significant increase of the grain yield from 31 dt/ha up to 49 dt/ha, which corresponds to an increase of about 60%. As expected, the plant height decreased, while the grain yield increased significantly over the past 120 years [3]. These findings were supported by the fact that the plant height was inversely correlated with the grain yield (r = -0.87).

4.1.2 Protein content and composition

In addition to standard determinations (e.g. water, ash and crude protein), the proteins of all 60 wheat cultivars available from three harvest years each were extracted from the wholemeal flours according to the modified Osborne fractionation based on their solubility. The contents of the resulting fractions albumins/globulins, gliadins and glutenins, as well as the protein types and subunits, were determined by RP-HPLC.

The study showed that the cultivars had variable crude protein contents, with an average protein content of less than 10%. Generally, the amount and splitting of fertilization influences the grain protein content but this effect was avoided, because the cultivars were not fertilized [4]. The average over three years showed protein contents between 11.7% and 7.1% and tended to decrease slightly from 1891 to 2020. The decreasing tendency was also reported in studies from Germany [5], the UK [6] and the US [7], however increasing protein contents were observed in a study from Canada [8]. Further studies did not find any changes in the protein contents [9,10]. Since wheat is composed of many individual proteins, these must be used for the evaluation of the grain protein development and alteration over the past 12 decades.

This study confirmed changes in the protein distribution based on the trends of the median of five cultivars per decade, which showed a decreasing trend for gliadins and a simultaneous increase of glutenins and consequently no changes for the gluten contents. Previous studies already supported the hypothesis that the protein composition may have changed due to breeding and observed a decreasing tendency for gliadins [11, 12] and no changes in gluten contents [13]. The percentages of albumins/globulins in the total RP-HPLC content averaged over three years ranged between 25.5% and 16.9% and did not reveal a clear trend over time. The proportions of gliadins ranged between 62.0% and 45.7% and those for glutenins between 33.1% and 17.1%. It has to be noted that the contents of crude protein and protein types were

variable over all 60 cultivars and also within the five cultivars per decade irrespective of the harvest year. A strong negative correlation was determined between gliadin and glutenin proportions (r = -0.89), whereas no correlation was observed between the proportions of albumins/globulins to either of the other parameters. The decrease of gliadins and the simultaneous increase of glutenins led to a consistent gluten content and was also displayed in the gliadin/glutenin ratio, which decreased significantly with values ranging between 3.6 and 1.4. Varying ratios result in different bread properties, with the consequence that a surplus of glutenins tends to lead to higher bread volumes and a stronger gluten network [14]. No substantial changes were revealed for the proportions of ω5- and ω1,2-gliadins, with values ranging between 5.2% and 1.0% and from 6.4% to 3.1%, respectively. In contrast, we found a significant decreasing trend for α- and γ-gliadins with proportions between 30.3% and 19.7% and between 28.3% and 17.5%, respectively. Interestingly, the proportions of the glutenin subunits showed an increasing trend with the exact same values as the glutenin fraction. In contrast to our results, earlier findings suggested that ω5-gliadins decreased significantly, while α- and γ-gliadins remained unchanged in Italian durum wheat cultivars from the past century [12]. Concerning the glutenin subunits, a previous study reported increasing trends for both HMW-GS and LMW-GS [11] in line with our results, whereas another study observed an increasing trend for HMW-GS, but a decreasing trend for LMW-GS [7]. Essentially, we observed no correlation between gliadins and ω5- and ω1,2-gliadins. In contrast, α- and γ-gliadins revealed medium positive correlations with gliadins (r = 0.77 and r = 0.73, respectively) and additionally medium negative correlations with the glutenin proportions (r = -0.68 and r = -0.74). Furthermore, LMW-GS proportions correlated strongly negative (r = -0.90) and HMW-GS proportions medium negative (r = -0.73) with gliadins. The reason these findings are of importance is that both, gliadin types and glutenin subunits contain immunogenic peptides. While ω5-Gliadins are known for triggering wheat-dependent exercise-induced anaphylaxis (WDEIA) in sensitized individuals [15], α-gliadins are considered to be the most immunogenic protein type [16], because they contain the most immunoactive peptide, namely the 33-mer. The decreasing trend of the α-gliadins suggests that the amount of immunogenic peptides decreased as well, with the consequence that α-gliadin is not associated with the increased prevalence of CD and NCGS. This aspect is of special interest since the findings of previous studies are inconsistent, with one study reporting a correlation [17] between α-gliadins and immunogenic peptides and two studies

reporting no correlation [18, 19]. Our results show that wheat breeding over the past 120 years did not contribute to higher gluten or albumin/globulin contents, however breeding did contribute to an increase of glutenins and a decrease of gliadins and gliadin/glutenin ratios. Further, our findings confirmed the decreasing trends of α- and γ-gliadins reported earlier [11] but not the decreasing trend of ω-gliadins.

4.1.3 Influence of genotype and harvest year on the protein distribution

The determination of the protein fractions and types by means of RP-HPLC in the 60 wheat cultivars available from three different harvest years, allowed the analysis of the genotype (G), environment (E) and the G x E variability by means of a two-way ANOVA (Tukey's post hoc test, $p < 0.001$). Crude protein contents were significantly influenced by the harvest year ($F = 13778.0$). The cultivar had a lower impact ($F = 400.3$), followed by the interaction between both effects ($F = 280.3$). The harvest year was further the major determinant for the albumin/globulin and glutenin proportions ($F = 85.4$ and $F = 137.0$, respectively), because the variance between the harvest years was significantly higher than the variance within one particular harvest year. Interestingly, the proportions of gliadins were predominantly influenced by the cultivar ($F = 34.1$). Further, it was the harvest year, which had the highest impact on the proportions of ω1,2-, α-, and γ-gliadins and HMW-GS ($F = 1482.1$; $F = 219.8$; $F = 717.9$ and 1080.2). In contrast, ω5-gliadins and LMW-GS were more determined by the cultivar ($F = 321.6$ and $F = 78.6$). Our findings indicated that the variability in the protein composition is mainly affected by the harvest year, except for gliadins, ω5-gliadins and LMW-GS, which were more attributed to different cultivars. The harvest year as the major determinant for the protein composition was already reported in previous studies [20, 21].

4.2 Fingerprinting of wheat protein profiles for improved distinction between wheat cultivars and species

4.2.1 Differentiation of modern and old wheat cultivars by the common RP-HPLC integration method

For the distinction of the cultivars the protein fractions were integrated using the traditional way, namely the albumin/globulin fraction was not further subdivided, the gliadins were subdivided into ω5-, ω1,2-, α- and γ-gliadins and the glutenins into ωb-gliadins, HMW-GS and LMW-GS [22]. A principle component analysis (PCA) was applied to identify possible changes from old to modern cultivars. Very high correlation coefficients were determined between gliadin and glutenin contents (r = -0.885), but no correlation between albumin/globulin and gliadin or glutenin contents was identified. Although the cultivars somehow showed a cluster formation, there was a distinct overlap of the cultivars. Interestingly, due to comparatively high glutenin proportions three old cultivars, namely 20, 22 and 26 were located among modern cultivars. In contrast, due to comparatively high gliadin proportions the modern cultivars 31 and 44 were found among old cultivars. Further, showed less variability between one another, whereas old cultivars were distributed unevenly and thus displayed greater variability. However, this approach was not suitable for the differentiation between old and modern cultivars, mainly due to the loss of the information on individual peaks.

4.2.2 Development of a new integration approach for RP-HPLC chromatograms

Distinct changes in the peak patterns of the 60 wheat cultivars were observed for the protein fractions, yet these changes were not reflected in the analyzed contents [23].
The most noticeable differences were observed for the ω5- and ω1,2-gliadins, which are of great interest because they contain two CD-active peptides and moreover, ω5-gliadins are known to trigger WDEIA. To understand where the changes in the RP-HPLC profiles come from and also to extract more information from the profiles, a new method of peak integration was developed. Although there is information about the specific number of single proteins in the particular protein types [24], these numbers can change due to the effect of the genotype or environment [25]. The patterns were partitioned into sections of 20 s each for the new approach, which allowed to capture the individual peaks. Subsequently, 22 integration events were performed for albumins/globulins, 42 for gliadins and 46 for glutenins.

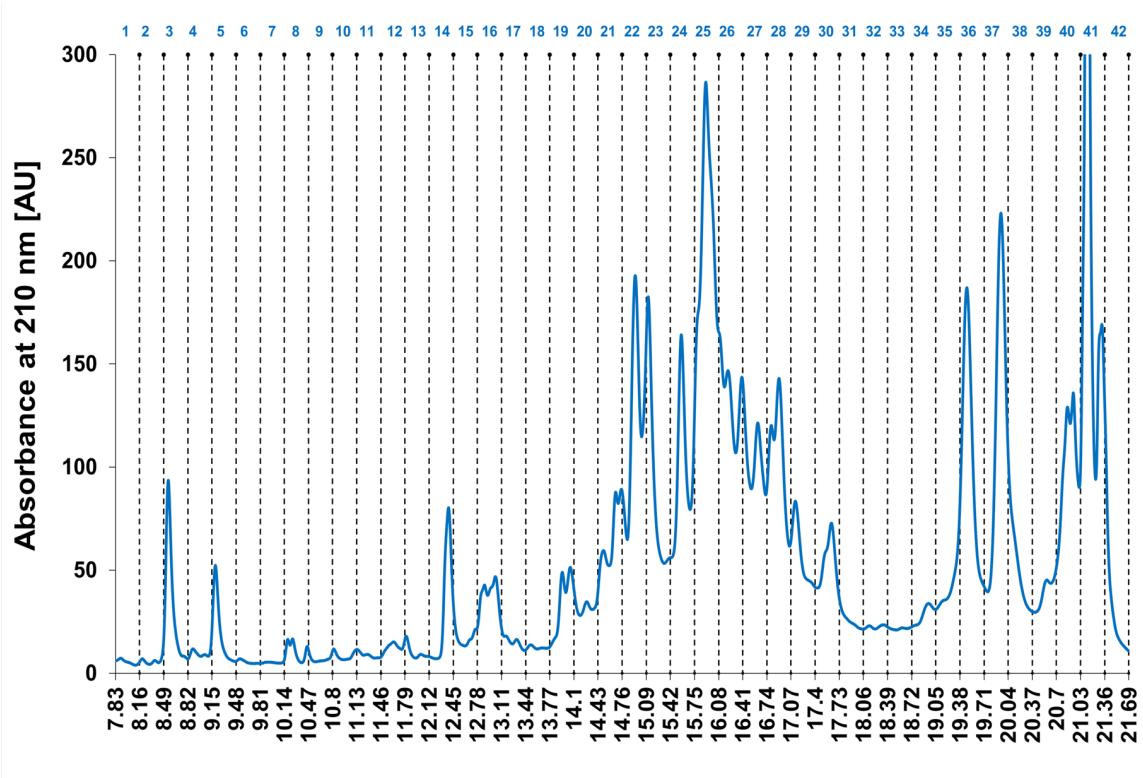

Figure 4.1: RP-HPLC pattern of the gliadin fraction of the sample 1 with the new integration approach. The integration events were carried out every 20 seconds with regard to the retention time, resulting in a total of 42 integration events.

4.2.3 Differentiation of modern and old wheat cultivars and additionally of different wheat species by the newly developed RP-HPLC integration method

To enable a better differentiation between wheat cultivars the results of the new integration approach were used to perform a PCA, which revealed three different and separated clusters. First cluster contained modern cultivars and was linked to glutenins, especially to the back parts of ω5- and ωb-gliadins, HMW-GS and LMW-GS, to the front part of albumins/globulins and the front part of γ-gliadins in terms of their RP-HPLC profiles. The second cluster consisted of old cultivars and of those which represented the transition from old to modern cultivars and was influenced by the middle part of albumins/globulins, the front parts of ω5- and α-gliadins and the back part of ω1,2-gliadins and also with ωb-gliadins, the front and middle parts of HMW-GS and the front part of LMW-GS. The third cluster was a mixed one, because it consisted of old and modern cultivars and was associated with the back parts of albumins/globulins, ω5-gliadins and α-gliadins and by the total of ω1,2- and γ-gliadins. The majority of the cultivars from one decade clustered together, with the exception of the cultivars, which were present in the mixed cluster, namely 35, 38, 40, 44, 51. In

addition, other wheat species, namely spelt, durum wheat, einkorn and emmer and common wheat cultivars from a different growing location were analyzed by the new method, in order to evaluate its applicability. In this context, common wheat and einkorn showed clear clusters, spelt and emmer were rather separated but with durum wheat samples in between. Essentially, this is due to the fact that the wheat species showed high variability within each species.

4.2.4 Elucidation of differences and similarities of the 60 wheat cultivars

Since gluten proteins have such a complex structure, it is of great importance to investigate their qualitative and quantitative composition and the corresponding protein fingerprints in detail to enhance the differentiation between different cultivars. Therefore, all integration events of the particular protein fraction of all wheat cultivars averaged over three harvest years were clustered by similarity using hierarchical clustering analysis.

Starting with the albumin/globulin fraction, the cultivars were classified into nine major groups, with similarities between the most modern cultivars (56-60) and the fairly modern ones (41-49) and also with the cultivars, which are part of the transition from old to modern (28,30,31). Although modern cultivars from one decade showed more similarities than old cultivars, all analyzed cultivars were rather distributed over the nine groups. However, it was possible to identify cultivars, which showed great disparities to other cultivars, e.g. 33, 35, 40, 44, 45, 51. Interestingly, these cultivars were always similar to each other, even correlated irrespective of the harvest year. Especially, cultivar 38 was always located among old cultivars with no correlation to all the other cultivars.

In contrast, the gliadin fraction showed a high correlation between the oldest cultivars (1-15) and the modern ones (36-60). Cultivars from one decade belonging to the old ones were rather located in the similar group, whereas the modern samples were distributed unevenly over the 10 identified groups for that fraction. However, cultivars 24 and 29 showed disparities to the other old cultivars. Concerning modern cultivars, the cultivars 33, 35, 38, 44, 51 and 59 were noticeable in terms of their protein composition.

10 groups were also determined for the glutenin fraction, in which old and modern samples were mostly correlating among themselves. However, some cultivars revealed major disparities, namely cultivars 33, 38, 44 and 59.

Overall, cultivars with unique peak distributions for all fractions were 33, 38 and 44.
Taken together, by means of the new integration techniques we gained a comprehensive insight into the distribution of the protein peaks, which indeed changed over the past 120 years. Further is was possible to emphasize the uniqueness of particular cultivars, which allowed us to classify those.
Our findings are of great interest, because only few studies focused on an in-depth characterization of protein profiles [26]. In fact, a recent study evaluated RP-HPLC peaks and observed changes in quality characteristics of gliadins over the past 100 years, but the methodology of the study did not show a cluster formation according to the release year of the cultivars [27].

4.3 Elucidation of the immunoreactivity of old and modern common wheat cultivars caused by breeding.

4.3.1 Quantitation of the immunodominant 33-mer peptide and peptides P1-P3 in old and modern wheat cultivars

The third part of the study should elucidate the changes of the immunoreactive potential of 60 wheat cultivars. Since gluten proteins are known to trigger adverse reactions in predisposed individuals because they contain CD-active peptides, it was important to determine whether the contents of these peptides have changed over the past 120 years due to breeding. Therefore, the contents of four CD-active peptides, including the one considered to be immunodominant, namely the 33-mer peptide, were analyzed by means of SIDA followed by targeted LC-MS/MS. While the 33-mer, P1 and P2 are present in α-gliadins, P3 is present in ɣ-gliadins.
For quantitation stable isotope labelled standards were required but not available for all peptides. This is why the peptides P2 and P3 were quantified based on the labelled *P1 standard. To identify possible changes in the contents of the 33-mer, P1, P2 and P3, their proportions were calculated as an average over three harvest years and based on the contents of α- or ɣ-gliadins, respectively.

First, the 33-mer was present in all cultivars with values ranging from 2.7% to 0.6% based on the contents of α-gliadins. The tendency from old to modern was rather increasing but due to the high variability within the five cultivars per decade no clear difference between the oldest (1891-1900) and the most modern cultivars (2001-2010) was determined. This is because the harvest year 2015 did not reveal a clear trend, whereas the harvest year 2016 showed a slight increasing trend and the harvest year 2017 even a significant increase between old and modern cultivars. The literature contains evidence that the content of the 33-mer is mainly affected by the growing environment and not by the cultivar and therefore explains the observed tendency [19]. We observed a large influence of the harvest year on the 33-mer content (F = 1959), whereas the influence of the cultivar was significantly lower (F = 190). Furthermore, the contents of 33-mer in modern and old cultivars were affected equally by the harvest year (F = 1258.9 and 1688.8, respectively).

Trends for the contents of the 33-mer over the past 12 decades are of special relevance, because on the one hand it contains six copies of three overlapping T cell epitopes and what is even more important, it was present in all analyzed cultivars.

In contrast, P1 was present in all cultivars except for cultivar 12 and its proportions based on α-gliadins were between 0.6% and 0.3%, without a wide variability of the cultivars. The observed trend from old to modern cultivars was clearly increasing. The content of P1 was also influenced more strongly by the harvest year (F = 3678.3) than by the cultivar (F = 154.5). We further observed that older cultivars were affected by the harvest year more strongly than modern cultivars in terms of their P1 contents (F= 3678.3 and 829.8, respectively), which consequently means that modern cultivars are less affected by environmental changes.

The peptides P2 and P3 were present in all cultivars and showed strong variability within the five cultivars per decade and also over all 60 wheat cultivars. Therefore, no clear trend for the proportions based on α- and ɣ-gliadins, respectively, was observed.

Since the 33-mer, P1 and P2 are present in α-gliadins, possible correlations between the contents were investigated. Interestingly, neither the 33-mer, nor the P1 contents were correlated with the α-gliadin contents, whereas a strong correlation coefficient was found for P2 (r = 0.75). Further, a very strong correlation was observed between γ-gliadin and P3 contents (r = 0.79).

Overall, the results showed that breeding did contribute to changes in the contents of two CD-active peptides, however high amounts were detected in both old and modern cultivars. This is why the environmental factors are far more important than the genetic effect.

Table 4.2: CD-active peptides analyzed. 33-mer, P1 and P2 derived from α-gliadins, P3 derived from γ-gliadins

Peptide	Amino acid sequence
33-mer	LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF
P1	LQLQPFPQPQLPYPQPQPF
P2	RPQQPYPQPQPQY
P3	LQPQQPQQSFPQQQQPL

4.3.2 Determination of the reactivity of protein fractions and types

In this study two different sandwich ELISA test kits, namely R5 and G12, were used to quantitate the gliadin and gluten contents, respectively, in all 60 wheat cultivars. According to Codex Alimentarius [28] the gliadin content determined by R5 ELISA is multiplied by the conversion factor 2 (2 x gliadins = gluten), in order to obtain gluten contents. Because this procedure was questioned lately, this work compared the results of multiplying with the factor 2 and also with the actual gliadin-to-glutenin ratios. In the next step both results were correlated with the actual gluten contents determined by RP-HPLC [23]. Medium correlation coefficients were found for both scenarios, however the correlation for the conversion factor 2 was higher ($r = 0.745$ and $r = 0.685$, respectively) and was applied for further analysis.

Comparing the two ELISA test kits, we observed that both overestimated the gluten contents, in particular the R5 test kit even more than the G12 test kit. The gluten contents determined by ELISA were further correlated with the contents of the gliadin types, since the monoclonal antibodies used in the test kits were raised against certain CD-active epitopes present in the gliadin types. The R5 test kit revealed a strong correlation with ω1,2-gliadins ($r = 0.824$) and a medium correlation with α- and γ-gliadins ($r = 0.667$ and $r = 0.685$, respectively), essentially because the R5 antibody recognizes the pentapeptides QQPFP and QLPFP present in these protein types. As expected, no correlation was determined for the ω5-gliadin contents. Although the G12

antibody was raised against the epitopes QPQLPY and QPQLPF present in α-gliadins [29], we found just a weak correlation between the parameters (r = 0.548). A strong correlation was found for the ω1,2-gliadins (r = 0.80) and no correlation with the ω5- and γ-gliadins. Further no correlation was observed for the 33-mer, although this peptide contains the epitopes the antibody was raised against.

Next the gliadins of 16 selected cultivars were extracted and analyzed by both test kits, in order to investigate possible changes from old to modern cultivars. Comparing the test kits, we found that the R5 test kit generally delivered higher absorption values but without significant differences between the fractions. Neither the G12 nor the R5 test kit allowed any distinctions between the old and modern gliadin fractions. Results of a previous study also did not show any tendencies for R5 or G12, because both test kits showed almost equal absorption values for all cultivars [30]. In the last step the gliadin types ω5-, ω1,2-, α- and γ-gliadins were extracted from one old (10) and one modern cultivar (51), but no differences were found between them. These findings do not support the study performed by Lexhaller et al. (2017), who identified a ranking among the gliadin types. In that case, ω1,2-gliadins showed the highest and ω5-gliadins the lowest sensitivities regarding the R5 and G12 test kits response [31].

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5 Future Research

In this work 60 hexaploid German winter wheat cultivars were grown in one location in three different harvest years with the objective to identify possible changes in the protein contents and their distribution. The obtained results laid the foundation for further questions, which should be addressed in the future.

First, to allow deeper insights into the progress of protein composition by plant breeding efforts in wheat and to allow a generalization of the results the sample pool should be expanded. Therefore, on the one hand wheat cultivars should be grown in different places with different soil properties and not only in one place, to include more environmental variations. On the other hand cultivars and entries from different countries and regions and not only from Germany should be considered.

Second, the connection between the obtained results for the protein composition and the baking properties of the individual cultivars should be established. It is of great interest how breeding and thus decreased GLIA/GLUT ratios and increased HMW-GS proportions affected bread-making performance in terms of dough strength and extensibility and bread volume.

Third, based on the insights from the novel peak integration approach specific peaks from the four identified outstanding cultivars should be isolated, in order to perform in-depth MS investigations and also immunological studies. This is especially important because the identified changes of two CD-active peptides were not necessarily related with changes in the protein composition. These details would allow a development of new wheat varieties with good baking properties and less immunoreactive components.

Fourth, the identified changes at the protein-and peptide level should be evaluated in medical examinations. In this context following questions should be addressed: (1) How and to what extent are these changes relevant regarding the increased prevalence of wheat-related disorders and (2) How the identified changes in the protein patterns are relevant concerning the sensitivities towards NCGS and CD.

6 Summary

The scientific literature contains evidence that the prevalence of celiac disease (CD) and non-celiac gluten sensitivity (NCGS) has increased in the population during the second half of the 20th century. There are numerous factors which can be attributed to the increased prevalence. First, the growing population results in an increasing demand for wheat with a consequence of new wheat processing techniques and agricultural practices in wheat cultivation. Second, the dietary habits have changed, including an increased ingestion of wheat and an increased awareness of the disease. Third, improved diagnostic techniques have been developed in the course of the years. Additionally, modern wheat breeding practices might have contributed to the increase in the prevalence, essentially due to changes of the protein content and composition of wheat cultivars over the past 120 years.

The aim was a profound investigation of 60 hexaploid German winter wheat cultivars from the past 120 years to form the basis for developing new wheat cultivars, which cause less harmful sensitivities in the population.
In the first part all cultivars were comprehensively analyzed for their quantitative protein composition and distribution. The second part emphasized the necessity of a new RP-HPLC pattern integration method for an in-depth elucidation of protein fingerprints. In the third part the focus was on the distribution of four CD-active peptides in all wheat cultivars.
Thus, the main hypothesis of this work was that the selection criteria for modern breeding may have led to an altered composition of wheat proteins and possibly to a higher immunostimulatory potential of modern cultivars (registered after the 1950s) compared to older cultivars.

The analyzed sample set consisted of 60 hexaploid German winter wheat cultivars, which were registered between 1891 and 2010. All cultivars were grown in a randomized field order without fertilization and harvested in three different harvest years (2015-2017) in Gatersleben, Germany.

The quantitation of the crude protein contents showed highly variable contents depending on the cultivar and harvest year. Taking the average over the three harvest

years, the crude protein contents ranged between 11.7% and 7.1% and showed a slightly decreasing trend based on the median of five cultivars per decade. Wheat proteins are characterized by a complex composition of various individual proteins, which are relevant for different functions in the grain.

Wheat proteins were extracted stepwise by means of the modified Osborne fractionation and measured by RP-HPLC in order to evaluate their alteration over the past 120 years. This work clearly demonstrated changes in the protein distribution based on the median of five cultivars per decade and averaged over three harvest years.

While the proportions in the total RP-HPLC content of albumins/globulins remained unaffected, the percentages of gliadins showed a clear decreasing trend and those of glutenins an increasing trend. The percentages for albumins/globulins ranged between 25.5% and 16.9%, those of gliadins between 62.0% and 45.7% and those for glutenins between 33.1% and 17.1%. These trends subsequently led to a change of the GLIA/GLUT ratio, which is known to contribute to the formation of a viscoelastic dough with a high gas holding capacity and therefore is important for the baking performance. We observed a significant decrease of the GLIA/GLUT ratio, with values ranging between 3.6 and 1.4.

Concerning the protein types ω5- and ω1,2-gliadins, no trends were revealed for their percentages, whereas the proportions of α- and γ-gliadins showed a significant decreasing trend. The values ranged between 5.2% and 1.0%; 6.4% and 3.1%; 30.3% and 19.7% and between 28.3% and 17.5%, respectively. In contrast, the proportions of the glutenin subunits showed an increasing trend as did the proportions of the glutenin fraction. Interestingly, we observed that the changes of the proteins were mainly influenced by the harvest year, except for gliadins, ω5-gliadins and LMW-GS, which were primarily affected by different cultivars.

Correlation analysis between the proportions of the protein fractions and types revealed that there was no correlation between albumins/globulins, ω5- and ω1,2-gliadins and the other parameters. However, very good correlation coefficients were determined between gliadin and glutenin proportions ($r = -0.89$) and also between gliadin and LMW-GS proportions ($r = -0.90$). Medium correlation coefficients were observed between gliadins and α- and γ-gliadins ($r = 0.77$ and $r = 0.73$, respectively)

and HMW-GS (r = -0.73) and also between glutenin proportions and α- and γ-gliadins (r = -0.68 and r = -0.74).
Additionally, the main goal of modern wheat breeding, namely high grain yields, was reflected in the trends for the plant height and yield for the breeding period of 120 years. A reduction of the plant height of about 40% and an increase of the grain yield of about 60% from old to modern cultivars were observed.
In line with the assumption that the composition of the wheat proteins has changed over the past 120 years, the results showed that the contents of the fractions as well as crude protein followed a specific trend although the contents were highly variable over all 60 cultivars and also within the five cultivars per decade irrespective of the harvest year.

Because of the high variability of the protein contents, a new approach of RP-HPLC profile integration was developed, in order to enable a better differentiation between wheat cultivars. For the novel approach, the integration was carried out every 20 s for all protein fractions and the fractions were no longer subdivided into protein types. Each event was further calculated as proportion relative to the total RP-HPLC content. For albumins/globulins the integration was performed from 5.6 min to 12.9 min and led to 22 integration events. The gliadin fraction was integrated from 7.8 min to 21.7 min and resulted in 42 integration events. The integration for glutenins was performed between 7.5 min and 22.5 min and comprised 46 integration events. This way of integration allowed us to consider the distribution of individual peaks without information loss. By means of principle component analysis (PCA) the common and the new integration techniques were compared, with the result that the novel approach allowed a better differentiation between old and modern cultivars. By means of hierarchical cluster analysis (HCA) it was possible to cluster similar cultivars and to identify cultivars with a unique protein composition. This is how an in-depth insight into the changes of the protein peak distribution of wheat cultivars over the past 12 decades was achieved.

It is known that wheat can cause adverse reactions in susceptible individuals because the gliadin and glutenin protein types contain CD-active peptides, but it is uncertain whether modern cultivars have higher or lower contents compared to old cultivars. In this study all wheat cultivars were analyzed for their contents of four immunodominant peptides, namely the 33-mer and peptides containing the DQ2.5-glia-α1a/DQ2.5-glia-

α2 (P1), DQ2.5-glia-α3 (P2) and DQ2.5-glia-γ1 (P3) epitopes by means of SIDA combined with targeted LC-MS/MS in order to identify the influence of breeding on the contents.

The values for the 33-mer and P1 proportions in α-gliadins ranged between 2.7% and 0.6% and between 0.6% and 0.3%, respectively, based on the median of five cultivars per decade and averaged over three harvest years and showed a slightly increasing trend from old to modern cultivars. However, the five samples per decade and all 60 cultivars showed a large variability. Interestingly, no correlation was observed between the absolute α-gliadin and 33-mer or P1 contents, since the α-gliadin contents increased and the contents of the 33-mer and P1 remained rather unaffected over the past 120 years. Concerning the P2 and P3 peptides present in α- and γ-gliadins, no trends, either in relative or absolute values were observed. This means therefore, that old and modern cultivars appear to have largely the same immunoreactive potential. What is more important is the effect of the harvest year, which influenced the contents of the CD-active peptides more strongly than the wheat cultivars.

7 Zusammenfassung

Die wissenschaftliche Literatur enthält Hinweise darauf, dass die Prävalenz von Zöliakie und Nicht-Zöliakie-Glutensensitivität (NCGS) in der Bevölkerung in der zweiten Hälfte des 20. Jahrhunderts zugenommen hat. Es gibt zahlreiche Faktoren, auf die die erhöhte Prävalenz zurückgeführt werden kann. Erstens führt die wachsende Bevölkerung zu einer steigenden Nachfrage nach Weizen, mit der Folge neuer Weizenverarbeitungstechniken und landwirtschaftlicher Praktiken im Weizenanbau. Zweitens haben sich die Ernährungsgewohnheiten geändert, einschließlich einer vermehrten Aufnahme von Weizen und einem gesteigerten Bewusstsein für Ernährung. Drittens wurden im Laufe der Jahre verbesserte Methoden zur Diagnose von Zöliakie entwickelt. Zusätzlich könnten moderne Weizenzuchtpraktiken zum Anstieg der Prävalenz beigetragen haben, was im Wesentlichen auf Veränderungen des Proteingehalts und dessen Zusammensetzung in den Weizensorten der letzten 120 Jahren zurückzuführen ist.

Das Ziel dieser Arbeit war eine umfassende Untersuchung von 60 hexaploiden deutschen Winterweizensorten aus den letzten 120 Jahren, um die Grundlage für die Entwicklung neuer Weizensorten zu schaffen, die weniger Reaktionen nach dem Konsum in der Bevölkerung hervorrufen.
Im ersten Teil wurden alle Sorten auf ihre quantitative Proteinzusammensetzung und -verteilung analysiert. Der zweite Teil unterstrich die Notwendigkeit einer neuen RP-HPLC-Musterintegrationsmethode zur detaillierten Aufklärung des Protein-Fingerabdrucks. Im dritten Teil lag der Schwerpunkt auf der Bestimmung von vier Zöliakie-aktiven Peptiden in allen Weizensorten.
Die Haupthypothese dieser Arbeit war daher, dass die Selektionskriterien für die moderne Züchtung zu einer veränderten Zusammensetzung der Weizenproteine und möglicherweise zu einem höheren immunstimulatorischen Potential moderner (registriert nach den 1950er Jahren) Sorten im Vergleich zu älteren Sorten führten.

Der analysierte Probensatz bestand aus 60 hexaploiden deutschen Winterweizensorten, die zwischen 1891 und 2010 registriert wurden. Alle Sorten wurden in einer randomisierten Feldreihenfolge ohne Düngung angebaut und in drei verschiedenen Erntejahren (2015-2017) in Gatersleben, Deutschland, geerntet.

Die Quantifizierung der Rohproteingehalte zeigte je nach Sorte und Erntejahr sehr unterschiedliche Gehalte. Im Durchschnitt über die drei Erntejahre lagen die Rohproteingehalte zwischen 11,7% und 7,1% und der Median von fünf Sorten pro Jahrzehnt zeigte eine leicht abnehmende Tendenz. Weizenproteine zeichnen sich durch eine komplexe Zusammensetzung von verschiedenen Einzelproteinen aus, die verschiedene Funktionen im Getreide erfüllen.

Die Weizenproteine wurden schrittweise mittels modifizierter Osborne-Fraktionierung extrahiert und mittels RP-HPLC gemessen, um ihre Veränderung in den letzten 120 Jahren zu untersuchen. Diese Arbeit zeigte deutlich Veränderungen in der Proteinverteilung, basierend auf dem Median von fünf Sorten pro Jahrzehnt und gemittelt über drei Erntejahre.
Während die Anteile der Albumine/Globuline am gesamten RP-HPLC-Gehalt unverändert blieben, zeigten die Anteile der Gliadine einen deutlich abfallenden Trend und die der Glutenine einen zunehmenden Trend. Die Anteile der Albumine/Globuline lagen zwischen 25,5% und 16,9%, die der Gliadine zwischen 62,0% und 45,7% und die der Glutenine zwischen 33,1% und 17,1%. Diese Trends führten in der Folge zu einer Veränderung des GLIA/GLUT-Verhältnisses, das bekanntlich signifikant zur Bildung eines viskoelastischen Teiges mit hohem Gashaltevermögen beiträgt und daher für die Backleistung wichtig ist. Wir beobachteten eine signifikante Abnahme des GLIA/GLUT-Verhältnisses, wobei die Werte zwischen 3,6 und 1,4 lagen.
Bei den Proteintypen ω5- und ω1,2-Gliadinen wurden keine Trends für deren Anteile festgestellt, während die Anteile der α- und γ-Gliadine einen signifikant abnehmenden Trend zeigten. Die Werte lagen zwischen 5,2 % und 1,0 %; 6,4 % und 3,1 %; 30,3 % und 19,7 % bzw. zwischen 28,3 % und 17,5 %. Im Gegensatz dazu zeigten die Anteile der Glutenin-Untereinheiten einen ansteigenden Trend mit den gleichen Werten wie die Anteile der Gluteninfraktion. Interessanterweise beobachteten wir, dass die Variabilität der Proteine hauptsächlich durch das Erntejahr beeinflusst wurde, mit Ausnahme der Gliadine, ω5-Gliadine und LMW-GS, die primär von verschiedenen Sorten beeinflusst wurden.
Die Korrelationsanalyse zwischen den Anteilen der Proteinfraktionen und -typen ergab, dass es keine Korrelation zwischen Albuminen/Globulinen, ω5- und ω1,2-Gliadinen und den anderen Parametern gab. Es wurden jedoch sehr gute Korrelationskoeffizienten zwischen Gliadin- und Glutenin-Anteilen (r = -0,89) und auch

zwischen Gliadin- und LMW-GS-Anteilen (r = -0,90) bestimmt. Mittlere Korrelationskoeffizienten wurden zwischen Gliadinen und α- und γ-Gliadinen (r = 0,77 bzw. r = 0,73) und HMW-GS (r = -0,73) und auch zwischen den Glutenin-Anteilen und α- und γ-Gliadinen (r = -0,68 und r = -0,74) beobachtet.
Zusätzlich spiegelte sich das Hauptziel der modernen Weizenzüchtung, nämlich hohe Kornerträge, in den Trends für die Pflanzenhöhe und den Ertrag für die Züchtungsperiode von 120 Jahren wider. Es wurde eine Verringerung der Pflanzenhöhe um etwa 40% und eine Erhöhung des Kornertrags um etwa 60% von alten zu modernen Sorten beobachtet.
Im Einklang mit der Annahme, dass sich die Zusammensetzung der Weizenproteine in den letzten 120 Jahren verändert hat, zeigten die Ergebnisse, dass die Gehalte der Fraktionen sowie des Rohproteins einem spezifischen Trend folgten, obwohl die Gehalte über alle 60 Sorten auch innerhalb der fünf Sorten pro Jahrzehnt unabhängig vom Erntejahr sehr unterschiedlich waren.

Wegen der hohen Variabilität der Proteingehalte wurde ein neuer Ansatz der RP-HPLC-Profilintegration entwickelt, um eine bessere Differenzierung zwischen Weizensorten zu ermöglichen. Für den neuen Ansatz wurde die Integration alle 20 s für alle Proteinfraktionen durchgeführt und die Fraktionen wurden nicht mehr in Proteintypen unterteilt. Jedes Ereignis wurde weiter als relativer Anteil am Gesamtgehalt über RP-HPLC berechnet.
Für Albumine/Globuline wurde die Integration von 5,6 min bis 12,9 min durchgeführt und führte zu 22 Integrationsereignissen. Die Gliadinfraktion wurde von 7,8 min bis 21,7 min integriert und führte zu 42 Integrationsereignissen. Die Integration für Glutenine wurde zwischen 7,5 min und 22,5 min durchgeführt und umfasste 46 Integrationsereignisse. Diese Art der Integration ermöglichte es, die Verteilung der einzelnen Peaks ohne Informationsverlust zu berücksichtigen. Mit Hilfe der Hauptkomponentenanalyse (PCA) wurden die gängigen und die neuen Integrationstechniken verglichen, mit dem Ergebnis, dass der neuartige Ansatz eine bessere Unterscheidung zwischen alten und modernen Sorten ermöglichte. Mit Hilfe der hierarchischen Clusteranalyse (HCA) war es möglich, ähnliche Sorten zu gruppieren und darüber hinaus Sorten mit einer einzigartigen Proteinzusammensetzung zu identifizieren. Auf diese Weise wurde ein tiefer Einblick

in die Veränderungen der Proteinverteilung von Weizensorten in den letzten 12 Jahrzehnten gewonnen.

Es ist bekannt, dass Weizen bei prädisponierten Individuen unerwünschte Reaktionen hervorrufen kann, weil die Gliadin- und Glutenin-Proteintypen Zöliakie-aktive Peptide enthalten, aber es ist nicht klar, ob moderne Sorten im Vergleich zu alten Sorten höhere oder niedrigere Gehalte aufweisen. In dieser Studie wurden alle Weizensorten auf ihren Gehalt von vier immunoreaktiven Peptiden mittels SIDA in Kombination mit gezielter LC-MS/MS analysiert, um den Einfluss der Züchtung auf den Gehalt zu identifizieren. Es wurden analysiert: das 33-mer und Peptide, die die Epitope DQ2.5-glia-α1a/DQ2.5-glia-α2 (P1), DQ2.5-glia-α3 (P2) und DQ2.5-glia-γ1 (P3) enthalten. Basierend auf dem Median von fünf Sorten pro Jahrzehnt und gemittelt über drei Erntejahre lagen die Werte für die 33-mer- und P1-Anteile in α-Gliadinen zwischen 2,7 % und 0,6 % bzw. zwischen 0,6 % und 0,3 % und zeigten einen leicht ansteigenden Trend von alten zu modernen Sorten. Die fünf Proben pro Jahrzehnt und alle 60 Sorten zeigten jedoch eine große Variabilität. Interessanterweise wurde keine Korrelation zwischen den absoluten Gehalten an α-Gliadinen und 33-mer oder P1 beobachtet, da die Gehalte an α-Gliadinen zunahmen, während die Gehalte an 33-mer und P1 in den letzten 120 Jahren eher unbeeinflusst blieben. Hinsichtlich der in den α- und γ-Gliadinen vorhandenen P2- und P3-Peptide wurden weder bei den relativen noch bei den absoluten Werten Trends beobachtet. Das bedeutet, dass alte und moderne Sorten weitgehend das gleiche immunreaktive Potential zu haben scheinen. Wichtiger ist der Einfluss des Erntejahres, das den Gehalt der CD-aktiven Peptide stärker beeinflusst hat als die Weizensorten.