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Influence of baking conditions on the detection and composition of gluten

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Am Ende wird alles gut. Wenn es nicht gut wird, ist es noch nicht das Ende.

-Oscar Wilde-

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1 Introduction

1.1 Wheat

Wheat is a staple crop for an estimated 40% of the world population. It provides one fifth of dietary calories in human diets globally (Giraldo et al., 2019). More than two thirds of global wheat are used for food, one fifth is used for livestock feed and the rest for other applications such as biofuels (Grote et al., 2021).

The major wheat species grown throughout the world (95%) is *Triticum aestivum ssp. aestivum,* a hexaploid species (AABBDD) usually called “common” or “bread” wheat. Among the minor wheat species, *Triticum turgidum ssp. durum* (AABB), also known as “durum” or “pasta” wheat, is the most prominent (Shewry and Hey, 2015; Wieser et al., 2023).

Many constituents play a role in wheat flour functionality. However, it is well established that the properties of its proteins (content and composition) are crucial for the baking quality of wheat.

1.2 Wheat proteins

The protein content of wheat usually ranges between 10 and 15% (Groos et al., 2003). Genetic and environmental factors influence the protein content and composition, which, in turn, affect protein quality for different applications. Genes regulating the protein content have been exploited in many breeding programs (Shewry and Hey, 2015). Among environmental conditions, nitrogen fertilization was found to be the most significant factor to affect protein content and composition, but other factors such as soil fertility, precipitation, temperature, and altitude also play a role (Triboï et al., 2003; Wieser et al., 2004; Altenbach, 2012).

The different compartments of the grain show varying protein contents. The highest contents are found in the germ (34%), followed by the aleurone (23%), the outer layers (11%) and the starchy endosperm (10%). However, considering the proportions of the compartments, most proteins (75%) are located in the endosperm. (Jensen and Martens, 1983)

For standard white flour production, the germ and bran (aleurone and outer layers) are removed during grain processing and milling (Codex Alimentarius). Wholegrain flour, which is made by milling the whole grain, contains all wheat proteins. It has a higher protein content by about 2% and a better balanced composition of essential amino acids than white flour (Shewry and Hey, 2015).

1.2.1 Classification

The protein fraction is a highly complex mixture of hundreds of different proteins (Skylas et al., 2000). They interact with each other but also with other flour constituents, which make them difficult to isolate and classify (Shewry, 2019).

Traditionally, wheat proteins have been classified according to their solubility properties which are often related to the molecular size. The first comprehensive fractionation scheme for wheat proteins was developed by Osborne (1907). It classified wheat proteins as albumins (soluble in water), globulins (soluble in dilute salt solution), gliadins (soluble in aqueous alcohol solution) and glutenins (soluble in dilute acid or alkali solution). Due to structural and functional similarities, albumins and globulins are often dissolved together with a dilute salt solution and combined in one fraction (albumins/globulins, ALGL). For better solubility, glutenins are nowadays extracted at increased temperatures in aqueous alcohols, containing disaggregating and reducing agents (Wieser et al., 1998).

Biochemical, genetic and mass spectrometry analyses have demonstrated that the classic Osborne fractionation does not really provide a clear separation of the wheat proteins that differ biochemically/genetically or in functionality (Goesaert et al., 2005). Also a small portion of structural and metabolic proteins, which are bound to membranes and cell walls, is not covered by the Osborne fractions and remains together with starch in the insoluble extraction residue (Wieser, 2007). Nevertheless, the Osborne fractionation is still extensively used to study cereal protein composition and functionality (Arendt and Zannini, 2013). Furthermore, due to its relative simplicity, this fractionation method often serves as an initial separation step to obtain semi-pure protein fractions.

Alternatively, wheat proteins are classified according to their functions as storage proteins (75 to 80% of total grain protein), metabolic and protective proteins (15 to 20% of total grain protein), and structural proteins (5% of total grain protein). Storage proteins are exclusively located in the starchy endosperm and provide the seedling with nitrogen, amino acids and energy during germination. They comprise all gluten proteins. Metabolic and protective proteins are involved in plant growth and defence, and are mainly present in the aleurone layer and the germ. Structural proteins are membrane proteins and lipoproteins maintaining the plant cells' shape (Wieser et al., 2020).

1.2.2 Albumins and globulins

The ALGL fraction as defined by Osborne (1907) accounts for around 20% of the grain protein (Merlino et al., 2009). The majority of them are monomeric with relative molecular masses (M_r) lower than 25 kDa. Other proteins of this fraction show M_r of up to 70 kDa (Singh et al., 2001)

(Figure 1). This fraction mainly consists of metabolic and protective proteins, such as enzymes and enzyme inhibitors (Wieser et al., 2023).

Metabolic proteins include enzymes like hydrolases cleaving carbohydrates (α- and β-amylase, β-glucosidase, cellulase, arabinoxylanase), proteins (peptidases) and lipids (lipases), as well as oxidoreductases, transferases, and further enzymes. They regulate cell metabolism and ensure germ nutrient supply (Brijs et al., 2009). Endogenous proteolytic and lipolytic activity has also been shown to affect the organoleptic and nutritional quality of bread and pasta (Ficco et al., 2014; Tomić et al., 2016; Žilić et al., 2016).

Enzyme inhibitors block hydrolytic enzymes of pest insects and pathogenic fungi. They belong to the family of amylase/trypsin inhibitors (ATIs). ATIs are also associated with the pathogenesis of baker's asthma, coeliac disease and non-coeliac gluten sensitivity (NCGS) (Weiss et al., 1997; Schuppan et al., 2015; Geisslitz et al., 2021).

A minor group of storage proteins such as polymeric globulins (triticins) is also present in the ALGL fraction (5% of the grain protein) (Singh and Shepherd, 1985; Singh et al., 1991). Though linked by interchain disulfide (SS) bonds, they are not considered to be part of the gluten-forming complex of proteins (Gianibelli et al., 2001). However, Goel et al. (2015) have indicated a role of triticins in wheat breadmaking quality such as dough stability and bread loaf volume. Other proteins related to storage proteins and soluble in the ALGL fraction are farinins and purinins (Kasarda et al., 2013).

In general, proteins of the ALGL fraction show higher nutritional values compared to the proteins present in the gliadin and glutenin fraction. Essential amino acids like lysine, tryptophan and methionine are present in relatively high amounts (Wieser et al., 2020).

1.2.3 Gliadins

The gliadin fraction contains proteins which are soluble in aqueous alcohols (e.g. 60% ethanol) and constitute 50 to 60% of the grain protein (Geisslitz et al., 2018) (Figure 1). The proteins are either monomeric or oligomeric.

The monomeric gliadins are differentiated based on their mobility upon acid polyacrylamide gel electrophoresis (PAGE) into ω5-, ω1,2-, α- and γ-gliadins (Wieser, 1996). A classification involving β-gliadins is obsolete, since the comparisons of amino acid sequences showed that α- and β-gliadins form a single group (Shewry, 2019). Recent work indicated a new class of wheat gliadins (δ-gliadins), which seem to be part of the wider family of γ-gliadins (Anderson et al., 2012; Huo et al., 2018).

Each gliadin type contains many closely related proteins with high amino acid sequence homology containing remarkably high proportions of glutamine and proline (Wieser et al., 2023).

ω5-Gliadins have M_r around 50 kDa, ω1,2-gliadins around 40 kDa. Both are composed of a large repetitive domain (B) with peptides such as QQQFP (ω5-gliadins) and QQPQQPFP (ω1,2-gliadins), and short nonrepetitive N-terminal (A) and C-terminal (C) domains with balanced amino acid compositions. ω5- and ω1,2-gliadins are characterized by an unbalanced amino acid composition with glutamine (53% and 42%), proline (20% and 29%) and phenylalanine (9% and 8%) accounting for around 80% of the total composition (Wieser et al., 2014). Unlike all other gluten proteins, ω-gliadins contain no cysteine residues with a very small portion of so-called glutenin-bound ω-gliadins (ωb-gliadins) being exceptional. The amino acid sequence of ωb-gliadins contains a cysteine residue at the domain C (point mutation) which is linked to glutenins by an SS bond. ωb-Gliadins can be solubilized only after reduction of SS bonds and appear in the glutenin fraction (Lutz et al., 2012).

The α- and γ-gliadins are smaller than ω-gliadins with M_r ranging from 28 to 35 kDa. They have a predominantly repetitive N-terminal and a nonrepetitive C-terminal domain (Kasarda et al., 1984). The N-terminal domain is abundant in glutamine, proline, phenylalanine and tyrosine (only α-gliadins), and unique for each type. In contrast to ω-gliadins, α-gliadins show lower contents of proline (14%), phenylalanine (4%) and in some cases also of glutamine (36%). Compared to α-gliadins, the γ-gliadins contain more proline (18%), phenylalanine (5%), methionine (2% vs. 1%) and lysine (0.7% vs. 0.4%) (Wieser et al., 2014). Typical repetitive sequences are QPQPF and PQQPYP in α-gliadins and QQPQQPFP in γ-gliadins. α-Gliadins contain an additional repetitive segment of glutamine residues. The nonrepetitive C-terminal domain is mainly equal for each type and has a more balanced amino acid composition compared to the N-terminal domain. Cysteine residues, which essentially determine the tertiary structure, are located in the C-terminal domains. α-Gliadins contain six cysteine residues and γ-gliadins contain eight cysteine residues, which form three and four homologous SS bonds via their thiol-groups. (Müller and Wieser, 1995; Grosch and Wieser, 1999; Wieser et al., 2020) In contrast to glutenins, no free cysteine residues occur in gliadins (Shewry et al., 1986).

The alcohol-soluble oligomeric proteins are known as high-molecular-weight (HMW)-gliadins. They are formed by modified gliadins with an odd number of cysteine residues, which are linked together as well as linked to glutenins by interchain SS bonds. Thus HMW-gliadins contain all types of gluten proteins with predominant portions of low-molecular-weight glutenin subunits (LMW-GS) (Schmid et al., 2016). The oligomers have M_r ranges between 70 to 700 kDa. (Shewry et al., 1983; Huebner and Bietz, 1993; Southan and MacRitchie, 1999)

1.2.4 Glutenins

The glutenin fraction represents the smallest fraction of the grain proteins (16 to 26%) (Geisslitz et al., 2018) (Figure 1). It consists of polymeric proteins which are in their native state insoluble in water, dilute salt or alcohol solutions. The protein monomers are linked by interchain SS bonds. The polymers are broken down into monomers by the addition of reducing agents such as dithiothreitol (DTT), which cleave the SS bonds. Increased temperature (e.g. 60 °C) and the addition of a disaggregating agents such as urea accelerate the dissolving process (Wieser et al., 1998). The solubility of the glutenin subunits is comparable to that of gliadins.

Based on the molecular masses of the monomers as determined by sodium dodecyl sulfate (SDS)-PAGE, glutenins are grouped into LMW- and HMW-GS. According to different amino acid sequences LMW-GS are further subdivided into B-, C- and D-type; HMW-GS into x- and y-type.

LMW-GS are related to α- and γ-gliadins in terms of amino acid composition and M_r ranging from 32-39 kDa. Similar to α- and γ-gliadins, LMW-GS have a unique mainly repetitive N-terminal domain, which is rich in glutamine and proline, and a nonrepetitive C-terminal domain with a more balanced amino acid composition. A typical repetitive sequences is QQQPPFS with a relatively high serine content (9%) characteristic for LMW-GS.

LMW-GS contain eight cysteine residues. Six residues are in positions homologous to α- and γ-gliadins and form three intrachain SS bonds. In contrast to gliadins, which only contain intrachain SS bonds, two cysteine residues of LMW-GS form also interchain SS bonds. In this way LMW-GS are incorporated in different protein types (modified gliadins, LMW-GS and HMW-GS) resulting in oligomeric HMW-gliadins and polymeric glutenins. LMW-GS of C- and D-type build a very minor group of LMW-GS and are actually modified α-, γ-gliadins (C-type) or ω-gliadins (D-type=ωb-gliadins). The odd numbers of cysteine residues of modified gliadins result from point mutations. The free SH groups are available for interchain SS crosslinking to other gluten proteins (Lutz et al., 2012).

The HMW-GS monomers are the largest grain protein subunits with M_r ranging between 67-88 kDa. HMW-GS are characterized by high contents of glutamine (32 to 36%), proline (11 to 13%) and glycine (18 to 20%) accounting for around 65% of the total composition. Besides, they contain more tyrosine (5.2 to 5.7%) and less phenylalanine (0.3%) than the other gluten proteins (Wieser et al., 2014). Similar to the primary structure of ω-gliadins, the amino acid sequences of HMW-GS contain a nonrepetitive N-terminal domain (A), a repetitive central domain (B), and a nonrepetitive C-terminal (C) domain (Shewry et al., 1992). The repetitive sequences consist of peptides such as PGQGQQ, which is often modified by single amino acid residues and separated by other peptides such as GYYPTSPQQ or GYYPTSLQQ, and GQQ

(x-type) or HYPASQ (y-type). The nonrepetitive domains are characterized by relatively balanced amino acid compositions, including most (y-type) or all (x-type) cysteine residues. Two cysteine residues located in domain A form intrachain SS bonds. Two other cysteine residues, one located in domain A and one located in domain C, form interchain SS bonds with other x-type HMW-GS (head-to-tail linkages). In comparison to the x-type, y-type HMW-GS have three additional cysteine residues. Two of them are adjacent in domain A and connected with corresponding residues of another y-type HMW-GS. The other cysteine residue is present in domain B and linked to the C-terminal domain of a LMW-GS (Wieser et al., 2023).

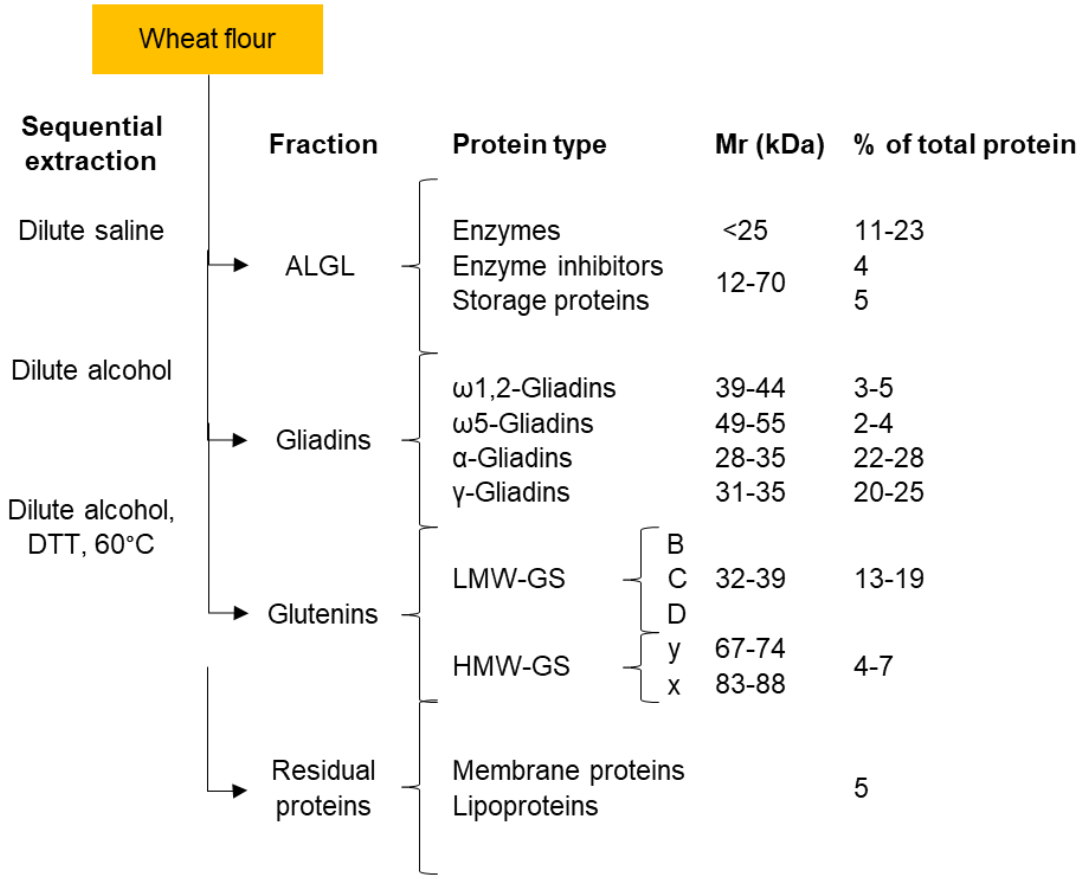

Figure 1. Classification of wheat proteins based on a modified Osborne fractionation according to Wieser et al. (2023). For each protein type, information on its molecular weight (Mr) and the percentage it represents in the total protein content is provided (Geisslitz et al., 2018). DTT, dithiothreitol; HMW-GS, high-molecular-weight glutenin subunits; LMW-GS, low-molecular-weight glutenin subunits.

1.2.5 Gluten

The term “gluten” usually describes the sticky residue left after washing wheat dough with water or saline to remove starch and water-soluble compounds (Beccari, 1745; Wieser et al., 2020). The Codex Standard 118-1979 defines gluten as the protein fraction present in wheat, rye, barley, oats, and their crossbred varieties and derivatives, that some individuals are intolerant of, and which is not soluble in water and 0.5 M NaCl.

Chemically, gluten is composed of gliadins and glutenins. Gliadins are present at higher levels compared to glutenins. The gliadin/glutenin ratio is dependent on the cereal species and variety. For common wheat it varies from 1.5 to 3.1:1 (Wieser and Koehler, 2009).

Gluten proteins make up around 80% of total grain protein, are located in the starchy endosperm and function as storage proteins. Their nutritional quality is poor, with high levels of glutamine (32% to 53%) and proline (11% to 29%), and low levels of essential amino acids like lysine (0.3% to 1.1%), tryptophan (0.0% to 1.0%), and methionine (0.0 to 1.8%). The amino acid composition is perfectly tailored to their biological function. Glutamine, containing two nitrogen atoms, serves as excellent nitrogen source for the germinating grain. The structure of proline allows dense packing of nitrogen in the endosperm (Tosi, 2012) and makes the proteins more resistant to degradation by external enzymatic attacks (Simpson, 2001). The presence of a relatively high amount of hydrophobic amino acids next to proline (valine, leucine, isoleucine, and phenylalanine as a sum up to 28%) is one of the reasons for the low water extractability of gluten proteins, that prevent the loss of proteins during germination. (Wieser et al., 2023)

Cysteines, which only make up about 2% of the amino acids in gluten, determine the polymer structure. Most cysteines are present in an oxidized state and form covalent SS bonds either within a protein (intrachain) or between proteins (interchain). Both kinds of SS bonds are structurally important.

The gluten polymer obtained when extracting proteins from wheat flour is mainly assumed to be built by glutenin subunits connected by interchain SS bonds at the N and C termini of the HMW-glutenin proteins (head-to-tail SS bonds), whereas the gliadins primarily show intrachain SS bonds. However, due to the difficulty to isolate gluten proteins without destroying the polymer, the precise native quaternary structure of gluten has not been fully understood yet. Current models suggest either HMW-GS (x- and y-type) or HMW-GS and LMW-GS to be the polymer backbone to which LMW-GS are attached. According to Lutz et al. (2012) LMW-GS (b- and c-type) are connected with y-type HMW-GS only.

The LMW-GS are supposed to be chain extenders (two or more cysteine residues available for cross-linking) and chain terminators (only one cysteine residue available for cross-linking). (Graveland et al., 1985; Masci et al., 1995; Lindsay and Skerritt, 1999). The covalent structure of the gluten network is further stabilised by non-covalent bonds, especially hydrogen bonds (Belton, 1999) (Figure 2).

Gluten forms one of the most complex protein aggregates in nature, with varying molecular masses ranging from 500.000 to over 10 million. (Wieser, 2007; Shewry, 2019; Wieser et al., 2023). The structural continuity due to this large size is important for its unique elastic properties (Delcour et al., 2012).

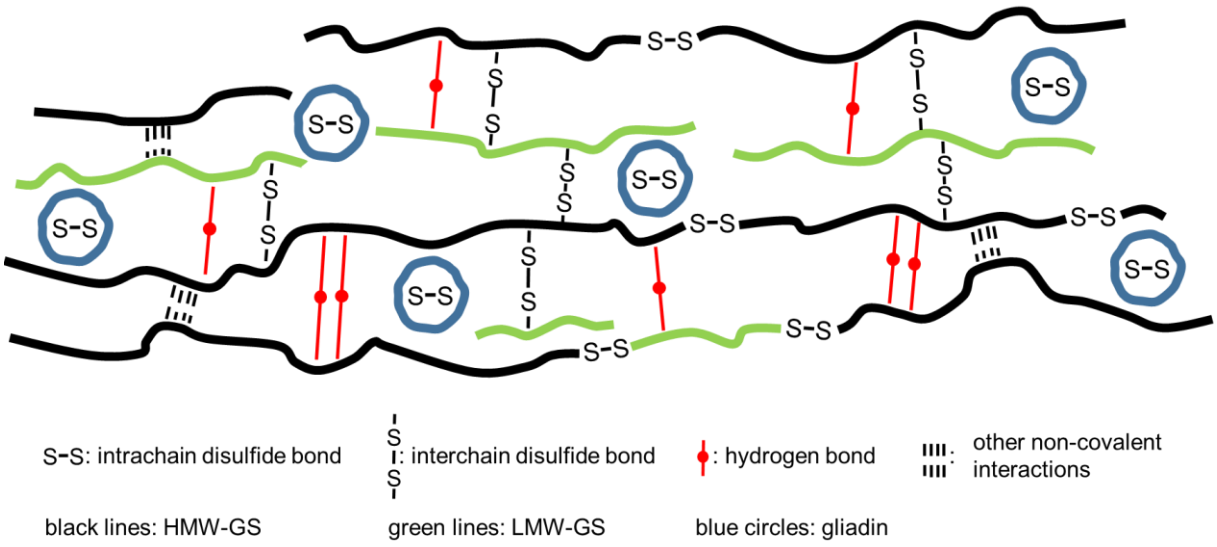

Figure 2. Gluten polymer adopted from Ortolan and Steel (2017). HMW-GS, high-molecular-weight glutenin subunits; LMW-GS, low-molecular-weight glutenin subunits.

1.3 Gluten network formation during food processing

The ability of the two fractions of gluten, gliadins and glutenins, to form a protein network as a result of water addition as well as mechanical and thermal energy input is crucial for the production of foods like bread or pasta.

The gluten polymer undergoes various changes during mixing of flour and water and further steps of processing. Although a tremendous amount of studies has been conducted, a full comprehension of the molecular associations and bond formation in dough during mixing, resting and thermal treatment has not been realized. The reasons are the complex structure of the gluten proteins and concomitant difficulties to extract the intact gluten polymer, but also the wide spectrum of chemical reactions that take place during wheat processing that add to the complexity of the gluten structure.

1.3.1 Hydration and mixing during dough formation

The addition of water (hydration) and mechanical energy (mixing) greatly influence the structure of gluten during dough formation. The behavior of dough is very dependent on water content and mixing time (Belton, 2005). Water acts as a plasticizer and increases the mobility of the system and therefore interactions of gluten proteins (Cuq et al., 2003). Hydration induces conformational changes allowing for hydrophobic interaction and also the solvation of hydrophilic and water soluble LMW-GS (Wang et al., 2015). With the aid of mixing, hydration will be facilitated. Mixing increases the opportunities for the interactions and conformational changes of gluten proteins. The energy input leads to the disruption of SS bonds (depolymerisation) and re-polymerisation of glutenin molecules to extensions beyond their equilibrium conformation (Weegels et al., 1997).

As a result of hydration and mixing, gluten proteins are transformed into a continuous cohesive visco-elastic gluten protein network (Singh and MacRitchie, 2001). During resting further re-polymerisation takes place (Weegels et al., 1997).

Oxidation of free SH groups and SH-SS interchange leading to SS bond formation between glutenins are the main background reaction for the rearrangement and build-up of the protein polymer structure during dough formation (Figure 3A,B) (Frater et al., 1960; Mauritzen and Stewart, 1963; Lindsay et al., 2000; Delcour et al., 2012). In the case of SH-SS interchange reactions, a free SH group carries out a nucleophilic attack on the sulfur atom of an SS bond. Because the pKa value of cysteine is about 8.5, this type of reaction is enhanced under alkaline conditions and inhibited under more acidic conditions (Lagrain et al., 2008b).

The formation of other covalent bonds such as tyrosine derivatives (Hanft and Koehler, 2005; Piber and Koehler, 2005; Peña et al., 2006) have been reported but their role in the structure of gluten in dough is poorly defined.

The role of the individual gluten components in dough functionality is very complex. The glutenin composition determines the glutenins structure and size distribution, which are correlated with the dough functionality (Gupta et al., 1993; Goesaert et al., 2005). Structural aspects that are responsible for its relevance are the number and distribution of the cysteine residues, which enable interchain SS crosslinking, and the repetitive motifs rich in glutamine residues. Latter can form hydrogen bonds within and between subunits, which contribute to the glutenin elasticity and overall stability of the network (Shewry et al., 1995; Belton, 1999). So far, more than 20 different HMW-GS and 40 different LMW-GS have been identified. There are about 3-5 HMW-GS and 7-16 LMW-GS in each wheat cultivar because not all possible proteins are expressed. This leads to an enormous variation in glutenin composition between different wheat cultivars. (Gupta and Shepherd, 1990; Shewry et al., 1992)

The intrachain SS bonds in gliadins are not involved in SH-SS interchange reactions during dough formation. They are retained in the network as monomers and either trapped in the polymer or connected by noncovalent bonds (Kuktaite et al., 2004; Iwaki et al., 2020).

In general, gliadins act as “plasticizer” for glutenins and increase the entanglement spacing (Singh and MacRitchie, 2001; Wieser, 2007). Gliadins mainly contribute to the viscosity and extensibility of the dough, whereas glutenins provide cohesiveness (dough strength) and elasticity (Delcour et al., 2012). The ratio of both fractions determines the balance between dough viscosity and elasticity (Veraverbeke and Delcour, 2002).

The covalent structure of the gluten network is superimposed by non-covalent bonds (hydrogen bonds, ionic bonds, hydrophobic bonds) (Domenek et al., 2003). Though these classes of chemical bonds are less energetic than covalent bonds, they are clearly implicated in gluten protein aggregation and dough structure (Kuktaite et al., 2004; Wieser, H., Bushuk, W., MacRitchie, F., 2006). Evidence for the presence of hydrogen bonds in gluten proteins are the

dough weakening effect of hydrogen bond breaking agents, e.g. urea, and the dough strengthening effect of heavy water compared with that of ordinary water (Tkachuk R., 1968) (Wieser, 2007). The importance of ionic bonds can be demonstrated by the strengthening effect of NaCl or of bipolar ions such as amino acids or dicarboxylic acids (Fu et al., 1996). Hydrophobic bonds contribute significantly to the stabilization of gluten structure (Kinsella and Hale, 1984; Iwaki et al., 2021). They are different from other bonds, because their energy increases with increasing temperature, which can provide additional stability during the baking process (Wieser, 2007). However, no method has been established to measure noncovalent bonds directly and changes in noncovalent bonds are still not well characterized in this context.

1.3.2 Thermal treatment of hydrated gluten during baking

At temperatures > 40 °C, the hydrophobicity of gluten increases due to conformational changes that expose hydrophobic groups of gluten. This is followed by the formation of covalent and non-covalent crosslinks between hydrated gluten proteins when heated to 50 °C and above (Guerrieri et al., 1996; Lagrain et al., 2005). As during dough formation, the key factor in further gluten polymerisation during subsequent heating is the formation of SS bonds due to SH oxidation and SH-SS interchange reactions. Hydrated gluten heated to ≥ 75°C leads to the formation of SS crosslinks between gliadins (mainly α- and γ-gliadins) and glutenins (Schofield et al., 1983; Singh and MacRitchie, 2004; Lagrain et al., 2008b; Ogilvie et al., 2021). This incorporation of gliadins into the glutenin structure results from SH-SS interchange reactions and follows a first-order rate law (Lagrain et al., 2008a).

Although most of the gluten crosslinking during heat treatment occurs through formation of additional SS bonds, crosslinks between other amino acids may also occur. Research by Tilley et al. (2001) found that bread baking led to an increase in dityrosine bonds (Figure 4C). Additionally, at high temperatures and pH ≥ 6, cysteine residues can undergo β-elimination to form dehydroalanine (DHA) and free SH groups in gluten proteins (Figure 3D) (Rombouts et al., 2010). Both reaction products can again participate in the formation of new crosslinks. For instance, DHA residues can react with cysteine to create the DHA crosslink lanthionine (LAN) (Figure 3C). At pH ≥ 10 lysinoalanine (LAL), a crosslink between DHA and lysine, can be formed between gluten proteins (Figure 4A) (Haraguchi et al., 1980; Rombouts et al., 2010). The free SH groups generated by β-elimination can also participate in oxidation or SH-SS interchange reactions, resulting in additional interchain SS bonds in gluten proteins (Lagrain et al., 2011).

Rombouts et al. (2011) indicated that heat treatment of gluten proteins in a dry state at neutral pH might also induce isopeptide bond formation between the ε-amino groups of lysine residues and the β- or γ-carboxamide groups of asparagine and glutamine residues (Figure 4B).

The Maillard reaction is yet another process that occurs during baking and involves the reaction between the ε-amino group of lysine and reducing sugars (Figure 4D). This reaction leads

to the formation of various Maillard reaction products, which play a significant role in the development of flavour and colour of foods. In some cases, Maillard reactions can crosslink two proteins by bridging amino acid side chains via a reducing sugar (Belitz et al., 2009). Identified Maillard-derived protein crosslinks in foods include histidino-threosidine (Dai et al., 2007), pentosidine, bispyrraline, and bisarg (Belitz et al., 2009). However, levels of Maillard-derived crosslinks of wheat proteins found in baked goods such as pretzels are very small (Henle et al., 1997).

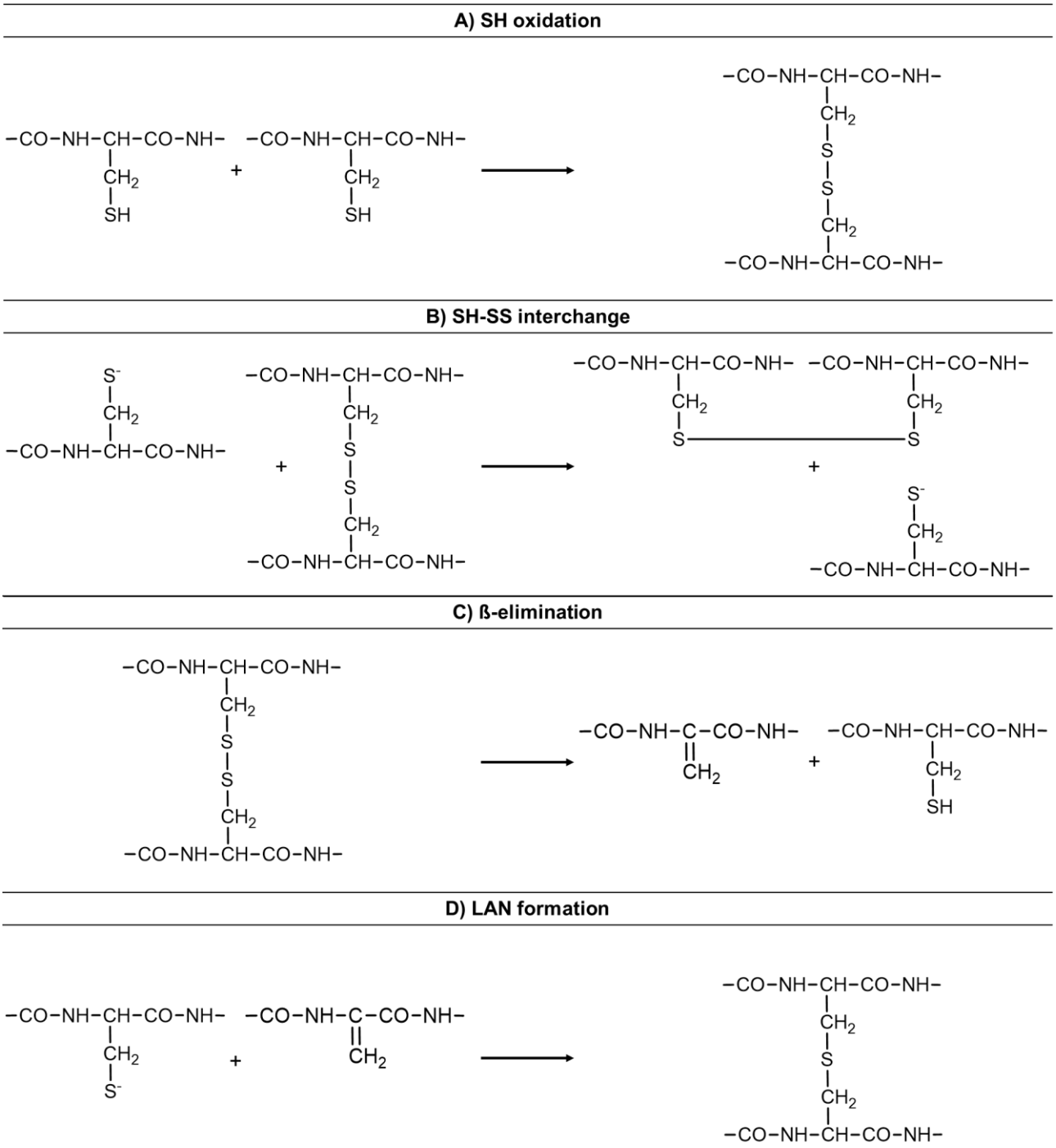

Figure 3. Schematic representation of reactions involving cyst(e)ine residues adapted from Rombouts et al. (2012). (A) Oxidation of cysteine residues with a free sulfhydryl (SH) to an intermolecular disulfide (SS) bond. (B) SH-SS interchange reaction transforming an intramolecular into an intermolecular SS bond. (C) Cleavage of an intramolecular SS bond by a β-elimination reaction leading to dehydroalanine (DHA) and cysteine residues. (D) Formation of a lanthionine (LAN) cross-link by the reaction between DHA and cysteine.

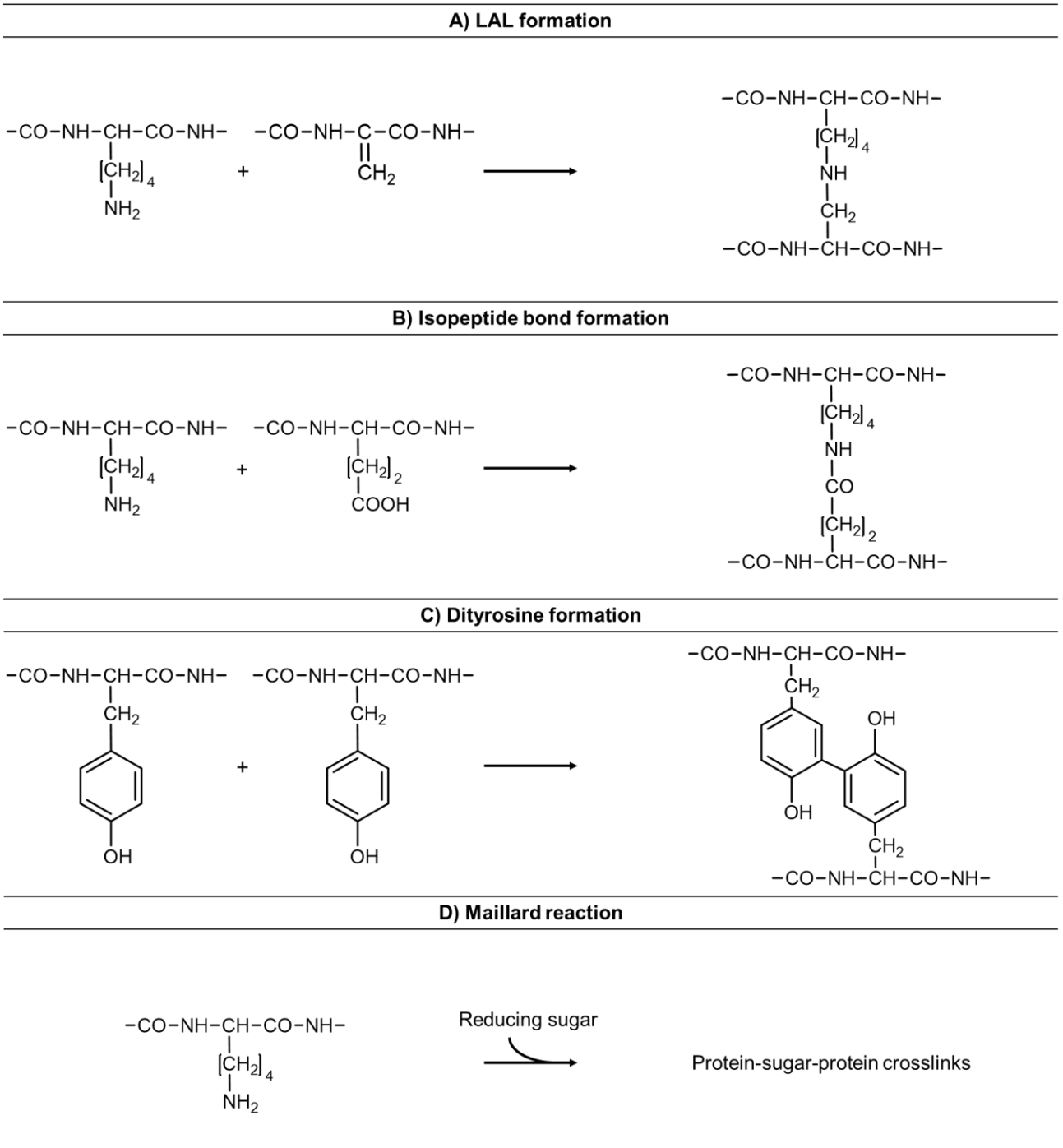

Figure 4. Schematic representation of reactions leading to covalent other than disulfide bonds. (A) Formation of a lysino-alanine (LAL) crosslink by the reaction between DHA and lysine residues. (B) Isopeptide bond formation between the ε-amino group of a lysine residue and the γ-carboxyl group of a glutamine residue. (C) Formation of dityrosine. (D) Maillard reaction involving the ε-amino group of a lysine residue and reducing sugars.

1.4 Wheat sensitivities

A small portion of the population has to avoid wheat-containing foods because of adverse immune reactions, commonly referred to as wheat sensitivities. Wheat sensitivities are a group of disorders with different clinical and pathogenic features, which are classified into three main forms: autoimmunogenic sensitivities including coeliac disease (CD), dermatitis herpetiformis, and gluten ataxia; allergic sensitivities including wheat-dependent exercise-induced anaphylaxis, immediate wheat allergy, respiratory allergy, and contact urticaria; and non-coeliac gluten sensitivity (NCGS) (Figure *5*) (Sapone et al., 2012).

Among them, CD is the most well-known form of wheat sensitivity. With an incidence of about 1%, it is one of the most frequent food sensitivities worldwide (Catassi et al., 2015).

The majority of wheat sensitivities are caused by consuming gluten-containing foods, which contain proline-rich sequences that are resistant to enzymatic breakdown in the gastrointestinal tract. As a result, relatively long peptides containing these sequences reach the lamina propria, where they elicit an immune response that leads to the development of the disease. The response can be initiated either by T-cell and antibody reactions (CD) or only by an antibody response (wheat allergy) (Scherf et al., 2016). The pathomechanism of NCGS is still poorly defined. Beside gluten, non-gluten proteins such as ATIs are suspected to play a role in its genesis (Biesiekierski et al., 2013; Fasano et al., 2015; Zevallos et al., 2017).

Individuals who have CD also need to avoid foods containing rye, barley, and sometimes oats, as these cereals contain sections of amino acid sequences that are homologous to wheat and hence may be harmful (Codex Standard 118-1979, 2008).

Accurate diagnosis and management of wheat sensitivities is challenging and requires a multidisciplinary approach involving gastroenterologists, dermatologists, and allergists.

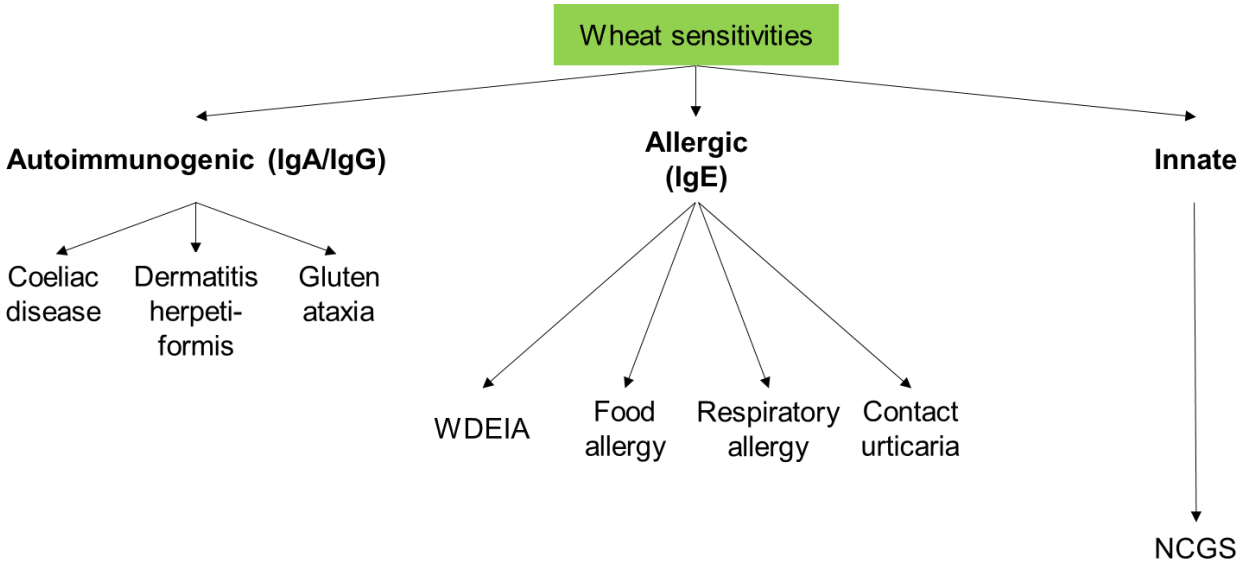

Figure 5. Overview of wheat sensitivities according to their pathomechanism. The classification is adopted from Sapone et al. (2012). Ig, Immunoglobulin; WDEIA, wheat-dependent exercise-induced anaphylaxis; NCGS, Non-coeliac gluten sensitivity.

1.5 Food processing and wheat sensitivities

Studies have shown that food processing such as baking reduces the gastrointestinal digestibility of gluten proteins (Simonato et al., 2001; Smith et al., 2015; Ogilvie et al., 2021). The release profile of immunogenic gluten peptides throughout in vitro digestion also significantly differs between flour and baked goods (Ogilvie et al., 2021).

However, the relationship between gluten protein structure, digestibility and immunogenicity is still poorly understood and there is a lack of clinical trials investigating the effects of these findings on individuals with wheat sensitivities.

Furthermore, recent research of Zimmermann et al. (2021) indicates that the protein composition and abundance of potentially allergenic proteins in flours from wheat, spelt, and rye rather depend on the grain type than on the processing conditions using different bread recipes as model system.

Food processing also has an impact on gluten detection, which can have consequences for managing the risks associated with foods containing gluten. The accuracy of gluten detection methods, such as the recommended (Codex Committee of Methods of Analysis and Sampling, 2015) and commonly used enzyme-linked immunosorbent assay (ELISA) can be influenced by the food matrix and processing conditions. The effect of thermal processing on gluten detection in wheat-based products by ELISA has been investigated by Bugyi et al. (2013), Gomaa and Boye (2013), Sharma et al. (2013), Török et al., (2014) and Pahlavan et al. (2016). In general, the detectability of gluten in processed foods such as bread or cookies is negatively

affected and processing conditions such as heating temperature and time are important factors. The reduced gluten recovery after baking is explained by loss of antibody-binding activity and/or solubility of gliadin due to the altered protein structure (Bugyi et al., 2013; Török et al., 2014; Pahlavan et al., 2016).

1.6 Methods for wheat protein characterization

1.6.1 Immunological methods

ELISA are commonly used for the detection of gluten in food products. The method is highly sensitive and specific, making it suitable for the detection of gluten traces (< 20 mg/kg) (Codex Standard 118-1979, 2008).

The principle of ELISA involves the specific binding of an antigen by antibodies, detection of the antigen-antibody complex through the addition of a labelled antibody, and quantitation of the complex via a colorimetric reaction.

In general, two types of ELISA methods are used for gluten detection: sandwich and competitive ELISA (Wieser et al., 2014). Sandwich ELISA is suitable for relatively large antigens such as intact gluten proteins, which have at least two antigen binding sites (epitopes), allowing for at least two different antibodies to bind. Competitive ELISA is used for the analysis of small-sized antigens such as gluten peptides, which have only one epitope. In gluten analysis, sandwich ELISA is typically used for raw materials or heat-processed products (e.g. flour, baked goods), while competitive ELISA is suitable for hydrolysed or fermented products (e.g. sourdough, beer) (Scherf et al., 2016).

The analytical technique of choice for this study is the sandwich ELISA. A monoclonal or polyclonal capture antibody with a known concentration is immobilized on a microtiter plate. The sample containing the antigen is added and allowed to incubate; during this time the capture antibody binds to the antigen. Next, an enzyme-labelled detection antibody is added, which binds to the second binding site of the antigen, thereby completing the sandwich antibody-antigen-antibody. Following another incubation, a substrate for the enzyme is introduced, which catalyses a colour reaction. Throughout the procedure, the microtiter plate is washed several times to eliminate any excess antigen and unbound detection antibodies. The resulting coloured end product is measured spectrophotometrically. The concentration of the antigen can be determined based on a calibration curve of a gluten reference material since the concentration is directly proportional to the absorbance in the sample.

Frequently used sandwich ELISA are based on the monoclonal antibodies (mAbs) 401.21 (Skerritt and Hill, 1990), G12 (Morón et al., 2008) and R5 (Valdés et al., 2003). The Codex Alimentarius recommended method for the analysis of gluten in food (Codex Standard 234-1999, 2014) is the Méndez method – a combination of R5 ELISA and a special, patented buffer (cocktail) for optimized extraction of gliadin from heat-processed and non-heated food samples (García et al., 2005; Méndez et al., 2005). R5 mAbs are raised against rye secalins and recognize the epitopes QQPFP, QQQFP, LQPFP and QLPFP (Kahlenberg et al., 2006), which

are present in many CD-toxic or immunogenic peptides of gliadins and corresponding prolamins from rye and barley (Tye-Din et al., 2010). R5 mAbs show only a very low reactivity towards glutenins and corresponding cereal glutelins (Lexhaller et al., 2016).

1.6.2 Gel-based method(s)

The qualitative wheat protein composition is routinely identified by SDS-PAGE. The method is based on the principle of separating proteins by their molecular weight using an electric field.

In order to compensate differences in molecular structure and charge, the proteins are totally unfolded and negatively charged by the treatment with the anionic detergent SDS (disruption of hydrogen bonds, providing negative charge density proportional to mass) and a reducing agent e.g. DTT (cleavage of SS bonds between cysteine residues).

The samples are loaded onto a gel made up of a matrix of crosslinked polyacrylamide molecules, which serves as a molecular sieve. An electric field is applied. Utilizing a buffer solution to conduct the current through the gel, the proteins migrate towards the positively charged electrode. The migration rate of each protein is determined by its molecular size. The smaller proteins can pass through the pores more easily, whereas the larger proteins take longer to pass through. For wheat protein analysis, a discontinuous Bis-Tris electrophoresis system with a neutral operating pH has been established (Kasarda et al., 1998; Lagrain et al., 2012).

After electrophoresis, the proteins are visualized by staining with e.g. Coomassie blue. The molecular weight of the separated proteins can be estimated by comparing their migration distance with a protein ladder, which is a mixture of known molecular weight marker proteins. The presence, intensity of the staining and "thickness" of protein bands are indicative of the relative protein abundance, which enables a semi-quantitative description of the results.

A typical SDS-PAGE of total protein extracts from wheat flour and products thereof is shown in Figure 6.

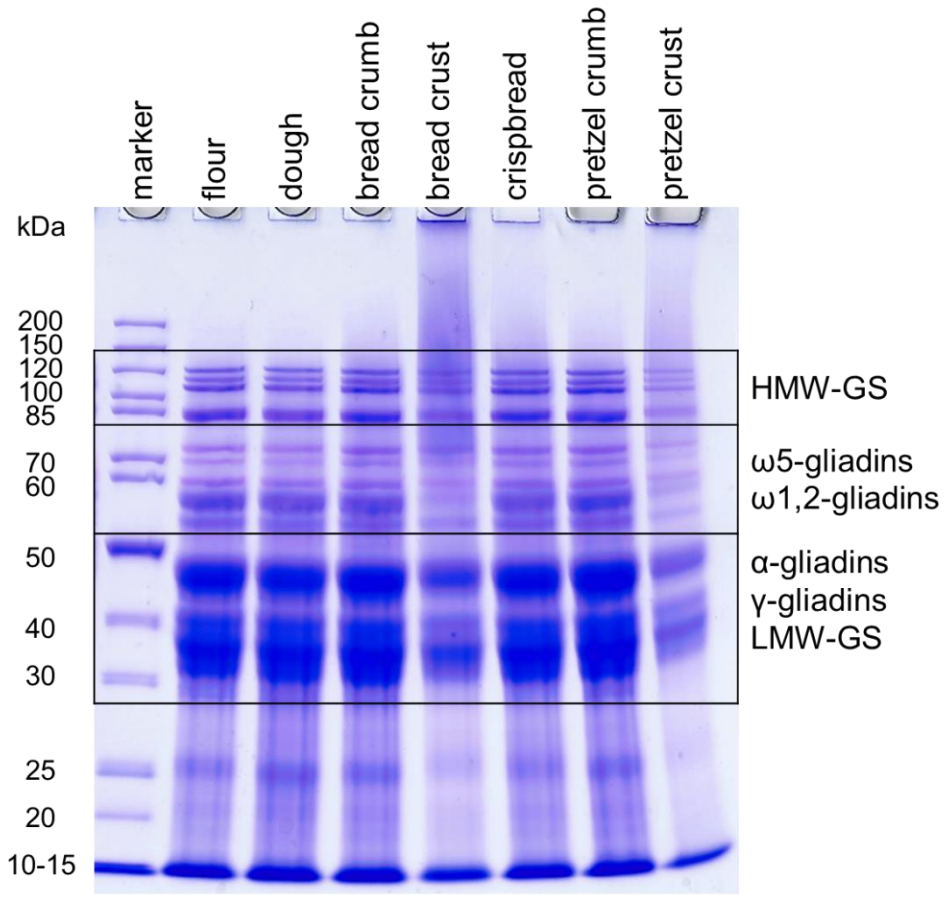

Figure 6. Typical SDS-PAGE (10%, Bis-Tris) of total protein extracts from wheat flour, dough and baked goods after staining with Coomassie blue. Gluten types are assigned according to Lagrain et al. (2012). HMW-GS, high-molecular-weight glutenin subunits; LMW-GS, low-molecular-weight glutenin subunits.

1.6.3 Chromatographic methods

The qualitative and quantitative wheat protein composition is usually determined by different types of high-performance liquid chromatography (HPLC). HPLC has several advantages over SDS-PAGE such as better resolution and automation (Labuschagne and Igrejas, 2020). The method utilizes a pressure-driven flow of a mobile phase through a column packed with a stationary phase. The mobile phase carries a liquid sample mixture through the column to the detector, and proteins separate due to different degrees of interaction with the stationary phase.

Different extraction protocols can be used for the HPLC analysis of wheat proteins. One commonly used method is the Osborne fractionation (Osborne, 1907). Following the protocol of Wieser et al. (1998), the different types of wheat proteins are sequentially extracted using phosphate buffered saline (ALGL), 60% ethanol (gliadins), and 50% 1-propanol under reducing conditions (DTT) at 60 °C (glutenins). The extracts are analysed using reversed-phase (RP)-HPLC, which separates proteins based on their hydrophobicity using a hydrophobic stationary phase made of silica gel coated with hydrophobic groups (alkyl chains, C18) (Figure 7). The more hydrophobic molecules will interact more strongly with the hydrophobic stationary phase, and will be retained on the column longer, while the more hydrophilic molecules will be eluted from the column more quickly.

RP-HPLC allows the separation of the gliadin extracts into ω5-gliadins, ω1,2-gliadins, α-gliadins, and γ-gliadins as well as ωb-gliadins and of the glutenin extracts into HMW-GS, and LMW-GS (Wieser et al., 1998) (Figure 7BC).

Another method for wheat protein extraction is the procedure of Thanhaeuser et al. (2014), which involves the use of a SDS-containing solution followed by an extraction with 50% 1-propanol solution under reducing conditions at 60 °C to obtain SDS-soluble and SDS-insoluble proteins. The extracts are analysed using gel permeation (GP)-HPLC, which separates proteins based on their molecular weight using a stationary phase made of a silica gel with well-defined pore sizes. The smaller molecules can enter the pores of the stationary phase and are therefore slowed down, while the larger molecules cannot enter the pores and are therefore eluted faster. A UV detector is used to measure the protein absorbance at 210 nm for both, RP- and GP-HPLC. The wheat protein content can be calculated using an external calibration with Prolamin Working Group (PWG) gliadin (van Eckert et al., 2006) for RP-HPLC analyses, and protein molecular weight markers for GP-HPLC analyses.

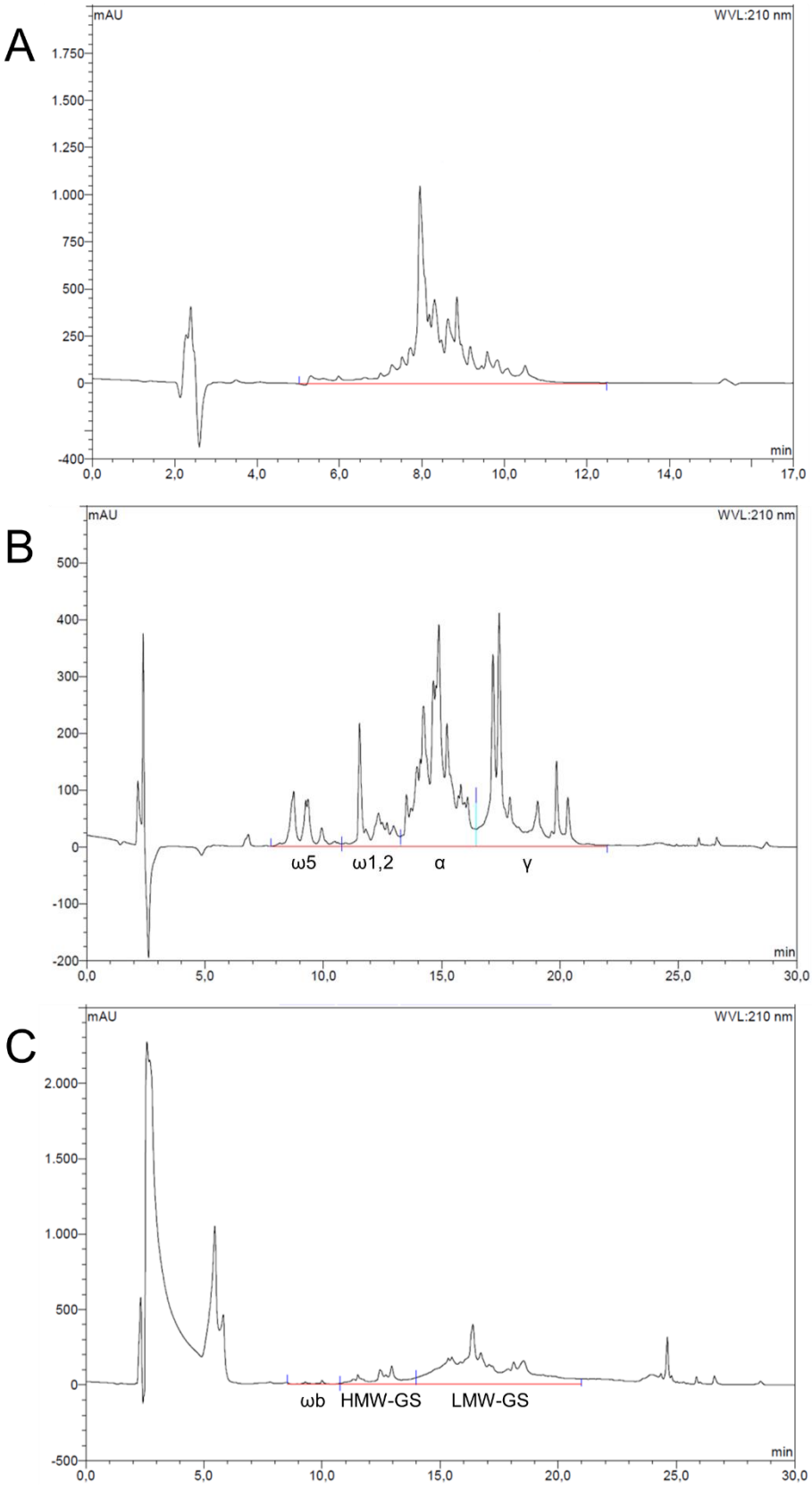

Figure 7: Exemplary RP-HPLC profiles of (A) albumins and globulins, (B) gliadins and (C) glutenins extracted from wheat flour via Osborne fractionation and measured at 210 nm. ω5, ω1,2, α, γ: gliadin types; ωb, glutenin-bound ω-gliadins; HMW-GS, high-molecular-weight glutenin subunits; LMW-GS, low-molecular-weight glutenin subunits.

1.6.4 Mass spectrometry-based methods

The most commonly used analytical methods to determine the qualitative and quantitative composition of wheat proteins (HPLC coupled to UV detectors, SDS-PAGE) are limited to the polarity (RP-HPLC) or the molecular weight (GP-HPLC and SDS-PAGE) of the analysed proteins. Meanwhile, bottom-up proteomics techniques based on mass spectrometry (MS) have been also well established and allow an advanced qualitative and quantitative characterization of wheat proteins.

In the traditional bottom-up approach, intact proteins are digested into peptides prior to introduction into the MS where they are then ionized, fragmented and detected.

Targeted liquid chromatography (LC)-tandem MS (LC-MS/MS) analyses selected peptides in multiple or parallel reaction monitoring mode, untargeted LC-MS/MS aims to analyse the totality of peptides in a sample. The quantitation can be classified according to the information that is provided into relative or absolute quantitation. Based on the underlying methodology, relative and absolute quantitation are label-based (stable isotopes labelling) or label-free (e.g. label-free or intensity based absolute quantitation). (Bantscheff et al., 2012; Nikolov et al., 2012)

The typical workflow for relative wheat protein quantitation by untargeted LC-MS/MS analysis includes the extraction of proteins, reduction of SS bonds, alkylation of cysteine residues, enzymatic hydrolysis of proteins to peptides and finally peptide analysis by (nano-)LC-MS/MS.

Due to its effectiveness, a modified Osborne fractionation is often used for wheat protein extraction (see 1.6.3). The extracts are concentrated before further workup. For the reduction of SS bonds either DTT or Tris(2-carboxyethyl)phosphine (TCEP) can be used. In contrast to DTT, TCEP does not react with the alkylation agent. For alkylation either iodoacetamide (IAA) or chloroacetamide (CAA) is used. Usually, chymotrypsin, pepsin, trypsin or a mixture of these enzymes are used for wheat protein hydrolysis (Colgrave et al., 2017). Chymotrypsin is able to cleave the peptide bonds on the C-terminal side of phenylalanine, tyrosine and tryptophan, which occur frequently in gluten and has been used by many researchers (Visioli et al., 2016; Bacala et al., 2020). The degradation of gluten proteins with chymotrypsin leads to a variety of peptides. In contrast, trypsin is only able to cleave at the carboxylic side of the amino acids lysine or arginine, which are not present in high amounts in gluten.

The peptide mixture is separated by RP-HPLC and introduced online into a hybrid MS via electrospray ionization, which converts the peptide into charged ions (Yamashita and Fenn, 1984).

For mass analysis, two different types of mass analysers are used. The mass analysis of the precursor ions resulting from the peptide ionisation is performed in a quadrupole ion trap. The

ion trap is based on trapping ions using radiofrequency electric fields. By changing the radiofrequency and direct current voltages, the ions can be manipulated. These manipulations include isolation of an ion with a specific mass to charge ration (m/z) and activation by putting kinetic energy into the ion with the specific m/z to cause fragmentation by collision with a background gas (collision induced dissociation) resulting in product ions. The mass analysis in the ion trap is done by selectively ejecting ions out of the trap into a pair of electron multipliers. This process is fast and allows for multiple tandem mass spectra to be obtained while a full scan (MS1) is being performed in the second type of mass analyser which is an Orbitrap.

The Orbitrap is a purely electrostatic trap, consisting of a cylindrical outer electrode and a barrel inner electrode that hold direct current potentials to trap the ions in circular orbits around the inner electrodes. The ions are collected in a device called a c-trap, which is an electrostatic trap in the shape of a C. The ions from the C-trap are then injected simultaneously into the orbitrap off axis. The ions than undergo axial motion at frequency related to the square route of the m/z of the ions. The frequency of the motion is measured to provide the m/z of the ions.

The precursor ions are selected either in a data-dependent acquisition (based on their relative intensity in the MS1 scan) or data-independent acquisition (all precursor ions within a defined m/z window) mode for fragmentation in the collision cell. (Perry et al., 2008; Zubarev and Makarov, 2013)

After the MS analysis, the experimental data are searched in silico against theoretical peptides created from a database using proteomics software e.g. MaxQuant (Tyanova et al., 2016). The protein database is typically a collection of protein amino acid sequences (e.g. in FASTA format) provided by resources such as UniProt.

For label-free quantitation (LFQ), the same peptides are compared in consecutive separate LC-MS/MS experiments. It is based on the observation that the amount of protein correlates well to the MS signal intensity of precursor ions belonging to the particular protein (MS1 based LFQ) (Cox et al., 2014). The signal intensities associated with a peptide peak are extracted based on the peptide precursor m/z generating an extracted ion chromatogram (XIC). An XIC is a plot of the intensity of a specific m/z value over the elution time. The area under the curve of the XIC is proportional to the abundance of the peptide and, subsequently, the proteins in the sample. The ratio of the XIC areas for each peptide is used to determine the relative abundance of each protein in the sample. (Rozanova et al., 2021)

In general, proteomics techniques based on MS have been successfully used to characterize wheat proteins in the context of grain development and quality as well as CD, which has been reviewed in detail by Alves et al. (2019), Labuschagne and Igrejas (2020) and Bacala et al. (2022). Untrageted LC-MS/MS analysis with Orbitrap instruments has been used for instance for the quantitation of ATIs in wheat (Geisslitz et al., 2022) or investigations on the allergenic

potential of bread (Ogilvie et al., 2020; Zimmermann et al., 2021). Vincent et al. (2022) presented a high-throughput label-free untargeted LC-MS/MS method suitable for exploring wheat flour proteoms.

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2 Objective of this thesis

Gluten network formation during processing results from the hydration of flour after water addition and input of mechanical energy during dough formation and further thermal treatment. It is responsible for the final product properties of wheat-based products and therefore of great interest concerning functional and quality aspects, but also physiologically relevant factors such as digestibility or allergenicity. Despite significant research, the underlying mechanism of inter- and intramolecular crosslinking is not yet fully understood. The complexity of the wheat proteome, which contains hundreds of very diverse proteins, makes its analysis challenging. In this context, proteomics techniques are promising but only little exploited analysis tools.

The conformational changes during wheat processing can also affect gluten analysis. Gluten analysis is important to determine whether products intended for individuals with CD are really gluten-free. Previous studies have explored the impact of food processing on gluten detection in wheat-based products using ELISA, the most common method for gluten detection. Generally, a decline in detectability has been observed after processing, such as heating, but the reasons for this are not yet fully understood.

The objective of this study was to elucidate the influence of processing on the qualitative and quantitative protein composition of wheat-flour based products. Therefore, wheat flour-based baked goods (bread, crispbread, and pretzels) were prepared to represent different degrees of processing. The study was divided into two parts: the first part of the study focused on the effect of the different baking conditions on the immunochemical detection of gluten by ELISA. ELISA analyses were combined with different extraction solvents and a comprehensive analysis of gluten extractability and composition (SDS-PAGE, RP-HPLC, GP-HPLC) was conducted to correlate to the ELISA results. Thiol, cysteine and glutathione concentrations of the samples were determined to examine SS and non-SS crosslinking.

The second part of the study investigated the effect of the different baking conditions on the proteome composition through an in-depth analysis of different protein fractions obtained by a modified Osborne fractionation. The application of SDS-PAGE, RP-HPLC and high resolution LC-MS/MS enabled the identification and quantitation of a large set of proteins and pointed out proteins with major abundance changes in comparison to their presence in flour. This is of special interest, since it not only indicates which particular proteins are involved in gluten network formation, but also highlights changes of solubility properties of wheat proteins that occur during processing.

3 Results

3.1 Influence of baking conditions on the extractability and immunochemical detection of wheat gluten proteins

The article “Influence of baking conditions on the extractability and immunochemical detection of wheat gluten proteins” was published in “Current Research in Food Science” (https://doi.org/10.1016/j.crfs.2022.100431) in 2022. The author of this thesis, Tanja Schirmer, developed the concept, generated and evaluated the data, wrote the manuscript, and revised it according to the reviewer comments.

Different wheat flour-based and incurred baked goods (bread, crispbread, pretzel) were prepared to investigate the influence of baking conditions on gluten quantitation by a commercial sandwich R5 ELISA. The incurred samples covered the threshold levels of gluten-free and low gluten foods (20, 50, 100 mg gluten/kg). Three extraction solvents (Cocktail, UGES, UPEX) for ELISA analysis were assessed and the Cocktail was found to be the preferred method. Regardless of the extraction solvent, less gliadin (20 to 50%) was detected in the baked goods than in the flour samples. Alkali treatment of the pretzel crust resulted in the lowest gliadin contents (< 12 mg/kg in 20 mg gluten/kg, < 33 mg/kg in 50 mg gluten/kg, < 66 mg/kg in 100 mg gluten/kg). Thus, common extraction solvents for ELISA do not achieve a complete gluten extraction from baked goods. However, the conversion factor of 2, which is usually applied to calculate the gluten content based on the gliadin content analysed by ELISA, would be sufficient for a correct labelling of most samples irrespective of the extraction solvent and processing condition. A comprehensive analysis of protein extractability and composition (SDS-PAGE, RP-HPLC, GP-HPLC) as well as of thiols and thiol-containing compounds (cysteine, glutathione) of the samples was conducted to establish a correlation with the ELISA results. SDS-PAGE revealed no clear differences in the qualitative protein composition. RP-HPLC was used to analyse Osborne extracts, GP-HPLC to analyse SDS-soluble and SDS-insoluble proteins. The findings from both RP- and GP-HPLC revealed that dough formation and baking decreased the overall protein extractability under both reducing and non-reducing conditions. The magnitude of the extractability losses was dependent on the processing conditions with dough formation resulting in a higher protein recovery (about 90%) than baking (75 to 83% recovery), and alkali dipping prior to baking resulting in the lowest protein recovery (58 to 61% recovery). This suggested that not only SS, but also non-SS crosslinking is involved in the formation of insoluble gluten proteins. The formation of non-SS crosslinks was further confirmed by a decrease of free SH (11 µmol to 5 µmol) and SS groups (120 µmol to < 88 µmol) and lower levels of total cysteine and glutathione in baked goods. There was no clear relationship between the protein composition and the ELISA results.

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Influence of baking conditions on the extractability and immunochemical detection of wheat gluten proteins

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ABSTRACT

Food processing conditions affect the accurate detection of gluten by ELISA, which is of importance for proper gluten-free labelling. We prepared different wheat flour-based and incurred baked goods (bread, crispbread, pretzel) to investigate the influence of baking conditions and alkali treatment on gluten quantitation by ELISA using different extraction solvents. Protein composition and extractability were determined (SDS-PAGE, RP-HPLC, GP-HPLC). The extraction solvents showed different performances; none of them could compensate the effect of baking on the detection. Dough preparation, baking and additional alkali treatment decreased protein extractability under reducing and non-reducing conditions. High temperature combined with alkali treatment resulted in the lowest protein extractabilities (<77% for bread crust, <61% for pretzel crust) due to the formation of disulfide and non-disulfide gluten crosslinks. There was no clear correlation between the protein composition and the extractability of alcohol- and SDS-soluble proteins of the baked goods. Thus, this research shows that gluten extractability rather than gluten composition is crucial for detection by ELISA in baked goods.

1. Introduction

Gluten, the storage proteins of wheat, consist of two major fractions: mostly monomeric and partially oligomeric gliadins and polymeric glutenins. Based on their relative molecular masses (M_r) and similar amino acid sequences, gliadins are subdivided into α-, γ- and ω-gliadins, and glutenins into low-molecular-weight (LMW) and high-molecular-weight (HMW) glutenin subunits (GS) (Wieser et al., 2020, 2022). When mixed with water they form a cohesive viscoelastic network and undergo further structural changes during thermal treatment. This property of gluten makes wheat suitable for the preparation of a great diversity of staple foods such as bread or pasta. Therefore, wheat is one of the most cultivated crops worldwide (FAOSTAT, 2022). Understanding gluten network formation is important for product quality as well as for analytical gluten detection methods, since there is at least 1% of the human population who suffer from celiac disease and need to follow a gluten-free diet (Lerner et al., 2019).

Many studies that investigated gluten network formation during dough preparation and baking are based on the solubility properties of gliadins and glutenins. Gliadins are soluble in aqueous alcohols. Solvents containing denaturing agents such as sodium dodecyl sulphate (SDS) and/or urea are also often used (Delcour et al., 2012). In contrast to gliadins, which contain only intramolecular or no disulfide (SS) bonds, glutenins are connected through intermolecular SS bonds. After reduction of the SS bonds, they become soluble in aqueous alcohol similar to gliadins (Wieser et al., 2022).

Heat treatment of gluten leads to large protein aggregates with further polymerisation of glutenins, formation of gliadin-glutenin bonds and oxidation of the free thiol groups (SH) of cysteine. The incorporation of gliadins into the glutenin structure via intermolecular SS bonds results from SH-SS interchange reactions (Wieser, 1998; Lagrain et al., 2008a). Besides SS crosslinking, gluten network formation also involves non-covalent interactions (hydrogen, ionic, hydrophobic) as well as covalent non-SS crosslinking (Wieser et al., 2022). Additional non-SS bonds between gluten proteins and other food matrix components formed during thermal processing are tyrosine-tyrosine (Tilley et al., 2001), tyrosine-dehydroferulic acid (Piber and Koehler, 2005), isopeptide (Rombouts et al., 2011), Maillard (Gerrard et al., 2003; Dai et al., 2007) and dehydro amino acid derived crosslinks (Rombouts et al., 2010).

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Currently, the recommended method for gluten analysis to assess whether gluten-free products test below the threshold of 20 mg/kg is the R5 ELISA using the so-called Cocktail as extraction solvent (CXS 118-1979, 2008). It contains a reducing agent (2-mercaptoethanol) and a disaggregating agent (guanidine hydrochloride) in aqueous buffer and was developed for a complete extraction of gliadins from both unprocessed and heat processed foods (García et al., 2005). Due to the toxicity and incompatibility of the Cocktail with other immunoassays (Doña et al., 2008; Mena et al., 2012), several alternative extraction solvents have been described (Gessendorfer et al., 2010; Mena et al., 2012; Ito et al., 2016; Segura et al., 2021), including universal gluten extraction solution (UGES) and universal prolamin and glutelin extractant solution (UPEX).

The effect of thermal processing on gluten detection in wheat-based products by ELISA has been investigated by Bugyi et al. (2013), Gomaa and Boye (2013), Sharma et al. (2013), Török et al. (2014) and Pahlavan et al. (2016). In general, the detectability of gluten in processed foods such as bread or cookies is negatively affected and processing conditions such as heating temperature and time are important factors. The decrease of gluten recovery after baking is explained by loss of antibody-binding activity and/or solubility of gliadin due to the altered protein structure (Bugyi et al., 2013; Török et al., 2014; Pahlavan et al., 2016). Furthermore, the sensitivity of the detection method to a specific antigen can be influenced by the extraction buffer and protocol applied (Gomaa and Boye, 2013). However, a lower detection of gluten does not necessarily indicate a decreased immunoreactivity for celiac disease or wheat allergic patients (Taylor et al., 2002; Sharma et al., 2013).

So far, studies on the influence of food processing on gluten detection by ELISA did not provide a concurrent analysis of their sample material, leaving a knowledge gap between the altered gluten structure and the detection ability.

Our aim was to contribute to a better understanding of the complex structural changes of gluten during processing leading to a decreased gluten detection. We prepared different baked goods (bread, crispbread, pretzel) and tested different extraction solvents (Cocktail, UGES, UPEX) to achieve the best possible ELISA results. This was followed by a comprehensive analysis of gluten extractability, composition and thiol concentrations to examine gluten network interconnectivity.

2. Material and methods

2.1. Material

Wheat, cultivar Akteur, harvested 2015, was kindly provided by I.G. Pflanzenzucht GmbH (Munich, Germany). The kernels were milled into flour using a Quadrumat Junior laboratory mill (Brabender, Duisburg, Germany) according to AACCI Method 26 - 50.01, sieved (mesh size 200 μm) and stored at 22 °C for 2 weeks before use. Gluten-free rice flour was donated by BFree Foods (Dublin, Ireland). Brauerei Wieninger (Teisendorf, Germany) provided fresh baker's yeast. R5 ELISA Ridascreen Gliadin (R7001) and Cocktail (patent WO 02/092633 A1) were from R-Biopharm AG (Darmstadt, Germany). UGES (patent ES 2 392 412 A1) was from Biomedal Diagnostics (Seville, Spain). PageRuler Unstained Protein Ladder and NuPage Bis-Tris Plus Gel (10%, pH 6.4, 1.0 mm × 10 wells) were from Thermo Fisher Scientific (Braunschweig, Germany). All chemicals were of analytical or higher quality and were from Merck KGaA (Darmstadt, Germany), Sigma-Aldrich (Steinheim, Germany) or SERVA Electrophoresis GmbH (Heidelberg, Germany). Prof. Dr. Koehler, Chairman of the Working Group for Prolamin Analysis and Toxicity, provided the reference material Prolamin Working Group (PWG)-gliadin (van Eckert et al., 2006). Water for chromatographic separations was purified using a Milli-Q Gradient A10 system (Millipore, Schwalbach, Germany).

2.2. Preparation of wheat flour-based products

Dough: Dough was made by mixing flour with 3% yeast and 2% NaCl using a kitchen aid (1 min, level 1) and addition of water as determined according to AACC method 54-50.01. The dough was kneaded for 3 min at level 1 and 3 min at level 3. One portion for analysis was immediately frozen and freeze-dried. The remaining dough was placed in a water-saturated proofer to rest for 20 min at 30 °C. *Crispbread*: The fermented dough was rolled as thin as possible and pricked all over with a fork before baking (9 min at 230 °C). *Bread*: About 300 g of the fermented dough was manually moulded, and put into a tin pan for proofing for 40 min (at 30 °C, 90% relative humidity) and baking (25 min at 230 °C with initial steam injection). *Pretzel*: Similar to the bread preparation, pieces of approx. 55 g of the fermented dough were hand-shaped into balls and proofed for 40 min (at 30 °C, 90% relative humidity). Afterward, the products were refrigerated (15 min, 5 °C) and shortly dipped in a 1.25 mol/L sodium hydroxide (NaOH) solution before baking (17 min at 230 °C with initial steam injection).

After baking, all baked goods were cooled for 2 h at 22 °C. Bread and pretzels were manually separated into crumb and crust. All baked goods were freeze-dried and milled (6000 rpm, 200 μm ring sieve) using an Ultra Centrifugal Mill ZM 200 (Retsch, Haan, Germany). The resulting sample material was stored air tight at 22 °C.

2.3. Preparation of wheat flour-incurred products

2.3.1. Wheat flour-spiked rice flours

Gluten-free rice flour was spiked with wheat flour (100.7 ± 1.4 mg gluten/g as determined by RP-HPLC) to obtain three spiked rice flour mixes containing 20, 50 and 100 mg gluten/kg, respectively. The spiking was performed in two steps. The rice flour was mixed with wheat flour to obtain a dilution of 1000 mg gluten/kg. This mixture was used to prepare the final dilutions. All spiked samples were mixed in a Turbula shaker-mixer (Glen Mills Inc., Clifton, USA) for 12 h to ensure sufficient homogeneity, as reported earlier (Schopf and Scherf, 2018).

2.3.2. Preparation of incurred baked goods

Crispbread, bread and pretzels were produced from each of the three spiked rice flour mixes (20, 50, 100 mg gluten/kg) as described above. The addition of water was 64% (w/w).

2.4. Crude protein and moisture content

The nitrogen content of wheat flour and wheat flour-based products was determined according to the method of Dumas using a TruSpec Nitrogen Analyzer (Leco, Moenchengladbach, Germany) and converted to crude protein content using a conversion factor of 5.7 as stated in ICC Standard 167 (International Association for Cereal Science and Technology, 2000). The moisture content of all flours and flour-based products was determined using an Infrared Moisture Analyzer MA35 (Sartorius, Goettingen, Germany) at 100 °C until the residual weight remained constant. All measurements were performed in triplicates.

2.5. R5 ELISA

The R5 ELISA Ridascreen Gliadin was performed according to the manufacturer's instructions. Wheat flour, wheat flour-based, wheat flour-spiked and wheat flour-incurred samples (3 replicates for samples containing 20 mg gluten/kg, 6 replicates for all other samples) were extracted with either 2.5 mL Cocktail, UGES or UPEX. Cocktail and UGES were purchased. UPEX (5 mmol/L tris(2-carboxyethyl)phosphine (TCEP), 2% (w/v) *N*-lauroylsarcosine in phosphate buffered saline (PBS), pH 7.0) was prepared according to Mena et al. (2012). TCEP and *N*-lauroylsarcosine were added to the PBS immediately before use. Samples were diluted with 60% (v/v) ethanol and in the last dilution step with the particular extraction solvent to final dilution factors of 500

for 20 mg gluten/kg of spiked flour and incurred materials, of 1000 for 50 mg gluten/kg of spiked flour and incurred materials, of 2500 for 100 mg gluten/kg of spiked flour and incurred materials, and of 2,500,000 for wheat flour and wheat flour-based products. The cubic spline function of the software RIDASOFT Win (version 1.93) was used to calculate the gliadin content from the absorbance at 450 nm. The dry matter of the sample material was considered.

2.6. SDS-PAGE

SDS-PAGE was carried out according to Lagrain et al. (2012) using a XCELL Surelock Mini-Cell electrophoresis system (Thermo Fisher Scientific, Braunschweig, Germany), a homogeneous NuPAGE Bis-Tris gel (10%) and a PageRuler Unstaind Protein Ladder as molecular weight marker. In brief, about 25 mg of wheat flour and wheat flour-based products were dissolved in 1 mL of extraction buffer and incubated under reducing conditions overnight at 22 °C. Then, the suspensions were shaken for 10 min at 700 rpm and 60 °C and centrifuged. The supernatants (10 μL) were loaded onto the gel. Gel electrophoresis was performed with a 3-(*N*-morpholino)propanesulfonic acid (MOPS) running buffer and the following running conditions: current: 115 mA; voltage 200 V, power: 30 W, time: 20 - 30 min. Proteins were fixed for 30 min with 12% trichloroacetic acid, stained for 30 min with 0.25% Coomassie Brilliant Blue R-250 and destained for 15 min with methanol/water/acetic acid (50/40/10, v/v/v) and in water/methanol/-acetic acid (80/10/10, v/v/v) overnight.

2.7. Determination of protein composition by Osborne fractionation

Modified Osborne fractionation was carried out according to Wieser et al. (1998). All samples were extracted in triplicate. In brief, wheat flour and wheat flour-based products (100 mg) were extracted twice with 1.0 mL 0.067 mol/L Na_2HPO_4/KH_2PO_4 buffer (pH 7.6) and 0.4 mol/L NaCl (salt-soluble proteins). Then, the residues were extracted three times with 0.5 mL 60% (v/v) ethanol (alcohol-soluble proteins). Last, the residues were extracted twice with 1.0 mL of 50% (v/v) propan-1-ol, 0.05 mol/L Na_2HPO_4/KH_2PO_4 (pH 7.5) and 1% (w/v) DTT under nitrogen atmosphere at 60 °C (alcohol-insoluble proteins).

An UltiMate 3000 instrument (Dionex, Idstein, Germany) with an Acclaim 300 C_{18} column (2.1 × 150 mm, Thermo Fisher Scientific, Braunschweig, Germany) and the software Chromeleon was used for reversed-phase high-performance liquid chromatography (RP-HPLC). The instrument was set to: column temperature: 60 °C, flow rate: 0.2 ml/min, injection volume: 20 - 50 μL (salt-soluble proteins), 10 - 20 μL (alcohol-soluble and alcohol-insoluble proteins), solvent: water/trifluoroacetic acid (TFA) (999/1, v/v) (A) and acetonitrile (ACN)/TFA (999/1, v/v) (B), gradient for salt-soluble proteins: 0 min 0% B, 0.5 min 20% B, 7 min 60% B, 7.1 min 90% B, 11.1 min 0% B, gradient for alcohol-soluble and alcohol-insoluble proteins: 0 min 0% B, 0.5 min 24% B, 20 min 56% B, 20.1 min 90% B, 24.2 min 0% B, detection: UV absorbance at 210 nm. PWG-gliadin in 60% (v/v) ethanol was used for an external calibration. The peaks for salt-soluble proteins had a retention time of 5.0 - 12.0 min. The peak profiles of alcohol-soluble and alcohol-insoluble protein extracts were further divided into four or three fractions, respectively, as described by Wieser (1998). ω5-Gliadins occurred from 7.0 to 10.8 min, ω1,2-gliadins from 10.8 to 13.2 min, α-gliadins from 13.2 to 16.5 min and γ-gliadins from 16.5 to 22.0 min. Glutenin-bound ω-gliadins (ωb-gliadins) were eluted from 7.0 to 10.8 min, HMW-GS from 10.8 to 14.0 min and LMW-GS from 14.0 to 22.0 min. Protein content is given as mg/g based on dry matter.

2.8. Determination of SDS-soluble proteins and SDS-insoluble proteins

The extraction protocol was adapted from Thanhaeuser et al. (2014). All samples were extracted in triplicate. Wheat flour and wheat flour-based products (100 mg) were extracted twice with 1.0 mL of 0.05 mol/L Na_2HPO_4/KH_2PO_4 buffer (pH 6.9) and 1% (w/v) SDS (SDS-soluble proteins). Then, the residues were extracted twice again with 1.0 mL of 50% (v/v) propan-1-ol, 0.05 mol/L Na_2HPO_4/KH_2PO_4 buffer (pH 7.5) and 1% (w/v) DTT under nitrogen atmosphere at 60 °C (SDS-insoluble proteins). The suspensions were centrifuged (20 min, 4600×g, 22 °C) and the resulting supernatants were combined. Supernatants of the first extraction were filled up to 5.0 mL, supernatants of the second extraction up to 2.0 mL with the corresponding extraction solution. All supernatants were filtered (Whatman, Spartan 13/0.45 RC, GE Healthcare, Freiburg, Germany) prior to analysis by gel permeation(GP)-HPLC.

An XLC instrument (Jasco, Gross-Umstadt, Germany) with a BioSep SEC S3000 column (4.6 × 300 mm, Phenomenex, Aschaffenburg, Germany) and the software ChromPass was used for the analysis of the protein fractions. The instrument was set to: column temperature: 22 °C, flow rate: 0.3 ml/min, injection volume: 20 - 60 μL (SDS-soluble proteins), 5 - 10 μL (SDS-insoluble proteins), solvent: water /TFA (999/1, v/v) (A) and ACN/TFA (999/1, v/v) (B), isocratic 50% A/50% B, detection: UV absorbance at 210 nm.

PWG-gliadin was used for an external calibration as described above. The peaks had a retention time of 6.0 - 13.0 min. The peak profiles of SDS-insoluble protein extracts were further divided into three fractions as described by Thanhaeuser (2015). HMW-GS appeared from 6.0 to 8.2 min, LMW-GS from 8.2 to 10.3 min and residual ALGL from 10.3 to 13.0 min. Protein contents are given as mg/g based on dry matter.

2.9. Determination of alcohol-soluble proteins by GP-HPLC

Wheat flour and wheat flour-based products (100 mg) were extracted twice directly with 1.0 mL of 60% (v/v) ethanol as described above. After splitting into two aliquots, one aliquot was further incubated under reducing conditions with excess DTT for 30 min at 60 °C. GP-HPLC analysis was performed with an Extrema LC-4000 instrument (Jasco, Gross-Umstadt, Germany) as described above. Retention times were assigned to relative M_r ranges by analysing a gel filtration M_r marker kit from Sigma-Aldrich (Steinheim, Germany). It contained the following proteins: β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), albumin (66 kDa), carbonic anhydrase (29 kDa) and cytochrome C (12.4 kDa). The M_r markers of 66 and 200 kDa eluted in one peak, to which proteins with M_r ranging from 60 to 200 kDa were assigned. All samples were extracted in triplicate.

2.10. Determination of free thiols and disulfide bonds

Wheat flour and wheat flour-based products (10 mg) were suspended in 900 μL of Na_2HPO_4/NaH_2PO_4 buffer (0.05 mol/L, pH 6.5, 3 mol/L urea, 0.001 mol/L EDTA and 2% (w/v) SDS), further referred to as sample buffer. The samples were shaken for 60 min at 500 rpm and 22 °C. Then, 100 μL of 5,5′-disulfanediylbis(2-nitrobenzoic acid) (0.1% (w/v) in sample buffer) was added and the mixtures were incubated for 45 min at 500 rpm and 22 °C. Samples were centrifuged (11,000×g, 5 min, 20 °C). The absorbance of the supernatants was immediately measured with an UV-2401PC spectrophotometer (Shimadzu, Kyoto, Japan) at 412 nm. Controls containing no sample were used to correct for background absorbance. Absorbance values were converted to levels of free SH (μmol SH/g protein) using a calibration with reduced glutathione (0 - 15 μmol). To quantitate SS bonds, samples were reduced with 200 μL of $NaBH_4$ (2.5%, w/v) and shaken for 60 min at 500 rpm and 50 °C. Excess $NaBH_4$ was degraded by adding 100 μL HCl (1 mol/L) and further shaking for 30 min at 500 rpm and 22 °C. Free SH were determined as described earlier. Oxidized glutathione was used for calibration (0 - 15 μmol). Levels of SS bonds (μmol SS/g protein) were corrected for the amount of free SH (subtraction of mean).

2.11. Determination of cysteine and glutathione

The concentrations of total glutathione (GSH) and cysteine (CSH)

were determined by a stable isotope dilution assay using LC-MS/MS as described by Schmid et al. (2016). For the reduction of SS bonds, 20 mg of wheat flour and wheat flour-based processed foods were suspended in 200 µL of an aqueous TCEP solution (5 mg/mL). Then, 20 µL of internal standard GSH ([$^{13}C_2$], [^{15}N]; c = 25 µg/mL) and CSH ([$^{13}C_3$], [^{15}N]; c = 16 µg/mL) dissolved in 5% (v/v) perchloric acid, as well as 200 µL TCEP (c = 5 mg/mL) were added. Samples were shaken for 30 min at 500 rpm and 22 °C in the dark. Then, 600 µL of iodoacetic acid (0.02 mol/L) in a H_3BO_3/LiOH buffer (0.5 mol/L, pH 8.5) were added. Samples were shaken for 30 min at 500 rpm and 22 °C in the dark. Afterward, 500 µL dansyl chloride (3.7 mmol/L in ACN) was added, and the mixture was shaken again for 1 h at 500 rpm and 22 °C in the dark. Last, 500 µL of dichloromethane was added, quickly mixed using a vortex mixer and centrifuged (16,000×*g*, 10 min, 22 °C). The supernatants were filtered and centrifuged in Vivaspin centrifugal concentrators (cut-off <3000 g/mol, 16,000×*g*, 22 °C) overnight. The permeates were analysed using a TSQ Quantum Discovery LC-MS/MS system coupled with a Finnigan Surveyor Plus HPLC system (Thermo Fisher, Braunschweig, Germany) with a Synergi Hydro-RP column (2 × 150 mm, 4 µm particle size, 8 nm pores, Phenomenex, Germany) and the software Skyline (version 4.2.0). The HPLC system was set to: flow rate: 0.2 ml/min, injection volume: 10 µL, solvent: water/formic acid (FA) (999/1, v/v) (A) and ACN/FA (999/1, v/v) (B), linear gradient: 0 min 0% B, 25 min 100% B. The MS was operated in electrospray ionization positive mode with capillary temperature: 290 °C, sheath gas pressure: 30 arbitrary units, auxiliary gas pressure: 10 arbitrary units, spray voltage: 4 kV, capillary offset: 35 V, collision cell pressure: 67 Pa, scan time 200 ms.

2.12. Statistical analysis

One-way analysis of variance (one-way ANOVA) with Tukey's test at a significance level of $p < 0.05$ was applied to determine significant differences between analysis results (SigmaPlot version 12.0, Systat Software, San Jose, USA).

3. Results

3.1. Investigation of different extraction solvents for ELISA

The wheat flour and wheat flour-based samples were extracted only with Cocktail since gluten analysis by ELISA is primarily relevant for low gluten products. Fig. 1 shows that the gliadin content of bread, crispbread and pretzel samples was significantly lower than the gliadin content of the wheat flour. Pretzel crust had the lowest detectable gliadin content (42 g/kg).

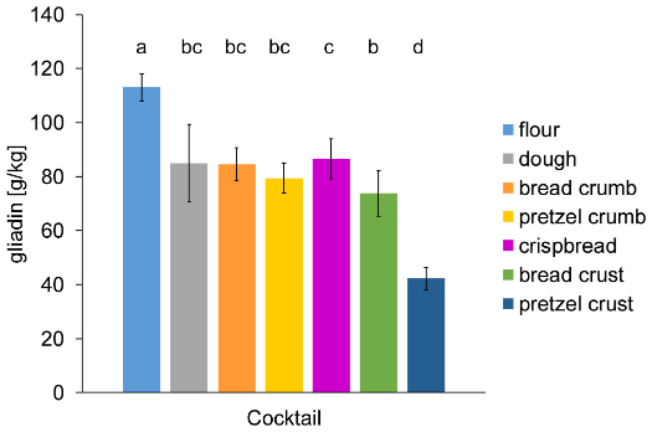

Fig. 1. Gliadin content of wheat flour, dough and baked goods quantitated by R5 ELISA after extraction with Cocktail. Data represented are the means ± standard deviation (n = 3). Means with different small letters are significantly different (one-way ANOVA, Tukey's test, $p \leq 0.05$).

Samples, which were spiked or incurred with different levels of wheat flour (20, 50, 100 mg gluten/kg) were additionally extracted with UGES and UPEX (Fig. 2). The gliadin recovery is expressed as gliadin content analysed relative to the spiked gluten protein content in the following. Alternatively, the gliadin content could be multiplied by 2, which is recommended as gliadin to gluten conversation factor by the Codex Alimentarius (CXS 118-1979, 2008). However, studies have shown that this factor is inaccurate in many cases leading either to over- or underestimation of the real gluten content (Wieser and Koehler,

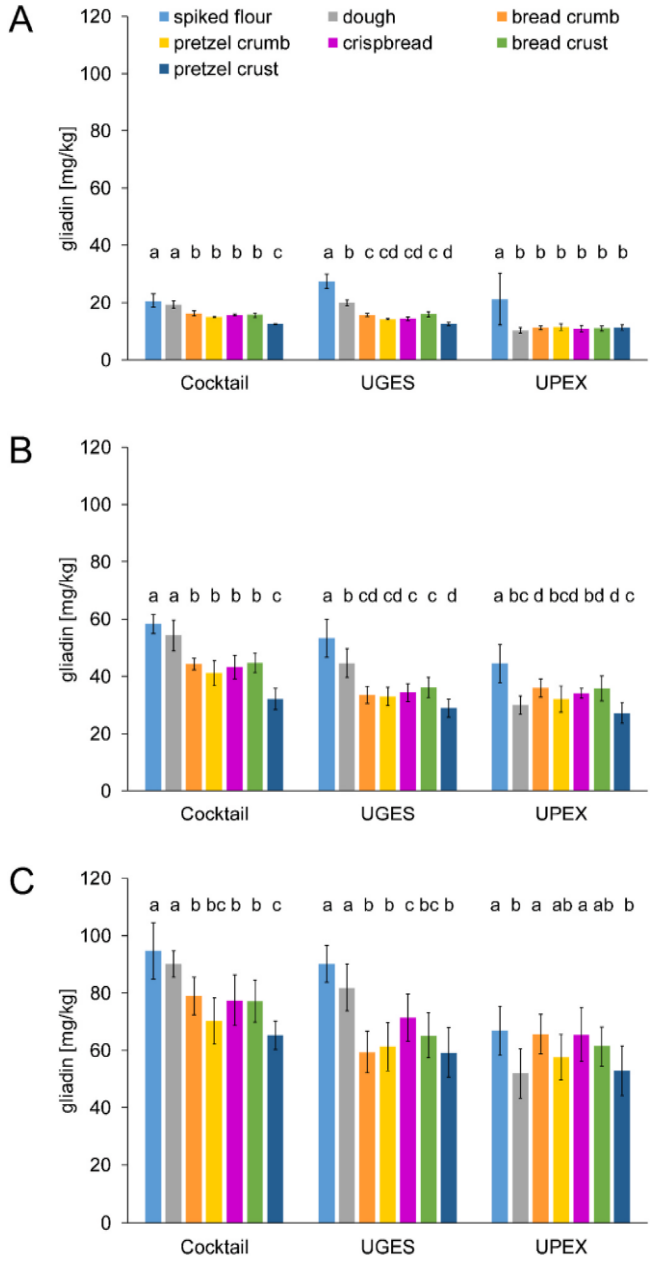

Fig. 2. Gliadin content of wheat flour-spiked rice flour, dough and baked goods quantitated by R5 ELISA after extraction with Cocktail, UGES (universal gluten extraction solution) and UPEX (universal prolamin and glutelin extractant solution). Target levels of 20 mg gluten/kg (A), 50 mg gluten/kg (B) and 100 mg gluten/kg (C). Data represented are the means ± standard deviation (n = 3). Different small letters represent a significant difference (one-way ANOVA, Tukey's test, $p \leq 0.05$) between samples extracted with the same extraction solvent.

2009).

All extraction solvents provided a good gliadin recovery (>80%) for the flours spiked with 20 and 50 mg gluten/kg. Sufficient gliadin was also extracted with Cocktail and UGES from their corresponding doughs. The gliadin content of all baked goods incurred with 20 and 50 mg gluten/kg was significantly lower than that of the spiked flours (Fig. 2 AB). No extraction solvent could compensate the effect of baking on the detection. UPEX showed the lowest extraction efficiencies, especially for the baked goods incurred with 20 mg gluten/kg, but also the dough samples. Results for samples spiked and incurred with 100 mg/kg gluten were similar to those with 20 and 50 mg gluten/kg (Fig. 2 C). However, the extractability of gliadin from flour spiked with 100 mg gluten/kg was less with UPEX in comparison to the other extraction solvents (67 mg/kg in comparison to > 90 mg/kg). For this reason, effects of the baking process were less significant if extracted with UPEX. In general, gliadin levels of bread, crispbread and pretzel samples ranged from about 16–45% less in Cocktail extracts, about 21–54% less in UGES extracts and about 2–48% less in UPEX extracts in comparison to their corresponding flours. The extraction with Cocktail achieved a slightly better gliadin recovery from baked goods in comparison to the extraction with UGES and UPEX. There was no clear correlation between the gliadin content analysed and the degree of heating, because the gliadin contents analysed in the crumb, crispbread, and bread crust were in the same range. However, additional alkali treatment of the pretzel crust resulted in the lowest gliadin content as also seen for the wheat flour-based samples (<12 mg/kg in 20 mg gluten/kg, <33 mg/kg in 50 mg gluten/kg, <66 mg/kg in 100 mg gluten/kg).

3.2. Investigation of protein extractability

3.2.1. Qualitative protein composition

SDS-PAGE of protein extracts from wheat flour, dough, bread, crispbread and pretzel samples showed the expected band patterns typical for wheat proteins, cv. Akteur, as described by Lagrain et al. (2012) (Fig. 3). The band patterns of all samples were similar, indicating that the qualitative composition was comparable. Only the bread crust and pretzel crust showed a weaker intensity of some bands, especially bands related to α- and γ-gliadins and LMW-GS. Both crust samples also contained very large protein aggregates, which did not migrate into the gel.

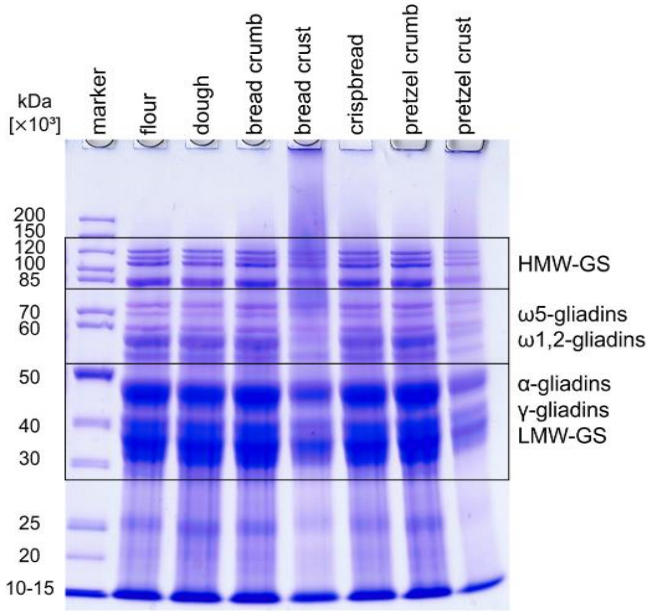

Fig. 3. SDS-PAGE (10% Bis-Tris) of total protein extracts from wheat flour, dough and baked goods. HMW-GS, high-molecular-weight glutenin subunits; LMW-GS, low-molecular-weight glutenin subunits.

3.2.2. Quantitative protein composition

The quantitative protein composition of the wheat flour, dough, bread, crispbread and pretzel samples was determined by RP-HPLC and by GP-HPLC (Fig. 4). Independent of the extraction protocol and the kind of HPLC analysis, dough preparation and baking decreased protein recovery, which is the protein content analysed relative to the total crude protein content. Baking (75 - 83% recovery) decreased protein extractability more than dough preparation (about 90% recovery). Additional alkali treatment further reduced protein recovery in the pretzel crust (58 - 61% recovery).

The proportion of salt-soluble, alcohol-soluble and alcohol-insoluble proteins of flour and dough was similar (Fig. 4 A). In contrast, baking decreased the levels of salt-soluble and alcohol-soluble proteins and increased the levels of alcohol-insoluble proteins. The composition of proteins extracted from the crumb and crust samples was similar ranging from 3.3 (bread crumb) to 5.5 (pretzel crust) mg salt-soluble protein/g, 7.4 (bread crumb) to 12.5 (bread crust) mg alcohol-soluble protein/g, and 86.8 (crispbread) to 99.8 (bread crumb) mg alcohol-insoluble protein/g. The crispbread showed a higher concentration of alcohol-soluble proteins (27.4 mg/g) than the other baked goods. The pretzel crust had the lowest protein recovery (58%), resulting in a lower content of

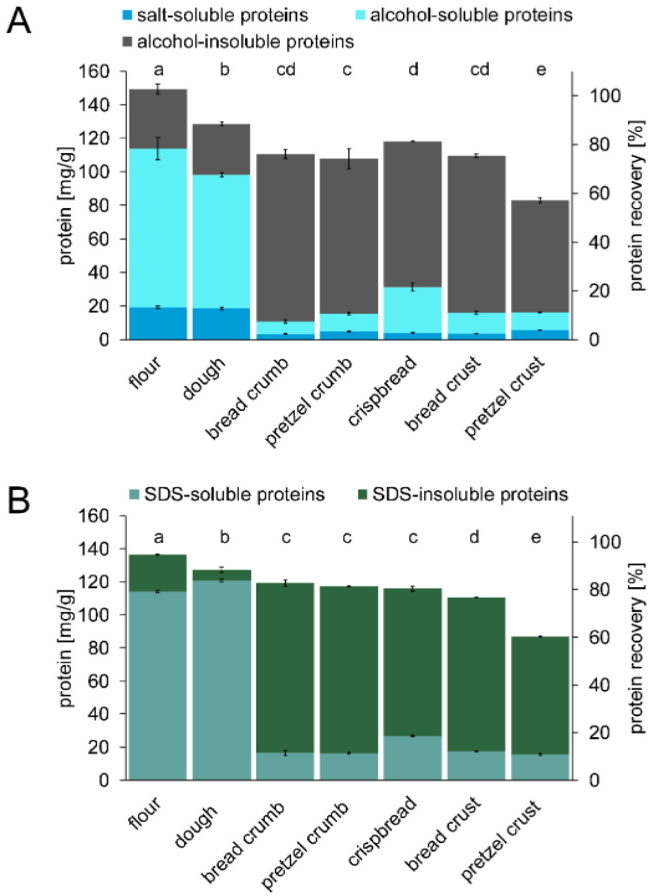

Fig. 4. Concentrations of salt-soluble, alcohol-soluble and alcohol-insoluble proteins determined by RP-HPLC (A), SDS-soluble and SDS-insoluble proteins determined by GP-HPLC (B), and protein recovery calculated as percentage of the extracted proteins relative to the total crude protein content. Data represented are the means ± standard deviation (n = 3). Means of protein recovery with different small letters are significantly different (one-way ANOVA, Tukey's test, $p \leq 0.05$).

alcohol-insoluble proteins (66.9 mg/g) in comparison to the other baked goods.

In general, the quantitation of SDS-soluble and SDS-insoluble proteins corresponded with the results of the modified Osborne fractionation. Protein recovery significantly decreased from 95% for flour to 61% for pretzel crust (Fig. 4 B). Dough preparation significantly increased the protein extractability in SDS buffer from 114.0 mg SDS-soluble protein/g flour to 120.8 mg SDS-soluble protein/g dough. As seen for salt-soluble, alcohol-soluble and alcohol-insoluble proteins, baking drastically changed the protein composition in favour of SDS-insoluble proteins. Levels of SDS-soluble proteins decreased by at least 72%. The amount of SDS-soluble and SDS-insoluble proteins extracted from the crumb and crust samples was similar ranging from 15.6 mg (pretzel crust) to 17.6 (bread crust) mg SDS-soluble protein/g and 71.3 mg (pretzel crust) to 102.6 mg (bread crumb) SDS-insoluble protein/g. Again, the crispbread showed a higher concentration of SDS-soluble proteins (26.8 mg/g). The pretzel crust had a lower content of SDS-insoluble proteins in comparison to the other baked samples due to the low protein recovery (61%).

The alcohol-soluble, alcohol-insoluble and SDS-insoluble protein fractions were further analysed as described above. The alcohol-soluble protein fraction was subdivided into ω5-, ω1,2-, α- and γ-gliadins (Fig. 5 A). The alcohol-insoluble protein fraction was subdivided into ω_b-gliadins, HMW-GS and LMW-GS, and the SDS-insoluble fraction into HMW-GS and LMW-GS as well as residual albumins and globulins (Fig. 5 BC). Flour and dough did not significantly differ within the gliadin types. In comparison to flour and dough (8% ω5-gliadins, 9% ω1,2-gliadins, 51% α-gliadins, 33% γ-gliadins), the baked goods had higher proportions of ω5-and ω1,2-gliadins with values of about 20% (crispbread) to 45% (bread crumb) and lower proportions of α- and γ-gliadins with values of about 10% (bread crumb) to 38% (bread crust) for α-gliadins, and 2% (bread crumb) to 25% (crispbread) for γ-gliadins. The ω_b-gliadins showed the lowest proportions overall (<4%), further reduced in the bread crumb, bread crust and crispbread.

The alcohol-insoluble HMW-GS and LMW-GS showed the same proportions for flour and dough (28% HMW-GS, 69% LMW-GS). In comparison to the flour, the relative proportions of HMW-GS in the baked goods were lower, ranging from 15% (bread crust) to 19% (crispbread) and higher for LMW-GS, ranging from 79% (crispbread) to 83% (bread crust).

In general, there was no clear effect of the additional alkali treatment (pretzel crust) on the composition of gluten protein types. The absolute levels of all alcohol-soluble gliadins decreased in the bread, crispbread and pretzel samples; α- and γ-gliadins to a greater extent than ω-gliadins. Concurrently, absolute levels of all types of alcohol-insoluble proteins were increased by the baking process. The quantitation of HMW-GS and LMW-GS of the SDS-insoluble protein fraction from flour, dough and crumb samples were in accordance with the results for the alcohol-insoluble protein fraction. However, there was no clear trend of HMW-GS, LMW-GS as well as the residual albumin and globulin proportions within the samples of the SDS-insoluble protein fraction.

To further investigate changes of the gliadin composition caused by bread, crispbread and pretzel making, an additional extraction of all alcohol-soluble proteins (including salt-soluble proteins) was performed and the proteins were analysed regarding their M_r distribution before and after reduction with DTT. It revealed that proteins with M_r of >66 kDa (15 - 23%) became alcohol-insoluble after the baking process (Fig. 6 A). These proteins could be reduced almost completely if treated with DTT (Fig. 6 B). The alcohol-soluble proteins extracted from bread, crispbread and pretzel samples showed higher proportions of proteins with M_r of <12.4 kDa and 29 - 66 kDa, and a lower proportion of proteins with a M_r of 12.4 - 29 kDa in comparison to the flour and dough. Most proteins with a M_r of 29 - 66 kDa could be reduced. After reduction, the crispbread had the highest proportion of proteins with a M_r of 12.4 - 29 kDa (74%) and the lowest proportion of very small proteins with a M_r of <12.4 kDa (23%) of all baked goods, thus were closest to the M_r distribution of flour in general. The opposite was true for the pretzel crust.

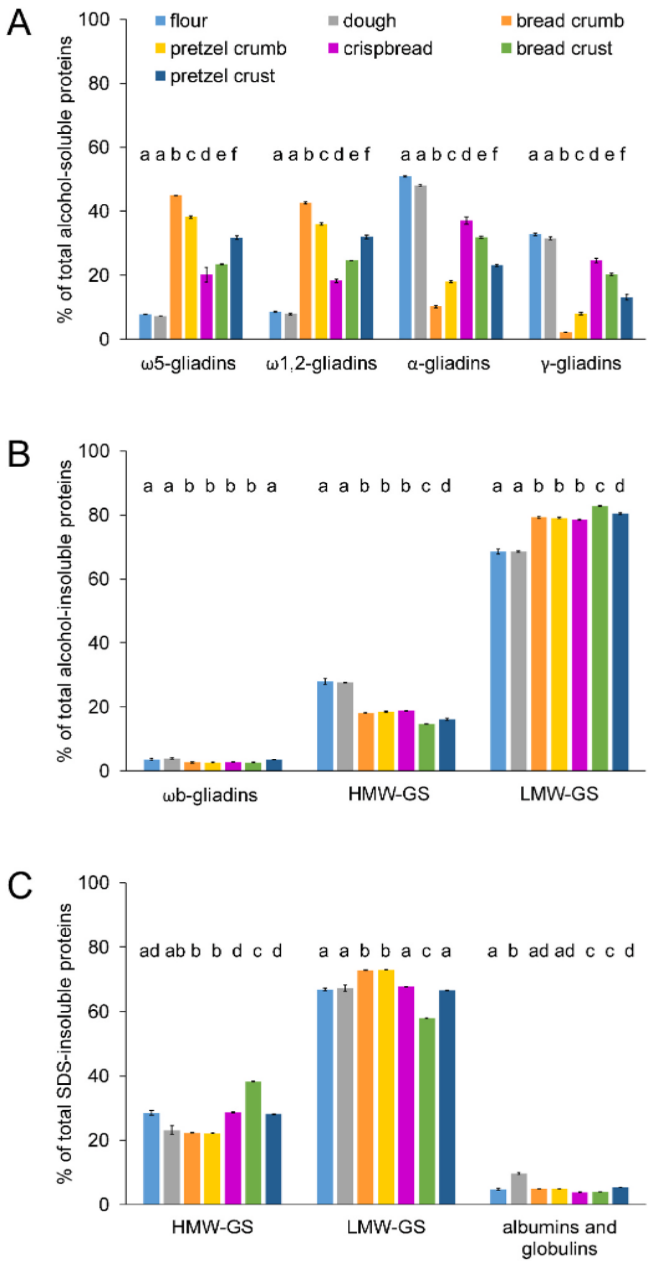

Fig. 5. Relative concentrations of ω5-, ω1,2-, α- and γ-gliadins from extracted alcohol-soluble proteins (A) and of ωb-gliadins, high-molecular-weight (HMW) and low-molecular-weight (LMW) glutenin subunits (GS) from extracted alcohol-insoluble proteins determined by RP-HPLC (B), as well as HMW-GS, LMW-GS, albumins and globulins from extracted SDS-insoluble proteins determined by GP-HPLC (C). Data represented are the means ± standard deviation (n = 3). Different small letters represent a significant difference (one-way ANOVA, Tukey's test, $p \leq 0.05$) between samples within one gluten protein type.

3.3. Content of S-containing groups and compounds

The content of free SH and SS bonds was analysed using the Ellmann method (Fig. 7). In bread, crispbread and pretzel samples both SH and SS bonds decreased from about 11 µmol SH/g (flour, dough) to about 5 µmol SH/g and from about 120 µmol SS/g (flour, dough) to less than 88

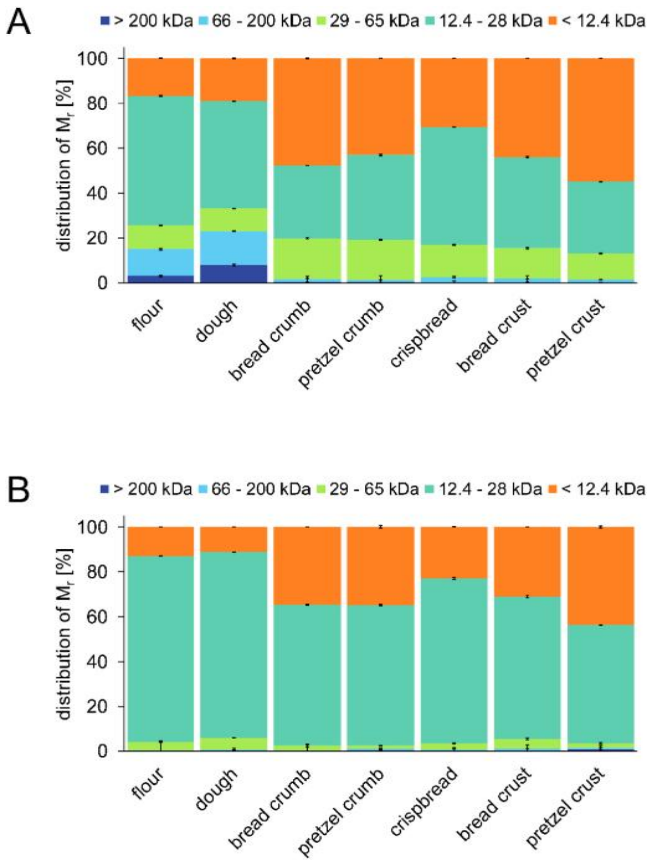

Fig. 6. Relative molecular mass (M_r) distribution of alcohol-soluble proteins (A) and alcohol-soluble proteins after reduction with DTT (B) determined by GP-HPLC. Data represented are the means ± standard deviation (n = 3).

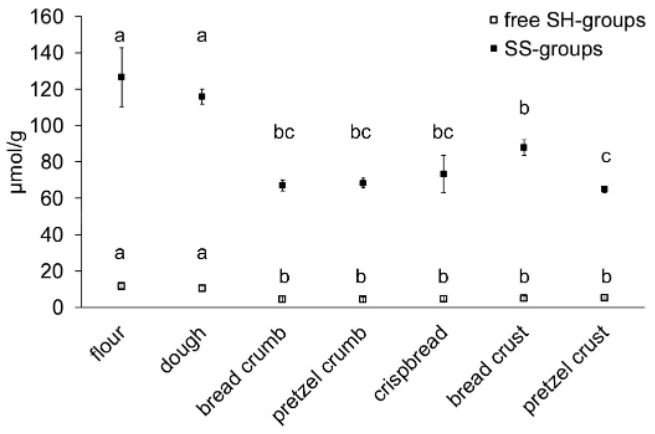

Fig. 7. Concentrations of disulfide (SS) and free thiol (SH) groups determined spectrophotometrically. Data represented are the means ± standard deviation (n = 3). Means with different small letters are significantly different (one-way ANOVA, Tukey's test, $p \leq 0.05$).

µmol SS/g. The content of free SH did not differ between the samples of baked goods. However, the crispbread had a higher content of SS bonds (87.8 µmol SS/g) than the other baked goods.

The content of total CSH and total GSH was determined by SIDA LC-MS/MS. Fig. 8 shows that the total CSH content decreased with the degree of baking from 306 nmol CSH/g in the flour to <72 nmol CSH/g in the bread crust and pretzel crust. After the addition of yeast, the

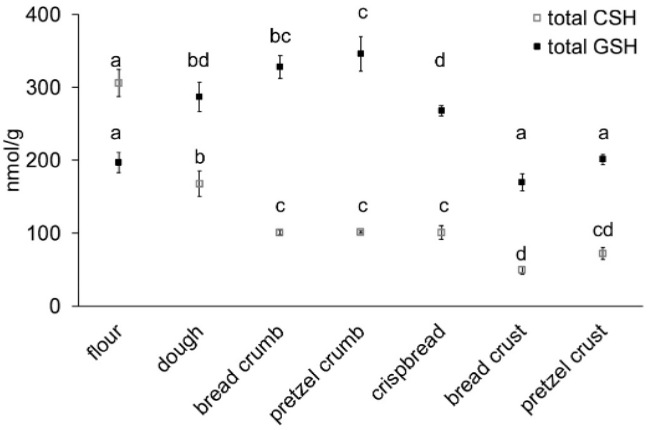

Fig. 8. Concentrations of total cysteine (CSH) and total glutathione (GSH) determined by stable isotope dilution assay LC-MS/MS. Data represented are the means ± standard deviation (n = 3). Means with different small letters are significantly different (one-way ANOVA, Tukey's test, $p \leq 0.05$).

content of total GSH in the dough (287 nmol GSH/g), bread crumb (328 nmol GSH/g), pretzel crumb (345 nmol GSH/g) and crispbread (268 nmol GSH/g) was higher than in the flour (197 nmol GSH/g). It is likely that the sample preparation with perchloric acid induced a release of GSH from yeast cells (Hahn, 1996). The total GSH concentrations of the flour and crust samples were the same, which hence corresponded to an actual decrease in the crust samples. Within the baked goods, there was no further effect of alkali treatment on the concentration on S-containing groups and compounds in the pretzel crust.

4. Discussion

We used different baked goods (bread, crispbread, pretzel) made from one dough as model system to reflect different degrees of processing. The bread and pretzel were separated into crumb and crust. These components had been exposed to different processing conditions, that is the baking temperature (Zanoni et al., 1993; Ogilvie et al., 2021), steam injection and alkaline dipping, favouring Maillard and caramelization reactions in the crust samples (Dessev et al., 2020). The crispbread baking conditions varied by a shorter baking time and no steam injection. According to an increasing degree of processing the sample material was ranked as follows: flour, dough, bread crumb, pretzel crumb, crispbread, bread crust and pretzel crust. To cover the legislative threshold levels of gluten-free and very low gluten foods as specified in the European Commission Regulation (EC) No 41/2009, also gluten-reduced versions (20, 50, 100 mg gluten/kg) were prepared.

A commercial sandwich R5 ELISA was used to determine the gliadin content after extraction with Cocktail, UGES and UPEX. The extraction solutions contained different disaggregating and reducing agents. Disaggregating agents used were the chaotropic guanidine hydrochloride (Cocktail), the amino acid arginine (UGES) and the detergent N-lauroylsarcosine (UPEX). Reducing agents used were 2-mercaptoethanol (Cocktail) and TCEP (UPEX). The reducing agent contained in UGES is not disclosed yet (Segura et al., 2021). The mode of action of the disaggregating agents as well as the strength of the reducing agents is different, which can explain the discrepancies of the extraction efficiency. The precise mechanism and interplay of the different agents in the context of gluten solubilisation needs further clarification, though.

In general, all extraction solvents achieved gliadin recovery rates, which were in the tolerated range of 80% - 120% (Abbott et al., 2010), for the flours spiked with 20 and 50 mg gluten/kg. In the extraction of flours spiked with 100 mg gluten/kg UPEX was the only extraction solvent which stayed below 80% (58%). This is only partly in agreement with previous studies (Mena et al., 2012; Segura et al., 2021), that found

a good accordance in the gluten content of (gliadin spiked) commercial foods analysed by R5 sandwich ELISA in combination with Cocktail, UGES, but also UPEX extraction protocols. However, the effect of processing on gluten quantitation has only been evaluated by means of spiked materials, although incurred materials are recommended for better commutability to actual food samples. We showed that less gliadin was detected in the incurred bread, crispbread and pretzel samples in comparison to their spiked flour samples regardless of the extraction solvent. Additional alkali treatment of the pretzel crust impaired the gliadin recovery even more. Overall, the extraction with Cocktail resulted in slightly but often significantly higher recovery rates than the extraction with UGES and particularly UPEX. Furthermore, only the use of Cocktail enabled a gliadin detection in dough comparable to flour. Thus, the extraction with Cocktail seems to be the method of choice, but also faces challenges to compensate for effects of processing like baking and alkaline pH.

In our study, the conversion factor of 2, which is usually applied to calculate the gluten content based on the gliadin content analysed by ELISA, would be sufficient for a correct labelling of most samples regardless of the kind of extraction solvent or degree of processing. Three samples (flour spiked with 50 mg gluten/kg if extracted with Cocktail or UGES; dough incurred with 50 mg gluten/kg if extracted with Cocktail) would be even unnecessarily labelled gluten-containing. Thus, the conversion factor is often also a kind of safety factor, which was not originally intended. However, since this factor needs to cover highly variable conditions such as different gliadin/glutenin ratios depending on the type of grain and raw material (flour, starch), or the degree of processing, it is very likely that there are some especially low gluten foods which still suffer from incorrect labelling. Additional data have to be generated in further studies to assess if a recovery correction could help to improve food safety in this respect. At this point, it does not seem possible to advocate for recovery correction, as long as recovery may be very different depending on the sample.

To better understand if gluten structure or gluten amount was responsible for the reduced detectability, further experiments were performed to analyse gluten composition and extractability. SDS-PAGE revealed no clear differences in the qualitative protein composition. The protein extracts of the bread crust and pretzel crust resulted in weaker bands in comparison to the other samples indicating a reduced protein extractability.

From the results of both RP- and GP-HPLC, it became clear that dough preparation, baking and additional alkali treatment of the pretzel crust decreased protein recovery under reducing and non-reducing conditions. The magnitude of extractability losses was dependent on the processing conditions with dough preparation resulting in a higher protein recovery than baking, and alkali dipping prior to baking resulting in the lowest protein recovery. Alkaline treatment further reduced the extractability of alcohol- and SDS-insoluble proteins (but not salt-, alcohol- and SDS-soluble proteins) remaining in the extraction sediment.

Baking strongly influenced protein extractability and resulted in a large decrease of salt-, alcohol- and SDS-soluble proteins, whereas dough preparation significantly increased the extractability of SDS-soluble proteins. It is still under debate whether this is due to depolymerisation, conformational rearrangement or better dissolution by a changed effective surface area of SDS-soluble proteins (Eckert et al., 1993; Weegels et al., 1997; Belton, 2005).

A better SDS-solubility of proteins, mainly of gliadins, could also explain why the gliadin content analysed by ELISA with the Cocktail extraction was similar for the spiked flours and incurred doughs, though their overall protein extractabilities were reduced. Nevertheless, this was not true for the extraction with UGES and UPEX, where the reduction of the total protein extractability also resulted in a reduced ELISA detection. Thus, the kind of disaggregating agent might play a role in ELISA, but it is difficult to compare since none of the ELISA extraction solvents uses SDS as disaggregating agent.

The reduction of SS bonds with DTT rendered the major proportion of proteins extractable in aqueous propan-1-ol. This indicated that salt-, alcohol-, and SDS-soluble proteins, which contain the major part of gliadins, became extractable with the alcohol-insoluble and SDS-insoluble protein fractions, which mainly comprises glutenins. It is known that this is due to gluten polymerisation by oxidation of the free SH groups of cysteine and/or SH-SS exchange reactions, which are favoured by mechanical and thermal treatment (Schofield et al., 1983; Kieffer et al., 2007; Lagrain et al., 2008b).

The relative accumulation of ω-gliadins, accompanied by a relative decrease of α- and γ-gliadins in our study supported the hypothesis that α- and γ-gliadins are the main drivers in gliadin-glutenin crosslinking during baking (Schofield et al., 1983; Weegels and Hamer, 1998; Lagrain et al., 2007, 2008a). α-Gliadins have three and γ-gliadins four intramolecular SS bonds, which can be incorporated via SS crosslinking into the alcohol- and SDS-insoluble glutenin fraction (Shewry et al., 1986; Shewry and Tatham, 1997). ω-Gliadins lack cysteine residues and therefore play a minor role.

A proportion of around 15 - 23% of the alcohol-soluble proteins consisted of oligomeric proteins with M_r of >66 kDa, known as HMW-gliadins (Wieser et al., 2020). They were completely involved in gluten polymerisation via SS crosslinking induced by the baking process.

The absolute levels of all glutenins increased, whereas only the relative proportion of LMW-GS increased. Thus SH-SS exchange of α- and γ-gliadins resulted in a shift of solubility and consequently detection together with LMW-GS. However, this could only be partially confirmed by the analysis of protein types of the SDS-insoluble protein fraction.

Of all baked goods, the crispbread most closely resembled the flour regarding the protein type composition. Since there was no clear correlation between the protein type composition and the processing conditions of the baked goods, the protein composition of the pretzel crust did not provide an explanation for the lowest detectability by ELISA.

Even the use of the strong reducing agent DTT did not restore the original protein extractability as in the flour, suggesting the contribution of not only SS but also non-SS crosslinking between gluten proteins. The decrease of free SH but also SS groups confirmed that the baking process led to non-SS crosslinks involving SH groups. Previous studies suggest the significance of lanthionine as non-SS crosslink of wheat gluten (Rombouts et al., 2016). Also, lower levels of total CSH and GSH induced by the baking process indicated the involvement of free SH compounds in this kind of non-SS crosslink formation. The losses of total CSH and GSH were dependent on the baking temperature. GSH loss occurred only in the crust samples, i.e. if samples were exposed to a very high temperature, CSH loss was highest in the crust samples. A decrease of total CSH and GSH after baking was described earlier by Reinbold (2011), who suggested free SH being hyperoxidized to their sulfinic and sulfonic acids. The levels of S-containing groups and compounds did not significantly differ between the baked samples. Thus, yet another type of non-SS crosslinks may be responsible for the lower gluten extractability of the pretzel crust samples. Previous studies have demonstrated the formation of lysinoalanine in pretzels (Sternberg et al., 1975; Raymond, 1980; Hasegawa et al., 1987; Rombouts et al., 2012). It is strongly favoured at higher pH conditions (Friedman, 1999). Obviously, also the presence of Maillard reaction derived crosslinks should be further elucidated in pretzel crust.

5. Conclusions

In conclusion, gluten protein extractability rather than the composition is crucial for gluten detection and quantitation by ELISA in baked goods. Here the focus needs to be on a complete extraction of both gliadins as well as glutenins. Because the formation of insoluble gluten polymers results not only from SS but also non-SS crosslinking during processing, none of the applied extraction solvents achieved a complete gluten extraction from the baked goods. The different baking conditions led to significantly reduced recovery rates (20 - 50%) of the gluten

incurred products analysed by ELISA. Additional alkali treatment of the pretzel crust resulted in the lowest recoveries accompanied by a reduced glutenin extractability. Though in our study the conversion factor of gliadin to gluten for ELISA is sufficient to compensate the reduced extractabilities, the influence of processing of especially very low gluten foods should be considered in risk assessment and risk management decisions. Further studies are needed to gain more understanding of gluten crosslinking during processing to optimise gluten extraction methods and therefore accurate gluten detection and quantitation.

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CRediT authorship contribution statement

Tanja Miriam Schirmer: Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. **Katharina Anne Scherf:** Conceptualization, Funding acquisition, 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.

Data availability

Data will be made available on request.

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3.2 Proteomic characterisation of wheat protein fractions taken under different baking conditions

The article “Proteomic characterisation of wheat protein fractions taken under different baking conditions” was published in the “Journal of Agricultural and Food Chemistry” (https://doi.org/10.1021/acs.jafc.3c02100) in 2023. The author of this thesis, Tanja Schirmer, developed the concept, generated and evaluated the data, wrote the manuscript, and revised it according to the reviewer comments.

Different wheat flour-based goods (bread, crispbread, pretzel) were used to investigate the influence of baking conditions on the composition of the ALGL, gliadin and glutenin fractions (Osborne extracts). The general characterisation (SDS-PAGE, RP-HPLC) of the fractions and extraction residues revealed that baking changed the protein content of all samples (lower in ALGL fractions, higher in gliadin and glutenin fractions, higher in extraction residues) as well as their protein type composition. The gliadin fractions showed a relative increase of ω-gliadins (18 up to 74%), and decrease of α- and γ-gliadins (82 up to 26%). The glutenin fractions showed a relative increase of LMW-GS (82 to 87%) and decrease of HMW-GS (16 to 11%). The changes compared to flour were much more pronounced in the samples of the baked goods than in the ones of dough. A subsequent in-depth proteome analysis of all fractions via label-free untargeted nanoLC-MS/MS and the proteomic software MaxQuant identified 1488 peptides assigned to 93 protein groups. The ALGL fractions contained 71 protein groups, the gliadin fractions 27 and the glutenin fractions 47. Many of the protein groups were found in more than one fraction. The majority of proteins in the ALGL fractions were metabolic and protective proteins, while the gliadin and glutenin fractions contained mostly storage proteins. With the exception of ω-gliadins only found in the ALGL and gliadin fractions, the same protein classes were identified in all fractions. A cluster analysis of the samples within the fractions demonstrated that the proteome changes were mainly temperature-dependent. Differential abundance analysis of the proteins in the bread crumb and crust fractions compared to flour showed that 82 proteins were significantly affected by baking. The changes are a result of the gluten network formation during bread making. It affected the solubility properties of the gluten proteins in different ways. Specific gliadins and glutenins were either increased or decreased in some fractions, which indicates that they were either incorporated into the gluten network during baking (solubility shift) or not (accumulation). Overall, the protein abundances in the fractions of the crumb and the crust very often changed in the same way, with greater changes observed in the crust fractions. This research is a further step towards a better understanding of the complex process of protein rearrangement during baking.

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Proteomic Characterization of Wheat Protein Fractions Taken at Different Baking Conditions

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ABSTRACT: Food processing conditions affect the structure, solubility, and therefore accurate detection of gluten proteins. We investigated the influence of dough, bread, and pretzel making on the composition of different wheat protein fractions obtained by Osborne fractionation. The albumin/globulin, gliadin, and glutenin fractions from flour, dough, crispbread, bread, and pretzel were analyzed using RP-HPLC, SDS-PAGE, and untargeted nanoLC-MS/MS. This approach enabled an in-depth profiling of the fractionated proteomes and related compositional changes to processing conditions (mixing, heat, and alkali treatment). Overall, heat treatment demonstrated the most pronounced effect. Label-free quantitation revealed significant changes in the relative abundances of 82 proteins within the fractions of bread crumb and crust in comparison to flour. Certain gluten proteins showed shifts or reductions in particular fractions, indicating their incorporation into the gluten network through SS and non-SS cross-links. Other gluten proteins were enriched, suggesting their limited involvement in the gluten network formation.

KEYWORDS: *baking, foodomics, gluten, label-free quantitation, mass spectrometry*

1. INTRODUCTION

Already in 1907, wheat proteins were first classified according to their solubility by T. B. Osborne.[1] Following a sequential extraction procedure, he isolated water-soluble albumins, salt-soluble globulins, and alcohol-soluble gliadins and called the leftover alcohol-insoluble fraction glutenins. Most albumins and globulins are structural or metabolic proteins, whereas gliadins and glutenins are storage proteins and referred to as gluten.[2] Although the so-called Osborne fractionation does not consider structural and functional aspects of the proteins, it has been and still is the starting point of countless studies to investigate the properties of wheat and other plant proteins. Meanwhile, diverse solubility approaches have been established, which draw conclusions on covalent and noncovalent interactions of wheat proteins resulting from turning wheat flour into foodstuffs such as bread or pasta.[3−8]

Wheat gluten forms a complex network during dough preparation and further processing. Thermal treatment as applied during baking leads to covalent aggregation products primarily associated with disulfide (SS) and dehydroamino acid-based cross-linking as well as bridged polypeptide structures deriving from Maillard reactions. Alkaline treatment as applied in pretzel making further promotes these reactions.[9−11]

Gluten polymerization is responsible for the dough-forming and final product properties and therefore of great interest concerning not only functional and quality aspects but also physiologically relevant aspects such as digestibility or allergenicity. The underlying mechanism of intermolecular and intramolecular cross-linking is still not fully understood, although an enormous amount of research has been conducted.[12] The complexity of the wheat proteome, which contains hundreds of extremely diverse proteins, makes its analysis challenging. The introduction of wheat proteomics using liquid chromatography with tandem mass spectrometry (LC−MS/MS) has offered a new tool for complex data analysis. Despite challenges such as the limited enzymatic digestibility with standard proteases, high sequence homologies, missing sequence annotations, and the lack of reliable curated sequence databases, it has been established for cereal research.[12−15]

Until now, most proteomic approaches have been used to associate the protein composition of wheat with its technological quality or to screen for immunogenic peptides in the context of celiac disease or wheat allergies.[16] Very few studies have described proteomics methodologies to investigate the influence of wheat-based food processing on the protein composition. Ogilvie et al.[17] analyzed the effect of baking time and temperature on the in vitro digestibility of specific immunogenic peptides by targeted MS. An untargeted approach was applied by Zimmermann et al.[18] to determine the proteome of flour and bread focusing on the quantitation of potentially allergenic proteins.

Food processing significantly reduced gluten extractability, which affected the detection by an enzyme-linked immunosorbent assay, and was accompanied by distinct compositional changes of different wheat protein fractions defined according to their solubility.[19] While gluten protein types of wheat, rye, and barley have been characterized by LC−MS/MS,[20] no study so far has evaluated the influence of food processing on the composition of different kinds of wheat proteins using bottom-

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up proteomics. This is of special interest, since it not only indicates which proteins are involved in gluten network formation but also highlights changes of solubility properties of wheat proteins.

The aim of this work is a fundamental and comprehensive characterization of different protein fractions obtained from flour, dough, crispbread, bread, and pretzel by modified Osborne fractionation demonstrating the effect of different baking conditions on their protein composition. The application of RP-HPLC, SDS-PAGE, and high-resolution LC−MS/MS enabled the identification and quantitation of a large set of proteins and pointed out proteins with major abundance changes in comparison to their presence in flour. Our holistic view is a further step toward a better understanding of the complex process of protein rearrangement during baking.

2. MATERIALS AND METHODS

2.1. Materials. Wheat, cultivar Akteur, harvested 2015, was kindly provided by I.G. Pflanzenzucht GmbH (Munich, Germany). The kernels were milled into flour using a Quadrumat Junior laboratory mill (Brabender, Duisburg, Germany) according to AACCI Method 26-50.01., sieved (mesh size 200 μm), and stored at room temperature (RT) for 2 weeks before use. Brauerei Wieninger (Teisendorf, Germany) provided the baker's yeast (*Saccharomyces cerevisiae*). PageRuler Unstained Protein Ladder and NuPage Bis-Tris Plus Gel (10%, pH 6.4, 1.0 mm × 10 well) were products from Thermo Fisher Scientific (Braunschweig, Germany) and α-chymotrypsin (from bovine pancreas ≥45 U/mg protein, TLCK treated) from Biomol (Hamburg, Germany). All chemicals used were of analytical or higher quality and were purchased from Merck KGaA (Darmstadt, Germany), Sigma-Aldrich (Steinheim, Germany) or SERVA Electrophoresis GmbH (Heidelberg, Germany). Prof. Dr. Koehler, Chairman of the Working Group for Prolamin Analysis and Toxicity, provided the reference material Prolamin Working Group (PWG)-gliadin.[21] Water for chromatographic separations was purified using a Milli-Q Gradient A10 system (Millipore, Schwalbach, Germany).

2.2. Preparation of Flour-Based Products. The flour-based products were prepared as described previously.[19] Flour, 3% yeast, and 2% NaCl were mixed in a kitchen aid (1 min at level 1). The dough was prepared by adding water (AACC method 54-50.01) and kneading (3 min at level 1 and 3 min at level 3). A dough piece was instantly frozen and freeze-dried for analysis. For the preparation of the baked goods, the dough was rested in a water-saturated proofer (20 min at 30 °C). For crispbread preparation, a part of the dough was rolled as thin as possible and pricked all over with a fork before baking (9 min at 230 °C). For bread making, about 300 g of the dough was shaped, proofed (40 min at 30 °C, 90% relative humidity), and baked in a tin pan (25 min at 230 °C with initial steam injection). The pretzels were made of about 55 g dough pieces, which were shaped into balls, proofed (40 min at 30 °C, 90% relative humidity), and refrigerated (15 min, 5 °C). The dough balls were shortly dipped in a 1.25 mol/L sodium hydroxide (NaOH) solution and baked (17 min at 230 °C with initial steam injection).

After baking, all baked products were cooled for 2 h at RT. Bread and pretzels were separated into crumb and crust. All baked products were freeze-dried and milled (6000 rpm, 200 μm ring sieve) using an Ultra Centrifugal Mill ZM 200 (Retsch, Haan, Germany). The resulting sample material was stored airtight at RT. In total, two baking trials were performed. The reproducibility of the sample material was confirmed by RP-HPLC analysis as described before.[19]

2.3. Preparation of Osborne Fractions. Flour and freeze-dried flour-based products (30 g) were defatted three times with 75 mL of pentane/ethanol (95/5, v/v) and once with 75 mL of *n*-pentane under continuous stirring (30 min, 22 °C).[22] The suspensions were centrifuged (3750 × g, 15 min, 22 °C), and the solvent discarded. The defatted material was air-dried overnight.

The extraction procedure was carried out according to Schalk et al.[23] Defatted flour and flour-based products (15 g) were extracted three times with 60 mL of 0.067 mol/L Na_2HPO_4/KH_2PO_4 buffer (pH 7.6) and 0.4 mol/L NaCl (albumins/globulins, ALGL). Then, residues were extracted three times with 60 mL of 60% (v/v) ethanol (gliadins). Last, residues were extracted twice with 60 mL of 50% (v/v) propan-1-ol, 0.05 mol/L Na_2HPO_4/KH_2PO_4 (pH 7.5), and 1% (w/v) dithiothreitol (DTT) under nitrogen atmosphere (glutenins). Samples were extracted by mixing at 12,400 rpm for 5 min at 22 °C using an Ultra-Turrax blender (IKA-Werke, Staufen, Germany). The extraction of the glutenins was continued with magnetic stirring for 30 min at 60 °C and cooling prior to centrifugation. The suspensions were centrifuged (25 min, 3750 × g 22 °C). The supernatants were combined, dialyzed (molecular weight cut-off: 12,000−14,000 Da, Medicell Membranes, London, UK) for 3 days against acetic acid (0.01 mol/L) and for 1 day against deionized water, and freeze-dried.[24] Due to the dialysis conditions, proteins with a molecular weight of <12 kDa were not included in this study. However, it was evaluated that this concerns only a very small portion of proteins with a molecular weight of around 11 kDa contained in the ALGL fractions. The extraction residues after the third extraction step were freeze-dried immediately. Both, extraction residues and isolated protein fractions, were stored in a freezer until use.

2.4. RP-HPLC. Protein fractions (10 mg of ALGL of crumb, crust, and crispbread samples, 1 mg of all other samples; 3 replicates) were dissolved in 1 mL of the corresponding extraction solutions (see Section 2.3). Samples were thoroughly stirred (Vortex mixer), sonicated for 10 min, again thoroughly stirred, and filtered (Whatman, Spartan 13/0.45 RC, GE Healthcare, Freiburg, Germany) prior to analysis by RP-HPLC. As described before,[19] an UltiMate 3000 instrument (Dionex, Idstein, Germany) with an Acclaim 300 C_{18} column (2.1 × 150 mm, Thermo Fisher Scientific, Braunschweig, Germany) and the software Chromeleon was used for RP-HPLC analysis with the following settings: column temperature: 60 °C, flow rate: 0.2 mL/min, injection volume: 20 μL (ALGL, glutenins), 10 μL (gliadins), solvent: water/trifluoroacetic acid (TFA) (999/1, v/v) (A) and acetonitrile (ACN)/TFA (999/1, v/v) (B), gradient for ALGL: 0 min 0% B, 0.5 min 20% B, 7 min 60% B, 7.1 min 90% B, 11.1 min 0% B, gradient for gliadins/glutenins: 0 min 0% B, 0.5 min 24% B, 20 min 56% B, 20.1 min 90% B, 24.2 min 0% B, detection: ultra violet absorbance at 210 nm. PWG-gliadin, dissolved in 60% (v/v) ethanol, was used for external calibration in the range of 5.8−23.3 μg. The peaks for ALGL had a retention time of 5.0−12.0 min, those for gliadins appeared from 7.0 to 22.0 min, and those for glutenins from 7.0 to 21.0 min (Figure S1). Gliadins were further divided into ω5-, ω1,2-, α-, and γ-gliadins and glutenins into glutenin-bound ω-gliadins (ωb-gliadins), high-molecular-weight glutenin subunits (HMW-GS), and low-molecular-weight glutenin subunits (LMW-GS) as described by Wieser et al.[25] One-way analysis of variance (ANOVA) with Tukey's test at a significance level of $p < 0.05$ was applied to determine significant differences between the protein contents (SigmaPlot version 12.0, Systat Software, San Jose, USA).

2.5. Crude Protein. The crude protein content of the extraction residues was determined using a TruSpec Nitrogen Analyzer (Leco, Moenchengladbach, Germany) with ethylenediaminetetraacetic acid as standard. It was calculated by multiplying the nitrogen content by a factor of 5.7 as stated in ICC Standard 167 (International Association for Cereal Science and Technology, 2000). One-way ANOVA with Tukey's test at a significance level of $p < 0.05$ was applied to determine significant differences between the protein contents (SigmaPlot version 12.0, Systat Software, San Jose, USA).

2.6. Total Starch. The Total Starch Assay (amyloglucosidase/α-amylase method) (Megazyme, Bray, Ireland) was performed according to the manufacturer's instructions for the KOH-format.[26] All protein fractions were analyzed in duplicate. Due to limited quantities of the isolated protein fractions, the amount of sample material was reduced from 100 to 10 mg and the quantities of reagents for starch solution and hydrolysis were scaled down by a factor of 10. If samples contained >10% starch (ALGL fraction), the extraction volume was adjusted to 10 mL. If samples contained <10% starch (gliadin/glutenin fractions), the samples were not further diluted; the final extraction volume calculated was 1.04 mL. Glucose oxidase plus peroxidase reagent (3 mL) was

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added as indicated by the manufacturer. Standardized regular maize starch and glucose controls were included.

2.7. SDS-PAGE. The SDS-PAGE was performed as described by Lagrain et al.[27] and Schirmer and Scherf[19] using a homogeneous NuPAGE Bis-Tris gel (10%) and a PageRuler Unstained Protein Ladder as a molecular weight marker. Protein fractions (ALGL, gliadins, glutenins) and the extraction residues corresponding to 1 mg of protein (protein content determined by RP-HPLC and Dumas (residues)) were dissolved in 1 mL of extraction buffer (293.3 mmol/L sucrose, 246.4 mmol/L tris(hydroxymethyl)aminomethane (Tris), 69.4 mmol/L SDS, 0.51 mmol/L EDTA, 0.22 mmol/L Coomassie Brilliant Blue R250, 0.177 mmol/L phenol red, and 0.105 mmol/L hydrochloric acid, pH 8.5) and incubated under reducing conditions (50 mmol/L DTT) overnight at RT. The suspensions were shaken for 10 min at 700 rpm and 60 °C and centrifuged for 5 min at 5000 × g and 20 °C. 5 μL (ALGL, glutenins, extraction residues) or 10 μL (gliadins) of the supernatants was loaded onto the gel.

Gel electrophoresis was performed with 2-(*N*-morpholino)-ethanesulfonic acid (MES) running buffer (100 mmol/L MES, 50 mmol/L Tris, 3.5 mmol/L SDS, and 1 mmol/L EDTA) for separating ALGL and with a 3-(*N*-morpholino)propanesulfonic acid (MOPS) running buffer (50 mmol/L MOPS, 50 mmol/L Tris, 3.5 mmol/L SDS, and 1 mmol/L EDTA, pH 7.7) for separating gliadins, glutenins, and the residues, both under reducing conditions (5 mmol/L DTT) and the following running conditions: current: 115 mA; voltage 200 V, power: 30 W, and time: 20−30 min.

Proteins were fixed for 30 min with 12% trichloroacetic acid, stained for 30 min with 0.25% Coomassie Brilliant Blue R-250, and destained for 15 min with methanol/water/acetic acid (50/40/10, v/v/v) and in water/methanol/acetic acid (80/10/10, v/v/v) overnight. The gels were analyzed using Image Lab software version 6.0.0 (Bio-Rad Laboratories, Feldkirchen, Germany).

2.8. Sample Preparation for Untargeted nanoLC−MS/MS. Protein fractions (three technical replicates) corresponding to 1 ± 0.03 mg of protein (protein content determined by RP-HPLC) were hydrolyzed with 1 mL of chymotrypsin solution (0.02 mg/mL α-chymotrypsin in 0.04 mol/L urea, 0.1 mol/L Tris−HCl, pH 7.8) for 24 h at 37 °C. Proteolysis was stopped by adding 3 μL of TFA.[28] Protein digests were cleaned-up by solid phase extraction (SPE) using Discovery DSC-18 SPE tubes (Sigma-Aldrich Inc., Steinheim). SPE cartridges were conditioned with methanol (1.0 mL) and equilibrated with 1.0 mL of 0.1% TFA (v/v). The digests (1.0 mL) were loaded, allowed to interact for 5 min, and washed with 0.1% TFA (v/v) (3 × 1.0 mL). Peptides were eluted with 1 × 1.0 mL of 40% ACN + 0.1% TFA (v/v). The eluates were dried in a vacuum concentrator centrifuge. For LC−MS/MS analysis, the samples were dissolved in 1.0 mL of 0.1% formic acid (FA) (v/v) by sonication (5 min). The peptide concentrations were determined by NanoDrop One (Thermo Fisher Scientific, Madison, U.S.A.) at 205 nm.

2.9. Untargeted nanoLC-MS/MS. A NanoLC-Ultra 1D+ system (Eksigent Technologies, Dublin, CA, U.S.A.) was coupled online with an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific). The peptides (1 μg) were loaded onto a trap column (ReproSil-Pur 120 ODS-3, 5 μm, 2 cm × 75 μm, Dr. Maisch, Ammerbuch-Entringen, Germany) using loading solvent (0.1% FA) at a flow rate of 5 μL/min. Then, the peptides were transferred to an analytical column (ReproSil-Gold 120 C_{18}, 3 μm, 40 cm × 75 μm, Dr. Maisch) and separated using a 53 min gradient from 4 to 32% solvent B at a flow rate of 300 nL/min (solvent A: 0.1% FA and 5% dimethyl sulfoxide (DMSO) in water, solvent B: 0.1% FA and 5% DMSO in ACN). The eluate was sprayed via a stainless-steel emitter (Thermo Fisher Scientific) into the mass spectrometer at a source voltage of 2.2 kV. The transfer capillary was heated to 275 °C. The mass spectrometer was operated in data-dependent acquisition and positive ion mode. MS1 spectra (360−1300 m/z) were acquired in the Orbitrap using a resolution of 60,000, and MS2 spectra were acquired in the ion trap. The 20 most intense precursor ions were automatically selected from the proceeding MS1 spectrum with an isolation window width of 2.0 m/z. Peptide fragmentation was carried out by collision-induced dissociation with a normalized collision energy of 35%. Dynamic exclusion was set to 45 s, and only precursor ions with charge states >1 were selected.

2.10. Proteomic Data Analysis. MS raw files were analyzed with MaxQuant (version 1.5.3.30) by searching MS data against a *Triticum aestivum* (4565) database (downloaded from UniProt February 2023, protein entries 143,674) using Andromeda.[29,30] Chymotrypsin+ was specified as proteolytic enzyme, and up to two missed cleavages were allowed. Oxidation of methionine and protein N-terminal acetylation were selected as variable modifications. Instrument parameters and match tolerances were left as Orbitrap defaults. A minimum peptide length of 7 amino acids was considered. Identifications were made at 1% false discovery rate (FDR) at peptide spectrum match and protein level. Label-free quantitation (LFQ)[31] was performed with a minimum peptide ratio count of "2" for protein quantitation (default setting).

The protein-level LFQ results were loaded for further processing and statistics into Perseus version 1.5.6.0.[32] Protein groups designated as reverse, contaminant, or only identified through a post-translational modification site were removed from further processing. To be considered valid, a protein group required nonzero intensity values for all three technical replicates from at least one sample. Intensity values were log base 2 transformed. The coefficient of variation was calculated for technical replicate measurements.

The relative quantitative distribution of protein classes was calculated by the addition of summed up intensity values for all protein groups over all samples belonging to a protein class relative to the sum of the intensity values of all protein groups per fraction.

For hierarchical clustering (HC) of protein groups and samples, as well as for heatmap representations, data were z-scored (rows) and replicates averaged. For principal component analysis (PCA), missing values were imputed using the functionality "replace missing values from normal distribution" (width 0.3, downshift 1.8) and replicates averaged. HC and PCA were performed using default settings. Differential protein abundances in the protein fractions of bread crumb and bread crust in comparison to the control (flour sample) were calculated by two-sided t-tests with permutation-based FDR of 0.05 using imputed missing values.

3. RESULTS AND DISCUSSION

Gluten network formation during processing results from the hydration of flour after water addition and input of mechanical energy during dough preparation and further thermal treatment. To reflect varying degrees of processing, we investigated a range of baked goods (bread, crispbread, and pretzel) made from a single dough. The dough sample represents the gluten network formed during the mixing process. Dough mixing time and intensity are critical parameters that have a large impact on the final product properties.[33,34] The bread and pretzel were divided into their crumb and crust because these components were subjected to different processing conditions, including varying baking temperatures,[35] steam injection, and alkaline dipping, which promotes Maillard and caramelization reactions in the crust samples.[36] In the case of crispbread, shorter baking times and the absence of steam injection were employed. Our sample material was ranked according to increasing levels of processing in the following order: flour, dough, bread crumb, pretzel crumb, crispbread, bread crust, and pretzel crust.

3.1. Characterization of Osborne Fractions. The protein content and composition of the different Osborne fractions isolated from the wheat flour, dough, bread, crispbread, and pretzel were determined by RP-HPLC. The results are referred to the respective isolated material for each fraction (Figure 1). Typical chromatograms with retention times related to the gluten protein types are shown in Figure S1. The baking process significantly affected the protein content, that is the solubility of the proteins of the ALGL and glutenin fractions (Figure 1A). The protein content of the ALGL fractions of the flour and

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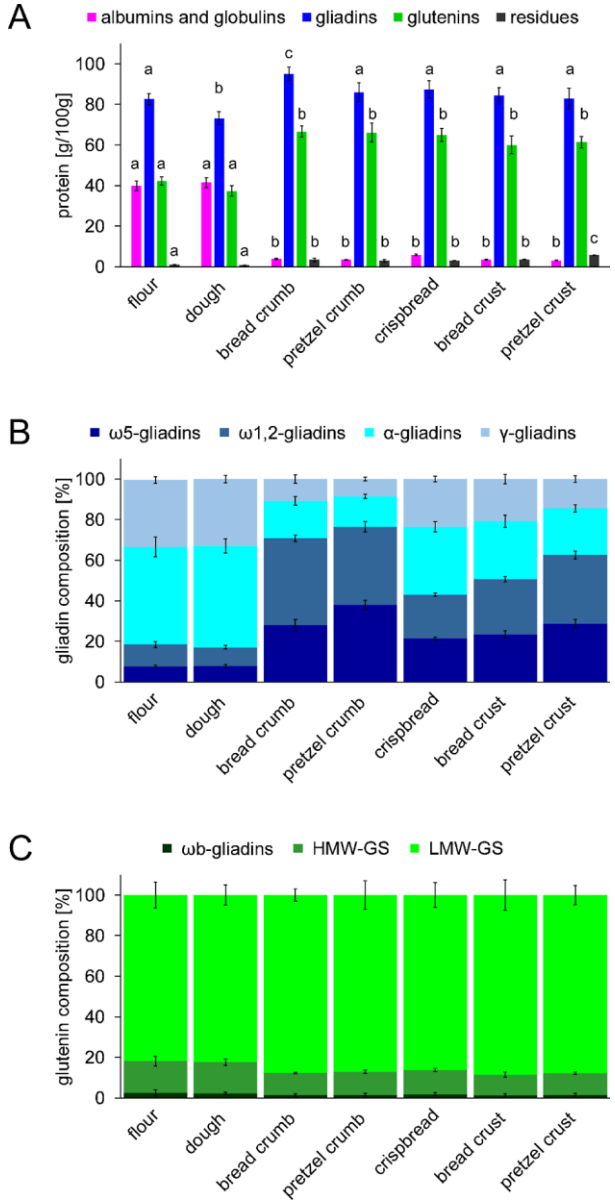

Figure 1. Protein content of the ALGL, gliadin, and glutenin fractions and the extraction residues isolated from flour, dough, and baked goods (A), and relative composition of protein types within the gliadin (B) and glutenin (C) fractions, determined by RP-HPLC. Data represented are the means ± standard deviation (n = 3) referred to the isolated fractions. Means of protein content with different small letters are significantly different within the fractions (one-way ANOVA, Tukey's test, $p \leq 0.05$).

dough (no heat treatment) was about 40/100 g. The ALGL fractions of the crumbs, crusts, and crispbread (baked goods) contained only very little protein (≤6/100 g) and consisted mainly of starch (39−62/100 g). The gliadin fractions were the richest in protein of all protein fractions ranging from 70/100 g (dough) to 98/100 g (bread crumb). There was no clear correlation between the protein content of the gliadin fractions and the sample treatment (mixing, heat, and alkali treatment). The protein content remained consistent between the flour and most of the baked goods. The protein content of the dough was slightly lower than that of the flour. The glutenin fractions of the baked goods were richer in protein (about 65/100 g) than the glutenin fractions of the flour and dough (about 40/100 g). Obviously, the extraction properties of the proteins contained in this fraction were changed by baking, which can be led back to a further polymerization of glutenins, also involving gliadins, via SS cross-linking.[4,6,17,37]

The starch content of all gliadin (<2/100 g) and glutenin (<7/100 g) fractions was low.

After extraction, a small portion of residual proteins such as membrane proteins and lipid proteins remains insoluble.[38] The protein content of the flour and dough extraction residues was very low (<1/100 g). The extraction residues of the baked goods had higher protein contents (3−6/100 g) indicating a decreased protein solubility due to aggregation and cross-linking of other than membrane or lipid proteins. Additional alkali treatment of the pretzel crust impaired protein solubility most. The formation of covalent cross-links other than SS cross-links favored by alkaline pH condition has been described as the reason for the decreased solubility.[39] These insoluble aggregates are very difficult to recover and analyze and account for a loss of information on the macromolecular network formation.

The composition of the gliadin and glutenin fractions of the flour and dough was similar but changed significantly if baked. In the gliadin fractions, the percentage of ω-gliadins (ω5, ω1,2) increased from 18% (flour) to up to 74% (crumbs), whereas the percentage of α- and γ-gliadins decreased from 82% (flour) to 26% (crumbs) (Figure 1B). The composition of the gliadin fractions of all baked goods changed in the same way, but the magnitude was different. A heat-induced reduced extractability of cysteine-containing α- and γ-gliadins has been also reported by Wieser[5] and Lagrain et al.[37] and attributed to thiol-disulfide (SH-SS) interchange reactions of gliadins with glutenins. As a consequence, the cysteine-free ω-gliadins (ω5, ω1,2) are supposed to accumulate in the gliadin fractions of the baked goods.

In the glutenin fractions, changes of the composition were also significant but less pronounced than in the gliadin fractions and in a similar range for all baked goods (Figure 1C). The percentage of HMW-GS decreased from 16% to about 11%; the percentage of LMW-GS increased from 82 to 87% in all baked goods. The percentage of ωb-gliadins (2%) remained constant. Looking at the RP-HPLC data, it is important to bear in mind that the interpretation of the chromatographic data as proposed by Wieser et al.[25] has certain limitations as it very much simplifies the complex composition of the single fractions.

3.2. SDS-PAGE. SDS-PAGE of the protein fractions confirmed the RP-HPLC results regarding the composition of the gliadin and glutenin fractions (Figure 2A,B). It further revealed significant changes within the composition of the ALGL fractions of the baked goods in comparison to the flour and the dough (Figure 2C). Remarkably, the predominant bands at about 13 kDa, most likely representing a class of α-amylase/trypsin inhibitors (ATIs) named 0.19 and 0.53,[40] diminished or completely disappeared in the ALGL fractions of the baked goods (Figure 2C). They showed a new, very prominent band for proteins with a molecular weight of about 26 kDa instead, which may indicate a dimerization of the ATIs due to the baking process. However, the identification of ATIs requires validation through additional experiments. In general, the band patterns of the ALGL fraction of all baked goods were similar with some variabilities depending on the kind of processing. The band patterns of the extraction residues visualized the decreased solubility of the proteins from the crusts, which could hardly be extracted (Figure 2D).

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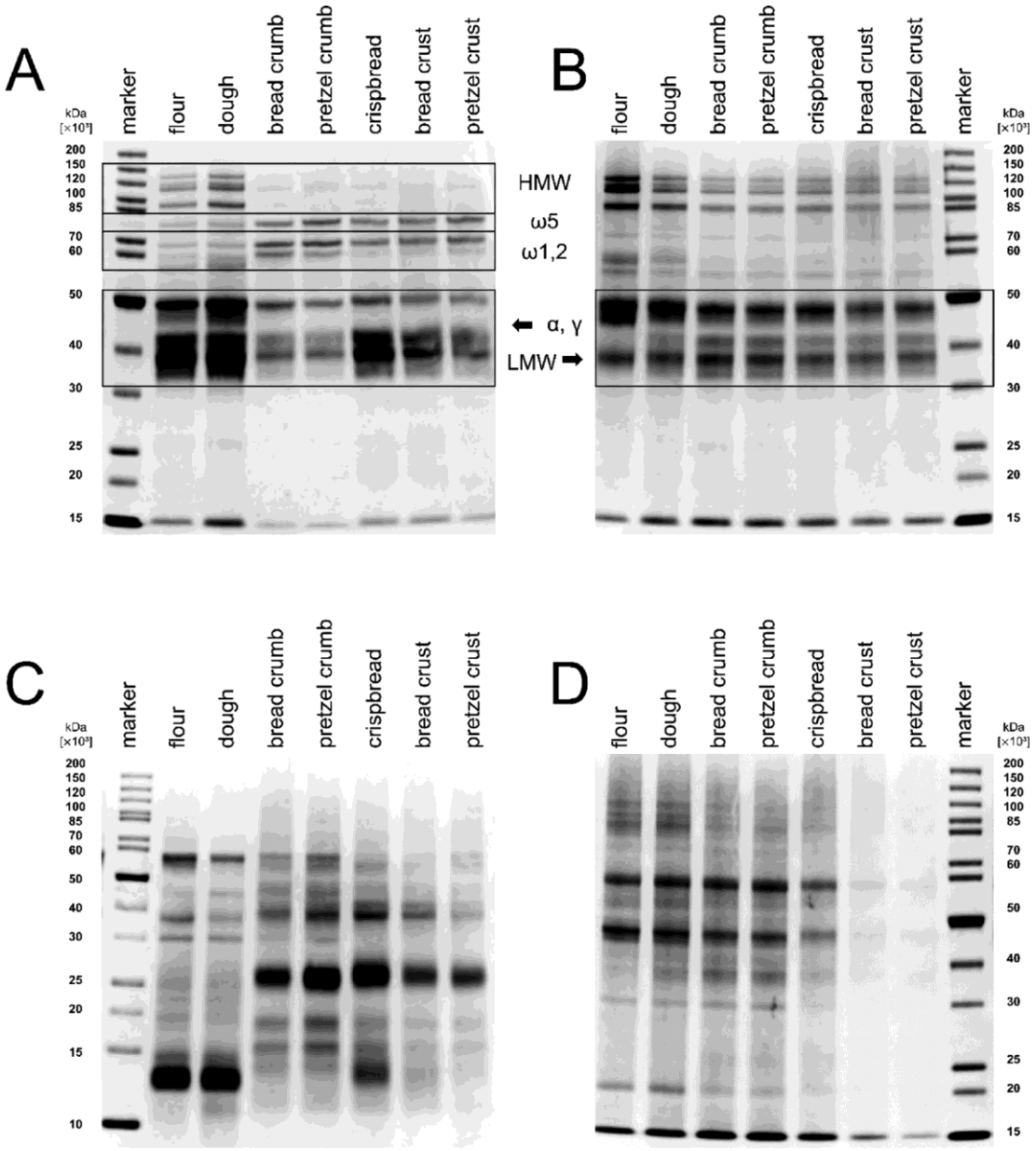

Figure 2. SDS-PAGE of the (A) gliadin, (B) glutenin, (C) ALGL fractions, and (D) the extraction residues from flour, dough, and baked goods, showing gliadin pattern (ω5-, ω1,2-, α-, and γ-type), HMW and LMW glutenin subunits.

3.3. Proteome Profiling. Label-free untargeted proteomics was used to establish a comprehensive profile of the proteomes in the ALGL, gliadin, and glutenin fractions of all samples. In total, 1488 peptides were identified, which could be assigned to 93 protein groups after quality filtering (Table S1). A protein group comprises all proteins, which were identified based on the same peptides and could not be distinguished by unique peptides.[41] The first accession number of each protein group was referred to as the representative protein accession number of the group and used for further reporting.

Figure 3 shows the distribution of total, shared, and exclusive protein groups, found in the three investigated protein fractions. Seventy-one protein groups were identified in the ALGL fractions, 27 in the gliadin fractions, and 47 in the glutenin fractions. Many protein groups identified in the glutenin or gliadin fractions were found in both fractions (22), and also in the ALGL fractions (17, 29). Sixteen protein groups were common in all fractions.

Protein groups were classified according to Wieser et al.[42] based on their different functions into storage proteins (α/β-, γ/δ-, and ω-gliadins; LMW-GS; HMW-GS; other), metabolic and protective proteins, which are involved in plant growth and defense, and structural proteins (Table S2). Structural proteins were not detected in any protein fraction. Due to their insolubility, they most likely remained in the extraction residues,[42] which we did not investigate using proteomics. The function of proteins annotated as "uncharacterized protein" in Uniprot is unknown; hence, they were classified as such. As seen in Figure 4, six protein classes were identified in all fractions, which further highlights the challenge of separating distinct wheat proteins based on solubility.[20,43] ω-Gliadins were one exception, as this protein class was only detected in the

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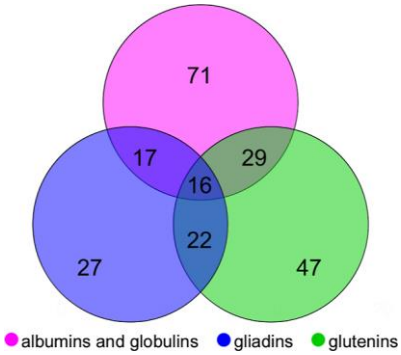

Figure 3. Venn diagram showing the number of proteins identified in all replicates (n = 3) of at least one sample (n = 7: flour, dough, bread and pretzel crumb, crispbread, bread, and pretzel crust) per protein fraction by untargeted LC−MS/MS.

ALGL and gliadin fractions, but not in glutenin fraction. The identified proteins in the ALGL fraction were composed of roughly two-third of metabolic and protective and one-third of storage proteins (Figure 4A). Storage proteins, which are mainly gliadins and glutenins (gluten), made up the majority of proteins in the gliadin (88%) and glutenin fractions (64%) (Figure 4B,C). Metabolic and protective proteins were also found in high quantities in the glutenin fractions (35%) and less in the gliadin fractions (12%). In general, the total number of identified proteins was in accordance with other proteomic profiling studies of wheat proteins.[20,44−46] Fallahbaghery et al.[46] found a similar number of gluten protein types (α/β-, γ/δ-, and ω-gliadins, LMW-GS, HMW-GS). However, the overlap of identified proteins with the same accession number found in previous studies was low (<20%), which can be explained by the different study designs in terms of sample preparation, LC−MS/MS setting, databases, or software tools. For example, it has been shown that the extraction method and the choice of enzyme very much affected the results.[46]

3.4. Clustering of all Fractions. For the relative quantitation of proteins, the MS intensities of all detected peptides per protein were used to compute a label-free quantitative protein intensity value. A PCA of this data is displayed in Figure 5. In general, samples clustered according to their fractions. The gliadin and glutenin fractions were more similar to each other than the ALGL fractions, which were more separated in principal component 1. The relationship of the gliadin fractions of the flour and dough to the baked goods was similar to their relationship to the glutenin fractions of the baked goods. As seen from the SDS-PAGE, this is likely due to the presence of HMW-GS and an increased amount of LMW-GS, α-, and γ-gliadins in the glutenin fractions of the baked goods in comparison to their gliadin fractions.

3.5. Clustering within the Fractions. A heatmap was created for each protein fraction to visualize the relationship between the samples based on their quantitative proteome composition (Figures 6, S2). The clustering of the samples demonstrated the influence of the different processing conditions including temperature and alkali treatment.

The protein fractions of the flour and the dough built a separate cluster each (no thermal treatment). Also, all fractions of the bread crumb and pretzel crumb clustered together, confirming the expected similarity of the protein composition of the crumbs. The ALGL and gliadin fractions of the crispbread, bread crust, and pretzel crust clustered apart from the crumbs in

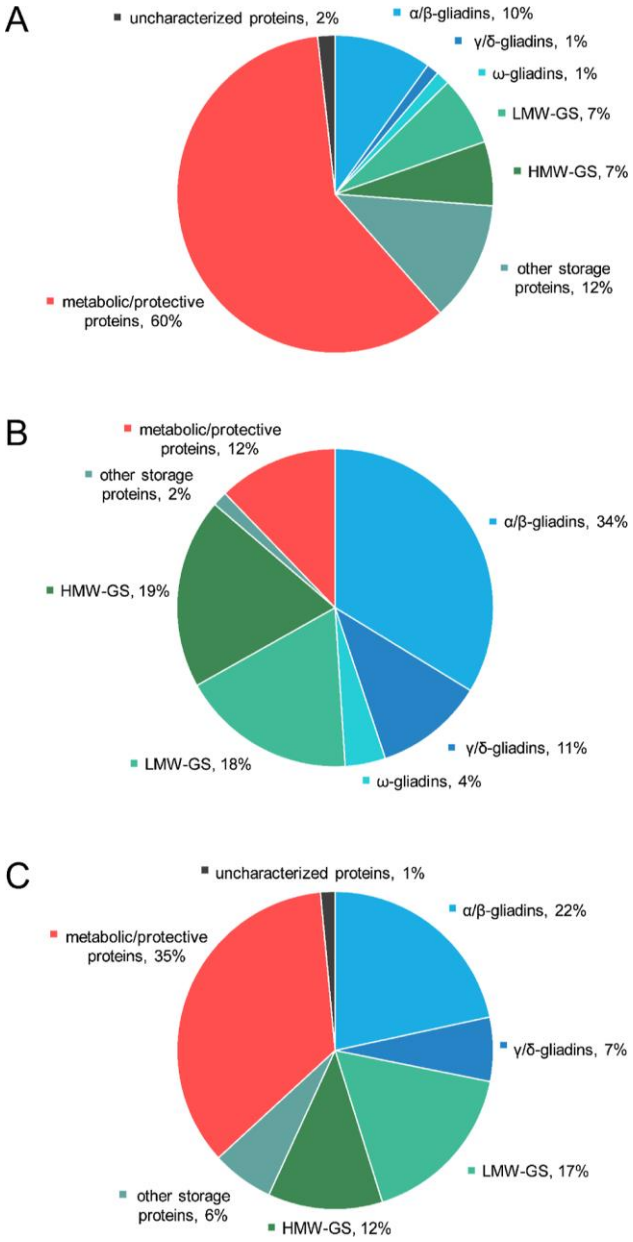

Figure 4. Relative quantitative distribution of protein types identified in the (A) ALGL, (B) gliadin, and (C) glutenin fractions. Proteins are identified in all replicates (n = 3) of at least one sample (n = 7) per protein fraction.

the same cluster in the same way: the fractions of pretzel crust and crispbread were more similar to each other than to the fractions of the bread crust. In contrast, in the glutenin fractions, the pretzel crust clustered away from the other heat-treated samples making it the most different one among the baked goods. This showed that the additional alkali treatment of the pretzel crust had a greater impact on the proteome composition of the glutenin fraction than on the proteome of the gliadin and ALGL fractions. In general, the extent of thermal treatment was the main factor for the cluster formation. Though the physicochemical properties of the gluten proteins are greatly modified during dough formation,[47] the proteomes of the dough fractions were closer to one of the flour fractions than to those of the fractions of the baked goods. Thus, the heat denaturation of the wheat proteins provoked the most significant structural

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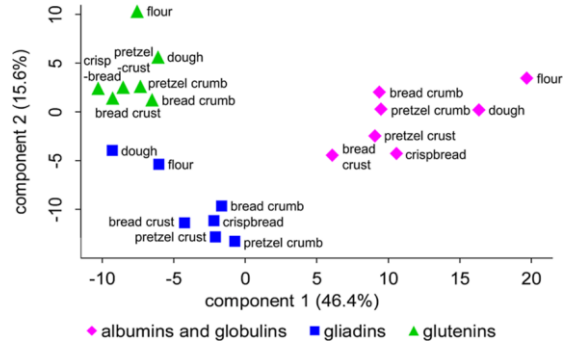

Figure 5. Principle component analysis of protein intensities measured over all fractions and sample types. For each sample, the mean over three technical replicates was computed.

changes during the bread making process. The heat-dependent formation of SS cross-links, rather than of non-SS cross-links favored by the alkali treatment of the pretzel crust is likely to mainly determine the proteome composition of the baked goods.

Protein groups were clustered according to their abundances in the samples. The α-gliadins, LMW-GS, and HMW-GS contained in the ALGL fractions were mainly comprised in the first cluster (Figure 6A). Their abundances increased with baking. The metabolic and protective proteins of the ALGL fractions clustered in the second cluster. Baking changed their abundances in different ways. Most HMW-GS und LMW-GS of the gliadin fractions also built a cluster with decreasing abundances in the baked goods (Figure 6B). The other cluster highlighted contained most gliadins (α/β, γ/δ, and ω) of the gliadin fractions. Their abundances tended to increase in the baked goods. The clustering of the protein groups of the glutenin fractions (Figure 6C) showed that all α-gliadins and all HMW-GS formed distinct clusters each. The abundances of the α-gliadins increased, and the abundances of the HMW-GS decreased if flour was processed. Many metabolic and protective proteins of the glutenin fractions also showed lower abundances after baking.

Heatmap representation further illustrated a considerable amount of missing values for protein intensities, especially for the baked goods. Previous studies[48−51] have shown that the baking process reduced the enzymatic digestibility of gluten proteins due to the heat-induced structural modifications, which is crucial for bottom-up proteomics. Alternatively, certain gluten proteins were not extracted from the baked goods due to molecular rearrangements during baking changing their solubility.[3] However, it remains unclear, if missing data resulted from the absence of the proteins due to a process-related changed solubility (no extraction) or hindered detection.

3.6. Differential Abundance Analysis between Protein Fractions. Since the PCA and HC analysis revealed that the differences between the fractions of the flour, crumbs, and crusts were the greatest, we decided to focus on this data for further analysis (Table S3). Proteins were selected that showed significant differential abundance within at least one fraction (ALGL, gliadin, and glutenin) of at least one processed sample (crumb or crust) in comparison to the flour. In total, the concentrations of 82 proteins were significantly affected by the baking process in at least one fraction. Those proteins belong to all kinds of protein classes. Figure 7 shows the log2-fold changes of gluten protein abundances in the different fractions of bread crumb and crust, compared to flour. The changes are a result of

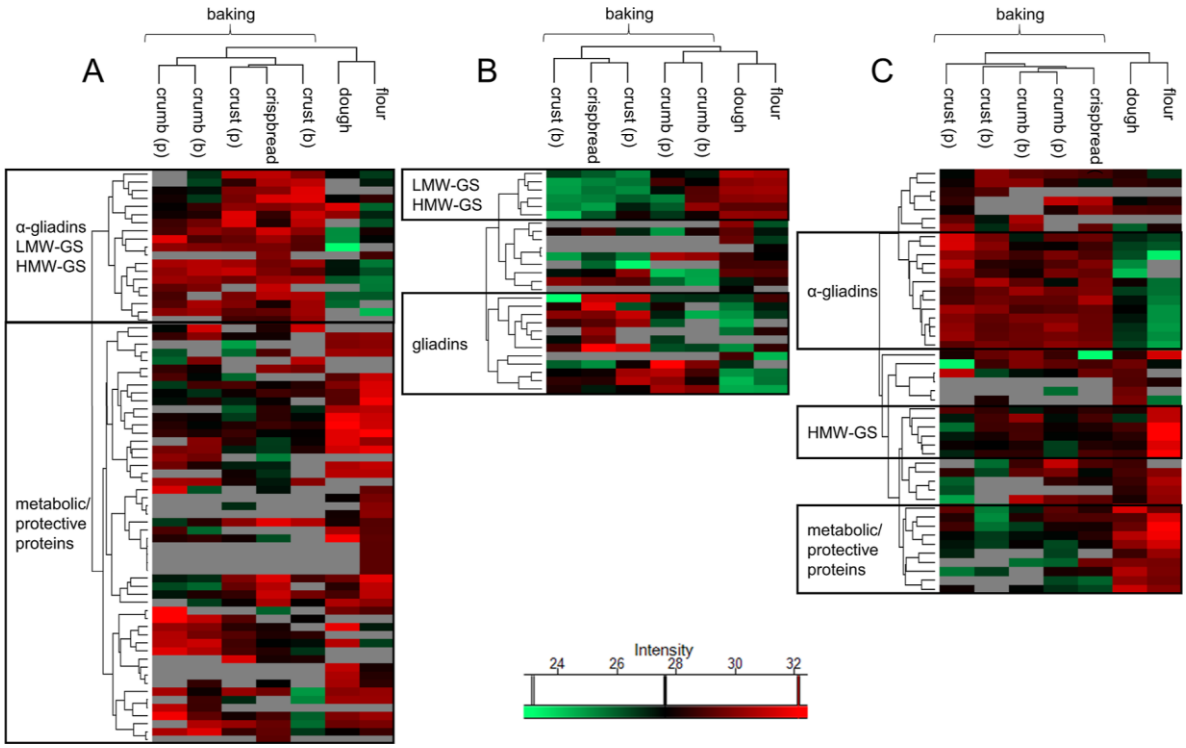

Figure 6. Hierarchical clustering of protein groups, (A) ALGL, (B) gliadin, and (C) glutenin fractions from flour, dough and baked goods, and heat map representation of z-scored label-free quantitation intensities ($n = 3$). The data matrix is color-coded according to the mean intensity values, which are proportional to the protein abundances. b, bread; p, pretzel. Missing values are in grey.

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Accession	Protein name	Crumb			Crust		
		ALGL	GLIA	GLUT	ALGL	GLIA	GLUT
A0A0K2QJU7	α/β-Gliadin	0.64	-1.02	5.47*	2.79	1.29	5.05*
A0A0K2QJA2	α/β-Gliadin	0.13	-0.12	4.20*	1.86	-0.37	4.04*
A5JSA6	α/β-Gliadin	-0.20	0.07	2.22*	0.57	-0.55	2.35*
D2T2K3	α/β-Gliadin (Fragment)	1.85	-2.38	0.37	0.78	-3.53	1.65*
A0A2P1H6A2	α-Gliadin	2.78*	-1.41	0.42	3.98*	-3.30	1.29*
A0A0K2QJX0	Pseudo α/β-gliadin	0.71	-1.96	1.49*	3.49*	-0.66	1.41*
A0A455QRK2	α/β-Gliadin (Fragment)	-0.57	-3.54	3.92*	5.98*	-2.64	2.71
A0A0E3Z516	α/β-Gliadin (Fragment)	0.32	-0.17	3.32	4.17*	-0.53	1.46
K7X0Q3	α-Gliadin	-1.15*	-3.47	0.32	1.99*	0.98	0.20
A0A2U8JD37	δ-Gliadin D1	0.63	1.07	-0.95	3.10*	0.78	0.09
B6UKM8	γ-Gliadin	1.07	0.16	-0.25	0.96*	-0.99	-0.09
R9XV62	γ-Gliadin	0.19	1.02	0.36	1.19*	2.86*	0.94
A0A060N0S6	ω-Gliadin (Fragment)	5.77*	4.53	0.92	5.71*	3.48	0.16
R4JFL5	LMW-GS (Fragment)	3.22	-4.39	-0.08	3.78*	-1.63	0.16
D6RVY4	LMW-GS (Fragment)	2.83*	0.23	0.48	2.57*	-0.91	0.43
A0A0S2GJT4	LMW-GS	0.45	-0.38	0.05	0.94*	-1.66	-1.33
B6ETR9	LMW-GS D-type (Fragment)	1.00	-0.05	1.99	1.86*	-1.45	1.42
I0IT56	α/β-Gliadin	-2.20	-3.50	0.86*	1.82*	-3.57*	1.29*
Q8W3W4	LMW-GS group 4 type II	0.50	-2.32	0.35	1.98*	-4.63*	0.31
R4JFP9	LMW-GS	-0.43	-0.73	-0.63	1.89*	-3.76*	-0.90
Q42451	HMW-GS Glu-B1-1b	0.41	-0.60	-1.01	1.56*	-1.63*	-0.95
Q0Q5D3	HMW-GS γ-type	3.11*	-2.31	-0.22	2.37*	-4.84*	-0.22
A9YSK4	HMW-GS	3.29*	-1.45	-0.71	3.16*	-3.04*	-0.69
Q03871	HMW-GS 1By9	3.80*	-1.91	-0.61	4.12*	-2.22*	-0.53*
A0A0X9BSF8	HMW-GS	4.40*	0.71	-0.55	4.45*	-0.76	-0.64*

Figure 7. Log2-fold changes of gluten protein abundances in the ALGL, gliadin (GLIA), and glutenin (GLUT) fraction from bread crumb and crust in comparison to flour quantitated by label-free quantitation. Negative values indicate a decrease of protein concentration in comparison to the flour sample; positive values indicate an increase of protein concentration in comparison to the flour sample. Color code: the more intensely red/green, the greater the increase/decrease of concentration. *Significant difference in comparison to flour (test *t*-test, $p < 0.05$).

the gluten polymerization during bread making, which affects the solubility properties of the gluten proteins. The gluten network formation mainly involves SH oxidation and SS-SH interchange reactions leading to SS cross-links between gluten proteins. For a comprehensive review of the physical and chemical changes of gluten proteins during processing, the reader is referred to Delcour et al.[47]

Most gliadins (α/β, γ/δ, and ω) were significantly increased in the glutenin and/or ALGL fraction of the crumb and/or crust. This can be explained by an accumulation, since most of the gliadins are not directly involved in the gluten network formation but are retained in the network as monomers.[52,53] Thus, these gliadins were enriched in certain fractions due to a reduced solubility of other proteins involved in gluten formation during baking. This was also seen for some LMW-GS (R4JFL5, D6RVY4, A0A0S2GJT4, and B6ETR9). Cysteine-free ω-gliadin (A0A060N0S6) showed the highest accumulation within the gliadin fractions.

On the other hand, the abundances of α/β-gliadin (I0IT56), LMW-GS (Q8W3W4 and R4JFP9), and HMW-GS (Q42451, Q0Q5D3, A9YSK4, and Q03871) decreased in the gliadin fraction of the crumb and especially the crust. HMW-GS (Q03871, A0A0X9BSF8) also significantly decreased in the glutenin fraction of the crust. Because the reduction was accompanied by an increase of abundance in other fractions, it could be partly attributed to a shift into other fractions. Interestingly, it was often a shift into the ALGL fractions, suggesting that distinct HMW-GS and LMW-GS become (partly) salt-soluble due to conformational changes induced by baking. Since the fold change decrease was often greater than fold change increase, the decrease of protein abundances is also likely due to their incorporation into HMW polymers, which become insoluble by Osborne fractionation and remain in the extraction residues. Non-SS cross-links have been demonstrated to have a significant impact in this context.[10,54]

The presence of most protective and metabolic proteins decreased in especially the ALGL fraction of the crumb and the crust but also in the glutenin fractions (Table S3). A few protective and metabolic proteins such as starch synthases (Q8W2G8 and Q9XEN9) or dehydrin (A0A3B6HW41) were increased in their abundance in the ALGL fractions of the crumb or crust. An explanation for the changes in protein abundance was found by detecting their presence exclusively in either the flour or the bread sample fractions. One should keep in mind that a considerable amount of non-protein material, particularly starch, was coextracted in the ALGL fractions of the baked goods. These potentially interfering substances could impair sample preparation resulting in a reduced detection of certain proteins. This further highlights the challenge of missing values and the ambiguity of whether they are due to the lack of the proteins in the samples or the inability to detect them. However, some protective and metabolic proteins such as beta amylase[55] or serpins[56,57] might also play a role as important components of the gluten polymer.

Overall, the protein abundances in the fractions of the crumb and the crust very often changed in the same way. The magnitude of the fold change was often greater in the crust than

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in the crumb, indicating heat-dependent SH-SS exchange reaction rates.[58]

One limitation of the current study is that the results are based on the analysis of protein fractions isolated from one single wheat cultivar. Although the choice of the cultivar was done carefully to select a representative sample for bread making, genetic and environmental factors and their interaction are known to influence the proteome composition of wheat.[43,59,60] The results obtained here thus only provide one snapshot and are expected to change depending on the flour sample. The overall procedure from milling to baking and isolation and analysis of different wheat proteins is rather time-consuming, cost- and labor-intensive. This is why the current study first focused on determining the influence of processing, prior to studying the variability arising from other factors.

All in all, this study provided novel insights into the compositional changes of different wheat protein fractions related to a bread and pretzel making process. Baking significantly changed their protein contents as well as the composition of the extracted proteins. The different baking conditions indicated that protein changes were mainly temperature-dependent. Protein concentration changes in bread crumb or crust in comparison to flour revealed which specific gluten proteins may play a greater role in gluten network formation during baking. The baking process also changed the abundances of many non-gluten proteins. Since compositional data alone cannot be used to determine precise reactivities, future work needs to complement the molecular description of the fractions with contextual information (higher order structure), focusing on distinct cross-linked peptides and protein−protein interactions. This will contribute to a better understanding as to which of the more than 100 very similar gluten proteins actually contribute to bread making quality and should be targets for future wheat breeding strategies.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.3c02100.

RP-HPLC profiles of ALGL, gliadin, and glutenin fractions; hierarchical clustering of protein groups with UniProt accession number and name within ALGL, gliadin, and glutenin fractions from flour, dough, and baked goods, and heat map representation of label-free quantitation intensities; and additional results of MS data analysis by Perseus software (PDF)

Overview of identified proteins (XLSX)

Log2 fold-changes of protein abundances in the ALGL, gliadin, and glutenin fractions of bread crumb and bread crust in comparison to flour (XLSX)

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Tanja Miriam Schirmer—Data curation, formal analysis, investigation, methodology, visualization, and writing—original draft. Christina Ludwig—Data acquisition and curation, resources, writing—review and editing. Katharina Anne Scherf—Conceptualization, funding acquisition, resources, supervision, and writing—review and editing.

Notes

The authors declare no competing financial interest.

The proteomics raw data, MaxQuant search results, and the used protein sequence database were deposited with the ProteomeXchange Consortium via the PRIDE partner repository[61] and can be accessed using the dataset identifier PXD041876.

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■ ABBREVIATIONS USED

AACC(I), American Association of Cereal Chemists (International); ALGL, albumins and globulins; ANOVA, analysis of variance; ATI, α-amylase/trypsin inhibitor; FDR, false discovery rate; HC, hierarchical clustering; HMW-GS, high-molecular-weight glutenin subunits; ICC, International Association for Cereal Science and Technology; LC-MS/MS, liquid chromatography tandem mass spectrometry; LFQ, label-free quantitation; LMW-GS, low-molecular-weight glutenin subunits; PCA, principal component analysis; PWG, Prolamin Working Group; RP-HPLC, reversed-phase high-performance liquid chromatography; RT, room temperature; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SH, sulfhydryl; SPE, solid phase extraction; SS, disulfide

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4 General discussion

Different wheat flour-based and incurred baked goods (bread, crispbread, pretzel) made from one dough were characterized using immunochemical, chromatographic, gel- and mass spectrometry-based methods. The samples were considered to reflect different degrees of processing (heat, alkali treatment) ranked according to an increasing degree of processing (flour, dough, bread crumb, pretzel crumb, crispbread, bread crust, pretzel crust); crumb samples were assumed to have been subjected to a very similar treatment. The study was composed of two main parts (Figure 8). The first publication focused on the influence of the different baking conditions on the immunochemical detection of gluten by ELISA. The second publication focused on the investigation of compositional changes within the Osborne fractions induced by the different baking conditions.

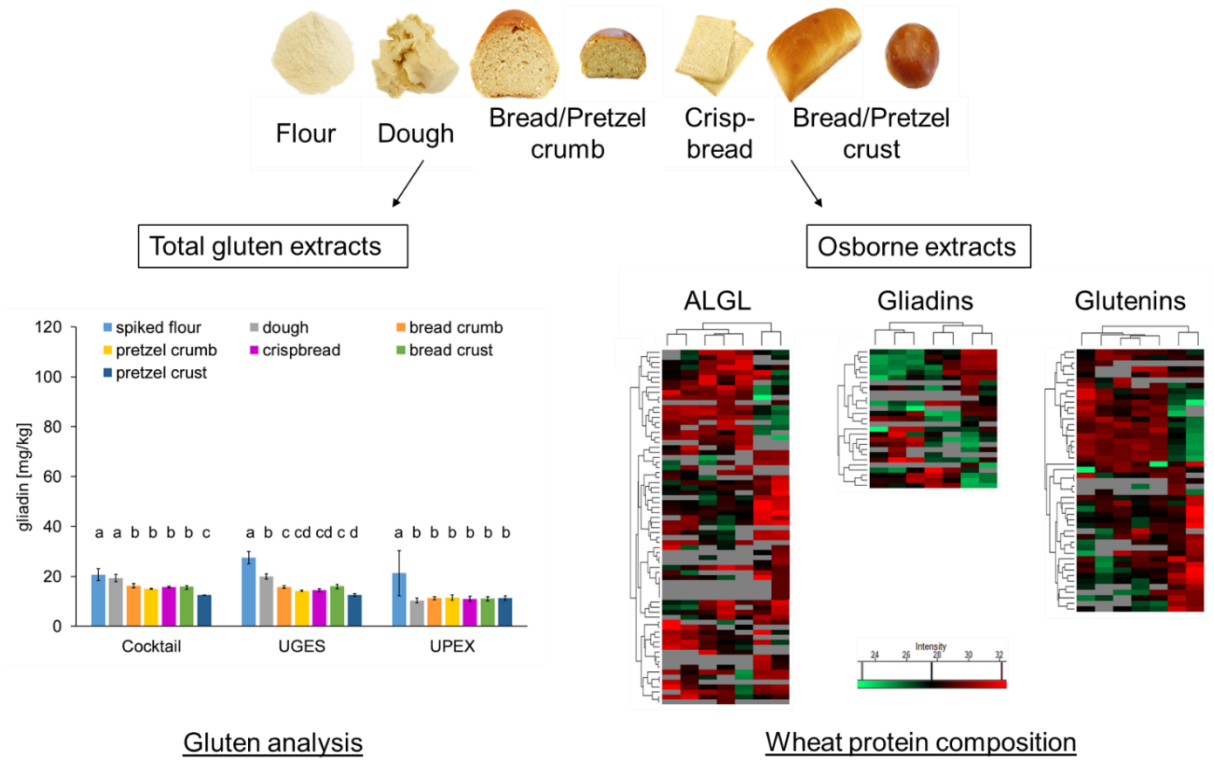

Figure 8. Graphical abstract. UGES, universal gluten extraction solution; UPEX, universal prolamin and glutelin extractant solution; ALGL, albumins and globulins.

4.1 ELISA analysis

A sandwich R5 ELISA was used to determine the gliadin content of the samples after extraction with Cocktail (patent WO 02/092633 A1), UGES (patent WO 02/092633 A1) and UPEX. The gliadin recoveries, calculated as the gliadin content analysed relative to the spiked gluten protein content, were between the tolerated range of 80 to 120% (Abbott et al., 2010) for most spiked flours regardless of the extraction solvent. Processing led to a decreased gliadin detectability in the incurred baked goods. The gliadin recovery of the incurred baked goods varied with the extraction solvents and gluten concentration and was about 16 to 45% less in Cocktail extracts, about 21 to 54% less in UGES extracts and about 2 to 48% less in UPEX extracts in comparison to their corresponding flours. There was no clear correlation between the gliadin content analysed and the degree of heat treatment, i.e. the gliadin contents analysed in heat-treated material were in the same range. Additional alkali treatment of the pretzel crust resulted in the lowest gliadin contents (< 12 mg/kg in 20 mg gluten/kg, < 33 mg/kg in 50 mg gluten/kg, < 66 mg/kg in 100 mg gluten/kg). Usually ELISA analysis is relevant for low gluten products. For completeness, wheat-based goods were also analysed with Cocktail extraction and the results were in agreement with the ELISA results for the incurred products. Overall, the extraction with Cocktail achieved for many samples a slightly, but significantly, better gliadin recovery for baked goods in comparison to the extraction with UGES and UPEX. Furthermore, only the use of Cocktail enabled a gliadin detection in dough comparable to flour. The variations in extraction efficiency can be attributed to the presence of different disaggregating and reducing agents in the extraction solutions (García et al., 2005; Mena et al., 2012; Biomedal Diagnostics, 2016). The mode of action of the disaggregating agents as well as the strength of the reducing agents vary. The precise mechanism and interplay of the different agents in the context of gluten solubilisation needs further clarification. Previous studies (Mena et al., 2012; Segura et al., 2021), found a good accordance in the gluten content of commercial foods spiked with PWG gliadin and analysed by R5 sandwich ELISA in combination with Cocktail, UPEX or UGES extraction protocols. However, only possible matrix effects (by means of spiked materials) but not effects of processing on gluten extraction efficiency were investigated. Our study confirms results of Méndez et al. (2005) and Lacorn et al. (2021) which were the base for the Codex Alimentarius decision to recommend the Cocktail extraction in combination with R5 ELISA for gluten determination (Codex Standard 118-1979, 2008). However, also the extraction with Cocktail faces challenges to compensate for effects of processing like baking and alkaline pH. An adverse effect on gliadin recovery determined by R5 ELISA in processed foods such as bread or cookies as a result of thermal processing has been widely reported. Bugyi et al. (2013) noted a lower gliadin content in PWG gliadin-incurred cookies (10 and 50 mg/kg) compared to the spiked flour and cookie dough with several ELISA kits. A study by Gomaa

and Boye (2013) using gluten-incurred cookies (1000 mg/kg), also reported a decrease in gluten recovery measured by two different ELISA kits with an increase in the baking time and a decrease in the cookie size (gluten recovery 31% to 59% for baking times of 25 min at 177 °C). Sharma et al. (2013) described a reduced gluten recovery of wheat flour-incurred cornbread (20 to 1000 mg/kg) analysed by various ELISA kits in comparison to the corresponding dough samples. The gluten recovery was reduced with increased baking time for most ELISA kits. They also found that the stability and immunoreactivity of gluten proteins were not adversely affected by the baking conditions. This is an important finding since it shows that a lower detectability does not indicate a decreased immunoreactivity. The finding aligns with the research of Scibilia et al. (2006) within the frame of a double-blind placebo-controlled food challenge study on wheat allergy. They demonstrated no significant difference in clinical reactivity after consumption of raw and cooked wheat test meals. Using the same raw and cooked meals, Pastorello et al. (2017) provided further evidence that several IgE-binding proteins in the different wheat protein fractions maintained their IgE-binding capacity after cooking. Török et al. (2014) showed that different ELISA kits achieved a gliadin recovery which was less than 60% in comparison to the powder mixture of the raw materials using PWG gliadin-incurred cookies (50 mg/kg). The literature review also revealed that in general the impact of food processing on gluten detectability was greater than the analytical variance among various ELISA kits. The decrease of gluten recovery after baking is explained by a loss of antibody-binding activity or solubility, i.e., extractability of gliadin or a combination of both. This is due to the altered protein structure induced by heat treatment and potential interaction of gliadin with other matrix components (Bugyi et al., 2013; Török et al., 2014). In contrast to our approach, none of the studies provided a comprehensive protein analysis of the sample material.

Another issue of gluten quantitation by ELISA is the recalculation of the gluten content based on the gliadin content measured by ELISA. The usually applied conversion factor of 2 (Codex Standard 118-1979, 2008) is based on the generalisation that the gliadin/glutenin ratio is 1. However, it has been shown that this factor is inaccurate in many cases leading either to over- or underestimation of the real gluten content (Wieser and Koehler, 2009; Török et al., 2014). In our study, the conversion factor of 2 would be sufficient for a correct labelling of most samples regardless of the kind of extraction solvent or degree of processing. In very few cases (flour spiked with 50 mg gluten/kg if extracted with Cocktail or UGES; dough incurred with 50 mg gluten/kg if extracted with Cocktail) an overestimation would be observed in our study, which may be important from an economical point of view by hampering potentially proper products to be declared as low gluten foods. As a result, the conversion factor often functions as a form of safety factor, despite not being its original purpose. However, given that this factor must account for highly variable conditions such as different gliadin/glutenin ratios depending

on the type of grain (Xhaferaj et al., 2023b, 2023a) and raw material (flour, starch), or the degree of processing, it is highly likely that especially low gluten foods may be incorrectly labelled. Further studies are needed to determine if a recovery correction can improve food safety in this regard. Currently, it is not feasible to recommend a recovery correction as the recovery rate may vary greatly depending on the sample.

4.2 Protein extractability

Overall the protein recovery, which was defined as the protein content analysed by RP-HPLC and GP-HPLC with different extraction protocols relative to the total crude protein content, decreased in processed goods (including dough) under reducing and non-reducing conditions. The degree of food processing (mixing, baking, baking + alkali treatment) had an effect on the protein recovery. The higher the degree of processing, the lower the recovery (90% in dough, 75 to 83% recovery in baked goods without alkali treatment, 58 to 61% recovery with alkali treatment). The protein recovery was related to altered solubility properties induced by baking. Proteins were classified by their solubility in salt solution (ALGL), alcohol (gliadins), and SDS containing buffer (ALGL+gliadins), and after SS reduction with DTT in alcohol, and SDS containing buffer (glutenins). Those were named as alcohol- and SDS-insoluble proteins. Decreased levels of salt-soluble (3.3-5.5 mg/g vs. 19.3 mg/g flour), alcohol-soluble (7.4-27.4 vs. 94.5 mg/g flour), and SDS-soluble (15.6-26.8 mg/g vs. 114.0 mg/g flour) proteins and increased levels of alcohol-insoluble (66.9-99.9 mg/g vs. 35.6 mg/g flour) and SDS-insoluble (71.3-102.6 mg/g vs. 22.4 mg/g flour) proteins were analysed in the baked goods. In contrast, the amount of alcohol- and SDS-insoluble proteins extracted from dough was decreased (30.4 mg/g, 6.4 mg/g), and SDS-soluble proteins were increased (120.8 mg/g). The increase of protein SDS extractability during dough formation corresponded to data published by Tanaka (1973b, 1973a), Eckert et al. (1993), Weegels et al. (1997), Belton (2005) and Lagrain et al., (2007). Lagrain et al. (2007) related the increase to an increased extractability of SDS-soluble glutenins. Whether the increase of extractability of SDS-soluble proteins during dough formation is due to gluten depolymerisation, conformational rearrangement, and/or improved dissolution by altered effective surface properties and/or area is still subject to debate.

There was no clear relationship between the solubility properties and degree of processing within the baked goods, with the exception that the decrease of protein recovery of pretzel crust (alkali treatment) was related to a decreased solubility of alcohol- and SDS-insoluble proteins. In general, the results were in agreement with previous studies investigating the extractability of proteins from wheat gluten and flour after thermal treatment under (non) reducing conditions by HPLC (Wieser, 1998; Lagrain et al., 2007; Rombouts et al., 2012b).

The explanation for a reduced extractability and changed solubility of wheat proteins in salt solution and under (non) reducing conditions in alcohol, and SDS-containing buffer, lies in the nature of the bread making process. In bread making, dough is produced by mixing flour, water, salt, and yeast, and then allowing it to ferment and bake (Goesaert et al., 2005). This process involves a series of complex chemical and physical transformations, with gluten proteins playing a crucial role. As the flour is mixed with water at the beginning of the bread making process, the mechanical energy from the mixer causes the gluten proteins to be disrupted and uncoiled, allowing them to interact with each other (Delcour et al., 2012). Gluten proteins contain a high concentration of hydrophilic amino acids, particularly glutamine, which leads to the formation of hydrogen bonds between protein strands. In addition, the SH groups from the amino acid cysteine, which is contained in almost all gluten protein types (Wieser et al., 2023), forms SS crosslinks. These two reactions are the base for the transformation of the gluten proteins into a continuous cohesive network surrounding the starch granules (Singh and MacRitchie, 2001). The network is essential for dough formation and rising, as the yeast generates and metabolizes sugars from the flour's starch, producing carbon dioxide and ethanol. During the baking process, the dough undergoes irreversible and obvious structural transformations, resulting in a light, porous product. Starch gelatinization occurs at the typical bread baking temperatures, starting above 50 °C (Belitz et al., 2008), and moisture levels, withdrawing water from the gluten proteins and stiffening the air cell walls (Delcour et al., 2010). Furthermore, gluten undergoes strain hardening due to gas cell expansion (Delcour and Hoseney, 2010). As the temperature rises during baking, the formation of further SS crosslinks is promoted (Lagrain et al., 2008) as shown by the decrease in alcohol-soluble and SDS-soluble proteins and the increase of alcohol-insoluble and SDS-insoluble proteins. Glutenins first polymerize through the oxidation of free SH groups (Veraverbeke et al., 1999; Lagrain et al., 2008). Later, gliadin-glutenin crosslinking occurs via SH-SS interchange reactions initiated by free SH groups (Lagrain et al., 2007).

Despite the use of a potent reducing agent (DTT), the protein recovery i.e. extractability from the processed samples was not fully restored, implying also non-SS crosslinking between gluten proteins. Wieser (1998) also did not achieve a complete recovery the extractability of total gluten proteins from bread samples following DTT reduction. The decrease in free SH (11 µmol to 5 µmol) and SS (120 µmol to < 88 µmol) groups and lower levels of total CSH and GSH in baked goods confirmed that non-SS crosslinking involving SH groups occurred during the baking process. Previous research has shown that lanthionine (reaction of DHA with cysteine) is such a notable non-SS crosslink in wheat gluten (Rombouts et al., 2016). However, earlier studies on the quantitation of GSH and CSH in cereal products suggested that free SH groups were hyperoxidized to their sulfinic and sulfonic acids causing a decrease in their levels after

baking (Reinbold, 2011). Within the baked goods, there was no further effect of alkali treatment (pretzel crust) on the concentration of thiols and thiol-containing compounds. Thus, another form of non-SS crosslinking may be responsible for the lower gluten extractability in the pretzel crust. Previous studies have demonstrated the presence of lysinoalanine formation (reaction of DHA with lysine) in pretzels (Sternberg et al., 1975; Raymond, 1980; Hasegawa et al., 1987; Rombouts et al., 2012a). It is highly favoured under higher pH conditions (Friedman, 1999) such as given during pretzel making. The presence of Maillard reaction-derived crosslinks was not further investigated in pretzel crust.

An in-depth analysis of the salt-soluble (= ALGL fraction), alcohol-soluble (= gliadin fraction) and alcohol-insoluble (= glutenin fraction) proteins extracted from the samples using label-free untargeted proteomics approach followed. The identification of gluten proteins involved in gluten network formation and therefore reduced extractability during baking were done by a differential abundance analysis based on LFQ values generated by LC-MS/MS. Because the heat denaturation of the wheat proteins provoked the most significant proteome changes during the bread, respectively pretzel, making process, the number of samples was reduced for the differential abundance analysis: protein abundances in the different fractions of bread samples (crumb, crust) were compared to flour.

The concentrations of 82 proteins (thereof 25 gluten proteins) were significantly changed in at least one fraction of at least one processed sample (crumb or crust) in comparison to the flour. Most gliadins (α, γ, ω) were found to be enriched in specific fractions (ALGL/glutenin), of the crumb and/or crust suggesting that they were not directly involved in gluten network formation. This finding aligned with existing literature, indicating that most gliadins are preserved within the network as monomers and are either captured within the polymer or linked through non-covalent interactions (Kuktaite et al., 2004; Iwaki et al., 2020). This was also seen for some LMW-GS (R4JFL5, D6RVY4, A0A0S2GJT4, B6ETR9), which can be explained with a very close structural similarity of some LMW-GS with gliadins. Cysteine-free ω-gliadin (A0A060N0S6) showed the highest accumulation within the gliadin fractions.

On the contrary, the abundances of α-gliadin (I0IT56), LMW-GS (Q8W3W4, R4JFP9), and HMW-GS (Q42451, Q0Q5D3, A9YSK4, Q03871) showed a decline in the gliadin fractions of the bread crumb and particularly the crust. HMW-GS (Q03871, A0A0X9BSF8) also significantly decreased in the glutenin fraction of the crust. This reduction may be partially attributed to a shift into other fractions, as an increase in abundance was observed in those fractions. The decrease in protein abundances was often greater than the corresponding increase, suggesting that the decline in protein abundances is also likely due to their integration into insoluble HMW polymers. These HMW polymers cannot be extracted by Osborne fractionation and

remain in the residual extraction material. This change of solubility reflected by a shift between specific fractions indicated an incorporation into the gluten network via SS crosslinks and non-SS crosslinks (Rombouts et al., 2011; Rombouts et al., 2016) as discussed before. Overall, the protein abundances in the fractions of the crumb and the crust very often changed in the same way. The magnitude of the fold change was often greater in the crust than in the crumb indicating heat-dependent SH-SS exchange reaction rates (Lagrain et al., 2008).

With over 100 closely related gluten proteins present in wheat, distinguishing the key players responsible for gluten network formation is crucial not only for analytical reasons but also with regard to bread making quality. Our work is another step toward a better understanding of which specific gluten proteins significantly contribute to gluten network formation. However, also the abundances of many non-gluten proteins in the Osborne fractions changed in response to processing. Some of them such as beta amylase (Wang et al., 2020) or serpins (Ostergaard et al., 2000; Li et al., 2018) are discussed to also play a role in gluten network formation, which adds to its complexity and should be considered in future research.

4.3 Protein composition

Solubility changes induced by baking also affected the protein type composition as shown by further analysis of the alcohol-soluble, and alcohol- and SDS-insoluble proteins. Therefore, the GP- and RP-HPLC profiles were assigned to different gluten protein types. In general, the composition of alcohol-soluble proteins changed in the same way, but the magnitude was different. There was a relative increase in ω-gliadins (ω5, ω1,2) (16% to 38-88%) and a relative decrease in α-gliadins (51% to 10-38%) and γ-gliadins (33% to 2-25%). The changes in composition of the alcohol-insoluble fraction were also significant, but less pronounced and within a similar range for all baked goods. The changes included a relative decrease in ωb-gliadins (4% to 3%) and HMW-GS (28% to 15-19%) and a relative increase in LMW-GS (69% to 79-83%). In the SDS-insoluble fraction there was no consistent change in the HMW-GS, LMW-GS, and alcohol-insoluble ALGL across the baked goods. This can be explained by the different extraction protocols. A higher proportion of LMW-GS was extracted from crispbread and crust samples using a SDS containing buffer as compared to salt and alcohol solutions under non reducing conditions. A better solubility of gluten protein in dilute SDS solution compared to dilute alcohol solution has been described before (Danno et al., 1974; Dupont et al., 2008).

To conduct an LC-MS/MS analysis of the sample material, greater quantities of salt-soluble, alcohol-soluble, and alcohol-insoluble proteins were isolated and referred to as ALGL, gliadin, and glutenin fractions, respectively. These fractions were characterized again using RP-HPLC. As expected, changes in the protein type composition of the gliadin and glutenin fractions were

similar. In the gliadin fractions, there was a relative increase in ω-gliadins (ω5, ω1,2) (18% to 43-74%) and a relative decrease in α-gliadins (49% to 15-33%) and γ-gliadins (33% to 9-24%). In the glutenin fractions, there was a constant level of ωb-gliadins (1%) and a relative decrease in HMW-GS (16% to 11%) and a relative increase in LMW-GS (82% to 87%).

As reported before (Wieser, 1998), although the absolute concentration of all gliadin types was decreased in the baked good, cysteine-free ω-gliadins were less affected than α- and γ-gliadins bearing intramolecular SS bonds, which contributed to the relative increase of ω-gliadins in the gliadin fractions. The absolute levels of all glutenins increased, whereas only the relative proportion of LMW-GS increased. Thus SH-SS interchange reactions of α- and γ-gliadins mainly occurred with LMW-GS. However, this could only be partially confirmed by the analysis of protein types of the SDS-insoluble protein fraction.

SDS-PAGE analysis of fractions and extraction residues made changes as determined by HPLC also visible and provided further information about changes in the ALGL fractions, where the protein composition was also significantly affected by baking. The SDS-PAGE of the total protein extracts from flour, dough and the baked goods showed the expected band patterns typical for wheat proteins, cv. Akteur, (Lagrain et al., 2012) and indicated a reduced protein extractability of the crust samples. However, due to the presence of numerous proteins with similar Mr resulting in indistinct bands, the analysis of the total protein extracts did not reveal clear differences in the protein composition in contrast to the SDS-PAGE of the protein fractions.

Proteome profiling of all fractions by untargeted LC-MS/MS and the proteomics software MaxQuant led to the identification of 1488 peptides assigned to 93 protein groups. The ALGL fractions contained 71 protein groups, the gliadin fractions 27 and the glutenin fractions 47. Sixteen protein groups were common in all fractions. For the relative quantitation of proteins, the MS intensities of all detected peptides per protein were used to compute a label-free quantitative protein intensity value. The majority of proteins in the ALGL fractions were metabolic and protective proteins (60%), while the gliadin (88%) and glutenin (64%) fractions contained mostly storage proteins. With the exception of ω-gliadins only found in the ALGL and gliadin fractions, the same kind of proteins (α/β-, γ/δ- and ω-gliadins, LMW-GS, HMW-GS, other storage proteins, metabolic and protective proteins) were identified in all fractions.

The results highlight the challenge of the common approach to separate distinct wheat proteins based on solubility (Osborne fractionation) and the subsequent classification of protein types according to their hydrophobicity (RP-HPLC). The reasons for this are the heterogeneity of gluten proteins, but still similarity of structures, comparable Mr and RP-HPLC retention times of certain protein types as well as their partly polymeric nature. Vensel et al. (2014) and Boukid

et al. (2019) have reported the coextraction of other protein types by solubility-based separation before. Vensel et al. (2014) found an accumulation of gliadins containing odd numbers of cysteine residues in the glutenin fraction, supporting the hypothesis that gliadins serve as chain terminators of the polymer chains. Lexhaller et al. (2019) showed that even gluten protein types isolated using preparative RP-HPLC contained significant proportions of other proteins than the targeted protein type.

In general, the total number of identified (gluten) proteins aligns with the findings of other proteomic profiling studies on wheat proteins (Colgrave et al., 2015; Bromilow et al., 2016; Fallahbaghery et al., 2017; Lexhaller et al., 2019). Fallahbaghery et al. (2017) found a similar number of gluten protein types (α/β-, γ/δ- and ω-gliadins, LMW-GS, HMW-GS). However, the overlap of identified proteins with the same accession number found in previous studies was relatively low (< 20%), which can be attributed to a range of factors, such as the different study designs in terms of sample preparation, LC-MS/MS settings, databases (Kumar et al., 2017), and software tools. For example, it has been shown, that the extraction method and the choice of enzyme very much affected the results (Fallahbaghery et al., 2017). Certain buffers can enrich for specific proteins which may be more amenable to digestion by a particular protease.

In general, trypsin is the standard enzyme in the context of proteomic experiments (Dau et al., 2020). This is due to its specific cleavage on the C-terminal side of lysine and arginine residues. However, the lysine/arginine content in gluten proteins is very low, limiting the utility of trypsin. While lysine/arginine content can range from approximately 1%–8% for different gluten proteins, the average content is between 2.2 and 3.0% across gluten proteins compared to 14.1% for bovine serum albumin, the most widely used control sample in proteomics (Bacala et al., 2022). Thus, multiple enzymatic digestion or less specific enzymes have been used for cereal proteomics (Alves et al., 2019; Bacala et al., 2022). Chymotrypsin, as used in our study, has been used frequently (Bromilow et al., 2016; Visioli et al., 2016; Bacala et al., 2020). It cleaves the peptide bonds on the C-terminal side of phenylalanine, tyrosine and tryptophan, which are abundant in gluten proteins. However, chymotrypsin is considered less specific than trypsin in terms of its cleavage preferences. While it predominantly cleaves after aromatic amino acids, it can also cleave after other amino acids like methionine and leucine, although to a lesser extent (Colgrave et al., 2017). However, the increased complexity of peptide fragments generated by chymotrypsin can also pose challenges for MS data acquisition and analysis (Dau et al., 2020).

A PCA for clustering of all fractions showed that samples clustered according to their fractions. A heatmap visualized the clustering within the fractions and demonstrated the influence of the different processing conditions. As the previous analysis had already shown, proteome

changes were mainly temperature-dependent. Thus, heat-dependent formation of SS crosslinks, rather than of non-SS crosslinks favoured by the alkali treatment of the pretzel crust is likely to mainly determine the proteome composition of the baked goods. However, additional alkali treatment had the greatest impact on the glutenin fraction.

The heatmap visualization also indicated how the protein abundances were changed by processing. The abundances of all gliadins and most LMW-GS and HMW-GS present in the ALGL fractions increased with baking. Baking induced distinct changes in the abundances of metabolic and protective proteins within the ALGL fractions in both directions (decreased and increased protein abundances).

The abundances of most HMW-GS und LMW-GS within the gliadin fractions decreased in the baked goods. Most gliadins (α, γ, ω) within the gliadin fractions exhibited an increase in abundance after baking. If these findings are related to the RP-HPLC data, it is reasonable to infer that the observed increase of ω-gliadins determined by RP-HPLC can be attributed to the increase in other types of gliadins as well. The decrease in α- and γ-gliadins determined by RP-HPLC may be also attributed to the decrease in HMW-GS and LMW-GS present in the gliadin fractions.

The clustering analysis of the protein groups within the glutenin fractions revealed that the abundances of all α-gliadins increased, while the abundances of all HMW-GS decreased upon flour processing. Many metabolic and protective proteins within the glutenin fractions showed lower abundances after baking. In relation to the RP-HPLC data, the observed increase of LMW-GS determined by RP-HPLC is also probably due to an increase of α-gliadins. The decrease in HMW-GS determined by RP-HPLC can be attributed to the reduced abundances of HMW-GS themselves, as well as the decreased levels of metabolic and protective proteins, which account for approximately 35% of the glutenin fractions.

The findings showed that gluten protein type characterisation by RP-HPLC, which separates proteins based on surface hydrophobicity, is limited since certain gluten protein types have very similar elution times leading to an imprecise RP-HPLC separation.

The heatmap representation further illustrated a considerable amount of missing values for protein intensities, especially for the baked goods. The occurrences of missing values in LC–MS based proteomics are often caused by biological and/or technical reasons (Hrydziuszko and Viant, 2012). Examples of biological factors are that proteins do not exist or that protein abundances are below the instrument detection limit. Analytical factors include sample loss in preparation, miscleavage of peptides during digestion, poor ionization efficiency and bad peptide-spectrum matches (Karpievitch et al., 2012). Previous research has indicated that the bak-

ing process leads to a decrease in the ability of enzymes to break down gluten proteins, primarily due to structural changes induced by heat (Pasini et al., 2001; Simonato et al., 2001; Smith et al., 2015; Wu et al., 2017). Furthermore, due to the molecular rearrangements of the gluten proteins during baking, it is also difficult to completely extract gluten proteins from baked goods (Wieser, 1998). However, it remains unclear, if the missing data resulted from the absence of the proteins due to a process-related changed solubility (no extraction) or hindered detection. Missing data reduces the power of a trial and introduces potential bias. Imputation of the missing values facilitates the statistical analysis and was done for the PCA and the differential abundance analysis.

4.4 Relationship between ELISA and protein analysis

The HPLC analyses did not reveal a clear correlation between the composition of gluten proteins including alcohol-soluble, alcohol-insoluble, and SDS-insoluble proteins and the results obtained from the ELISA. Instead, a relationship was observed between the extractability of gluten proteins and the ELISA results. There was no evidence of a connection between the extractability of alcohol- and salt-soluble proteins, mainly comprising ALGL and gliadins, and the ELISA results. However, a relationship was observed between the extractability of alcohol- and SDS-insoluble proteins with decreased solubility in baked goods and further reduction with alkali treatment and the ELISA results. The proteins of different solubility reflected the composition of the whole gluten extract (gliadins and glutenins), which was used for ELISA analysis. The alcohol-insoluble and SDS-insoluble proteins are supposed to contain the majority of glutenin proteins, to which the reactivity of R5 but also other ELISA antibodies such as G12 are known to be rather low (Lexhaller et al., 2016). However, these fractions also contain gliadins to which the ELISA is sensitive. The presence of a considerable amount of gliadins in the glutenin fractions (alcohol-insoluble proteins) of the samples was confirmed by the LC-MS/MS analyses. The LC-MS/MS analysis also provided additional evidence that the decreased extractability of the glutenin fractions, rather than their composition, is the primary factor responsible: though the proteome composition of the glutenin fractions demonstrated the strongest correlation with the degree of processing, the correlation was primarily attributed to an increase in α-gliadins and a decrease of HMW-GS as well as metabolic and protective proteins, which do not explain the observed decrease in ELISA detectability. Therefore, it is crucial to ensure the complete extraction of both gliadins and glutenins for ELISA analysis. Since the formation of insoluble gluten polymers arises from SS, but also non-SS crosslinking during processing, none of the recommended extraction solvents for ELISA analysis achieved a complete extraction of gluten from the baked goods. This could pose a concern for processed low gluten foods,

potentially leading to incorrect gluten-free labelling. Consequently, these findings should be taken into account in risk assessment and risk management decisions.

4.5 References

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5 Future Research

The results of the first part of the study revealed that a complete extraction of gluten from processed food for ELISA detection with common extraction solvents cannot be achieved. To optimise extraction protocols, a further understanding of gluten network formation during processing is necessary. Though the second part of the study contributed to a better understanding of which gluten proteins are involved in gluten network formation, this complex process is far from completely understood. There are further research questions arising from this study.

To begin with, it would be interesting to investigate in detail the nature of gluten protein crosslinking, in particular SS crosslinks, during processing. One potential approach could involve the identification of SS bonds using a crosslinking agent such as bismaleimidoethane. The crosslinker links two cysteine residues, which otherwise form a disulfide bond. Subsequently, the proteins are digested using proteases like trypsin or chymotrypsin. This enzymatic digestion gives rise to peptides and the crosslink, which connects two peptides via a thioether bond. Detection of these products could be accomplished through LC-MS/MS. Using suitable software tools for data analysis, it is possible to determine the number and location of the disulfide bonds.

Another focus could be placed on the detection of covalent non SS crosslinks such as dehydroamino acid-derived bonds.

Moreover, it is well known that gluten network formation is affected by both genotype and environment. Therefore, further proteomic work using more wheat varieties cultivated in different environments is needed in order to obtain a better understanding of gluten network formation in response not only to different processing conditions but also to the genetic background and growth conditions.

Finally, the importance of identified gluten proteins for the final product quality needs to be determined by combining proteomics results with rheological measurements and bread making properties in future research.

This knowledge will empower breeders to make informed decisions, prioritize desirable traits, and develop wheat varieties that meet the demands of the baking industry and consumer preferences.

6 Summary

Gluten network formation during processing plays a crucial role in determining the functional and quality aspects of wheat-based products. However, the underlying mechanism of cross-linking is not fully understood due to the complexity of the wheat proteome. Conformational changes of gluten proteins during processing of wheat flour also affect gluten analysis, which is important for determining a gluten-free status of food products.

This study aimed to investigate the influence of processing on the immunochemical detection of gluten proteins as well as on the qualitative and quantitative protein composition of wheat-flour based products.

Different wheat flour-based and incurred (20, 50, 100 mg gluten/kg) baked goods (bread, crisp-bread, pretzel) made from one dough were characterized using immunological, chromato-graphic, gel- and mass spectrometry-based methods. The samples were considered to reflect different degrees of processing (heat, alkali treatment) and ranked according to an increasing degree of processing as follows: flour, dough, bread crumb, pretzel crumb, crispbread, bread crust, pretzel crust.

First, the immunochemical detection of gluten in the sample material was examined. Previous studies investigating the impact of food processing on the immunochemical gluten detection did not include a concurrent analysis of the sample material, leaving a knowledge gap between the altered gluten structure and its detectability.

A sandwich R5 ELISA was used to assess the gliadin content of the samples, employing three different extraction solvents: Cocktail, UGES, and UPEX. Gliadin recoveries were determined by analysing the gliadin content relative to the spiked and incurred gluten protein content. The results showed that most spiked flours exhibited gliadin recoveries within the accepted range of 80 to 120%. Processing of the samples resulted in a decreased detectability of gliadin in the incurred baked goods. The gliadin recovery varied depending on the extraction solvents and gluten concentration. In comparison to their corresponding flours, the gliadin recovery was approximately 16 to 45% less in Cocktail extracts, 21 to 54% less in UGES extracts, and 2 to 48% less in UPEX extracts. No clear correlation was found between the analysed gliadin con-tent and the degree of heat treatment. The gliadin contents in the heat-treated samples fell within the same range. The additional alkali treatment of the pretzel crust led to the lowest gliadin contents (< 12 mg/kg in 20 mg gluten/kg, < 33 mg/kg in 50 mg gluten/kg, < 66 mg/kg in 100 mg gluten/kg), which was also visualized in the SDS-PAGE of the total protein extracts. Overall, the extraction with Cocktail yielded slightly better gliadin recovery for baked goods compared to UGES and UPEX extractions. This is probably attributed to the presence of dif-ferent disaggregating and reducing agents in the extraction solutions.

Challenges remain in compensating for processing effects such as baking and alkaline pH, even with the Cocktail extraction method.

The study also found that the recalculation of gluten content based on the gliadin content using a conversion factor of 2 is generally sufficient for accurately labelling most samples, regardless of the extraction solvent or degree of processing.

Different extraction protocols, including a modified Osborne fractionation, in combination with RP- and GP-HPLC analysis were employed to determine the protein recovery and solubility properties of the wheat proteins in the samples. Protein recovery referred to the protein content analysed relative to the total crude protein content. The results showed that the protein recovery decreased in the processed samples as the degree of food processing increased. For example, the protein recovery in dough was 90%, while in baked goods without alkali treatment, it ranged from 75% to 83%, and with alkali treatment (pretzel crust), it ranged from 58% to 61%.

The solubility properties of proteins under (non) reducing conditions were analysed and proteins were divided into salt-soluble (ALGL), alcohol-soluble (gliadins), SDS containing buffer-soluble (ALGL + gliadins), and alcohol-insoluble and SDS-insoluble proteins (glutenins). The levels of salt-soluble, alcohol-soluble, and SDS-soluble proteins decreased in baked goods compared to flour, while the levels of alcohol-insoluble and SDS-insoluble proteins increased. During dough formation, the extractability of SDS-soluble proteins increased, which corresponded to previous studies. No clear relationship was observed between the solubility properties and the degree of processing within the baked goods, except for the pretzel crust (alkali treatment), which showed a decreased solubility of alcohol- and SDS-insoluble proteins. Despite the use of a potent reducing agent, the protein recovery from processed samples was not fully restored, indicating the presence of not only SS, but also non-SS crosslinking between gluten proteins. The decrease in free SH groups and lower levels of total CSH and GSH confirmed the occurrence of non-SS crosslinking involving SH groups during the baking process.

The RP- respectively GP-HPLC profiles of the gliadin and glutenin fractions obtained by Osborne fractionation, as well as the glutenin fraction corresponding to the SDS-insoluble proteins, were further assigned to different gluten protein types. Thus it could be shown that the protein type composition of the different fractions was also changed by processing. The alcohol-soluble proteins showed a relative increase in ω-gliadins (ω5, ω1,2) and a relative decrease in α- and γ-gliadins. The alcohol-insoluble fractions exhibited a relative decrease in ωb-gliadins and HMW-GS, while LMW-GS increased.

The SDS-insoluble fraction did not exhibit consistent changes in HMW-GS, LMW-GS, and alcohol-insoluble ALGL proteins across the baked goods. The changes of the gluten type composition could not be clearly related to the decreased gliadin detectability by ELISA.

In a next step, an in-depth analysis of the proteome of the Osborne fractions was done. It was the first study which used RP-HPLC, SDS-PAGE and label-free untargeted LC-MS/MS analyses to develop a comprehensive profile of the ALGL, gliadin, and glutenin fractions of flour, dough and different baked goods. For this approach the wheat protein fractions were isolated in greater quantities. The characterisation by RP-HPLC was in agreement with the previous results. SDS-PAGE analysis of the fractions and extraction residues made changes as determined by HPLC visible and provided further information about changes in the ALGL fractions, where the protein composition was also significantly affected by baking.

The proteome profiling using LC-MS/MS identified a total of 1488 peptides assigned to 93 protein groups. The ALGL fractions contained 71 protein groups, the gliadin fractions contained 27 protein groups, and the glutenin fractions contained 47 protein groups. Only 16 protein groups were found in all fractions. The majority of proteins in the ALGL fractions were metabolic and protective proteins (60%), while the gliadin and glutenin fractions were predominantly composed of storage proteins (≥58 %).

It was noted that the common approach of separating wheat proteins based on solubility and hydrophobicity has limitations which can be explained by structural similarities and the polymeric nature of certain gluten protein types.

The involvement of specific gliadins and glutenins in gluten polymerization and network formation was observed through differential abundance analysis of proteins contained in the fractions of the bread samples compared to the flour. It revealed significant changes in the concentrations of 82 proteins, including 25 gluten proteins. In general, the changes of protein abundances in response to processing showed that glutenins are involved in gluten network formation via SS and non SS crosslinks and most gliadins rather via non covalent bonds.

The results of the HPLC analyses related the reduced detectability of gliadin/gluten by ELISA to a decreased extractability of gluten, especially the glutenin fractions, from the baked goods. The LC-MS/MS analyses showed the presence of a considerable amount of gliadins, to which ELISA is sensitive, in the glutenin fractions.

Our findings are important for a better understanding of the influence of food processing and the detection of gluten in processed food. Moreover, specific gluten proteins were identified that contribute to gluten network formation and, consequently, bread-making quality.

7 Zusammenfassung

Für die Funktionalität und Qualität weizenbasierter Produkte spielt die Ausbildung von Glutennetzwerken während ihrer Verarbeitung eine entscheidende Rolle. Der zugrundeliegende Mechanismus dieser Proteinaggregation und -vernetzung ist aufgrund der Komplexität des Weizenproteoms noch nicht vollständig verstanden. Die damit einhergehenden Konformationsänderungen von Glutenproteinen beeinflussen auch die Glutenanalyse, welche für die Kennzeichnung von Lebensmitteln als "glutenfrei" von großer Bedeutung ist.

Ziel dieser Arbeit war es, den Einfluss der Verarbeitung auf den immunchemischen Nachweis von Glutenproteinen sowie auf die qualitative und quantitative Proteinzusammensetzung von weizenmehlbasierten Produkten zu untersuchen.

Dazu wurden verschiedene glutenhaltige (Weizen) und glutenreduzierte (20, 50, 100 mg Gluten/kg) Backwaren (Brot, Knäckebrot, Brezel) mit immunologischen, chromatographischen, gel- und massenspektrometrischen Methoden charakterisiert. Die Proben spiegelten unterschiedliche Verarbeitungsgrade (thermisch, Alkalibehandlung) wieder und wurden nach Verarbeitungsgrad wie folgt eingestuft: Mehl, Teig, Krumen (von Brot und Brezel), Knäckebrot, Brotkruste, Brezelkruste.

Zunächst wurde der Glutengehalt des Probenmaterial mit Hilfe einer immunchemischen Methode bestimmt. Frühere Studien, welche den Einfluss der Lebensmittelverarbeitung auf den immunchemischen Glutennachweis untersuchten, hatten keine weiteren Analysen des Probenmaterials durchgeführt, wodurch der Zusammenhang zwischen veränderter Glutenstruktur und deren Nachweisbarkeit unklar geblieben ist.

Zur Bestimmung des Gliadingehalts der Proben wurde ein Sandwich R5 Enzyme-linked Immunosorbent Assay (ELISA) unter Einsatz von drei verschiedenen Extraktionslösungsmitteln (Cocktail, universal gluten extraction solution (UGES), universal prolamin and glutelin extractant solution (UPEX)) verwendet. Die Gliadin-Wiederfindung wurde definiert als analysierter Gliadingehalt im Verhältnis zum eingestellten Glutengehalt. Die Ergebnisse zeigten, dass die meisten mit Gluten versetzten Mehle eine Gliadin-Wiederfindung im Toleranzbereich von 80 bis 120 % aufwiesen. Die thermische Verarbeitung der Mehle (Backen) führte zu einer reduzierten Nachweisbarkeit von Gliadin. Die Gliadin-Wiederfindung variierte je nach Extraktionslösungsmittel und Glutenkonzentration. Im Vergleich zu den versetzen Mehlen war die Gliadin-Wiederfindung bei den Backwaren in den Cocktail-Extrakten 16 bis 45 %, in den UGES-Extrakten 21 bis 54 % und in den UPEX-Extrakten 2 bis 48 % geringer. Zwischen dem analysierten Gliadingehalt und dem Ausmaß der thermischen Behandlung (Einfluss Backtemperatur) konnte kein eindeutiger Zusammenhang hergestellt werden. Die Gliadingehalte der

Backwaren (Knäckebrot, Brotkrume, Brotkruste) waren ähnlich. Die zusätzliche Alkalibehandlung der Brezelkruste führte zur niedrigsten Gliadin-Wiederfindung (< 12 mg/kg bei 20 mg Gluten/kg, < 33 mg/kg bei 50 mg Gluten/kg, < 66 mg/kg bei 100 mg Gluten/kg). Übereinstimmend damit zeigte eine Natriumdodecylsulfat-Polyacrylamidgelelektrophorese (SDS-PAGE) der weizenmehlbasierten Proben ebenfalls eine deutliche Abnahme in der Bandenintensität für die Brezenkruste. Insgesamt erzielte die Extraktion mit Cocktail im Vergleich zu UGES- und UPEX-Extraktionen eine etwas bessere Gliadin-Wiederfindung für die Backwaren. Dies ist wahrscheinlich auf die Wirkmechanismen der Desaggregations- und Reduktionsmittel, durch welche sich die Extraktionslösungen unterscheiden, zurückzuführen. Aber auch mit der Cocktail-Extraktionsmethode bleibt die Kompensation von Verarbeitungseffekten wie Backen und alkalischem pH-Wert eine Herausforderung.

Die Arbeit zeigte außerdem auf, dass die Berechnung des Glutengehalts basierend auf dem Gliadingehalt des ELISA unter Verwendung eines Umrechnungsfaktors von 2 generell zu einer richtigen Glutenkennzeichnung (glutenfrei, sehr geringer Glutengehalt) führte, unabhängig vom verwendeten Extraktionslösungsmittel und dem Grad der Verarbeitung.

Verschiedene Extraktionsmethoden, darunter eine modifizierte Osborne-Fraktionierung, in Kombination mit Umkehrphasen (RP)- und Gelpermeations (GP)-Hochleistungsflüssigkeitschromatographie (HPLC) wurden eingesetzt, um die Wiederfindung und Löslichkeitseigenschaften der Weizenproteine in den Proben zu bestimmen. Die Protein-Wiederfindung bezog sich auf den analysierten Proteingehalt im Verhältnis zum Rohproteingehalt. Die Ergebnisse zeigten, dass die Protein-Wiederfindung in den verarbeiteten Proben mit zunehmendem Grad der Lebensmittelverarbeitung abnahm. Beispielsweise betrug die Wiederfindungsrate im Teig 90 %, während sie bei Backwaren ohne Alkalibehandlung zwischen 75 % und 83 % und mit Alkalibehandlung (Brezelkruste) zwischen 58 % und 61 % lag.

Die Löslichkeitseigenschaften der Proteine unter (nicht) reduzierenden Bedingungen wurden analysiert und diese in salzlösliche (Albumine und Globuline, ALGL), alkohollösliche (Gliadine), in SDS- und phosphathaltigen Puffer (SDS-PBS) lösliche (ALGL + Gliadine), und alkoholunlösliche und in SDS-PBS unlösliche Proteine (Glutenine) eingeteilt. Der Gehalt an salzlöslichen, alkohollöslichen und SDS-PBS-löslichen Proteinen nahm in den Backwaren im Vergleich zu Mehl ab, der Gehalt an alkoholunlöslichen und SDS-PBS-unlöslichen Proteinen nahm zu. Während der Teigbereitung erhöhte sich die Extrahierbarkeit von SDS-PBS-löslichen Proteinen, was mit früheren Studien übereinstimmte.

Mit Ausnahme der Brezelkruste, wurde kein eindeutiger Zusammenhang zwischen den Löslichkeitseigenschaften und dem Verarbeitungsgrad der Backwaren beobachtet. Die Alkalibehandlung der Brezenkruste führte zu einer verminderten Löslichkeit von alkohol- und SDS-

PBS-unlöslichen Proteinen. Auch unter Verwendung des starken Reduktionsmittels Dithiothreitol konnte keine vollständige Protein-Wiederfindung erreicht werden. Dies ließ darauf schließen, dass während der Verarbeitung sowohl Disulfidbrückenbindungen als auch andere kovalente Bindungen zwischen den Glutenproteinen gebildet wurden.

Eine Abnahme an freien Thiolgruppen und die geringeren gesamt Cystein und Glutathion-Gehalte bekräftigten die Hypothese der Ausbildung von kovalenten nicht Disulfidbrückenbindungen unter Beteiligung von Thiolgruppen während des Backprozesses.

Durch entsprechende Integration der RP- bzw. GP-HPLC Chromatogramme der durch Osborne-Fraktionierung erhaltenen Gliadin- und Gluteninfraktionen sowie der Gluteninfraktion, die den SDS-PBS unlöslichen Proteinen entsprach, konnten außerdem die verschiedenen Glutenproteintypen quantifiziert werden. So zeigte sich, dass auch die Proteintypzusammensetzung der verschiedenen Fraktionen durch die Verarbeitung verändert wurde. Die alkohollöslichen Proteinfraktionen zeigten einen relativen Anstieg an ω-Gliadinen (ω5, ω1,2) und einen relativen Rückgang an α- und γ-Gliadinen. Die alkoholunlöslichen Fraktionen zeigten einen relativen Rückgang an ωb-Gliadinen und hochmolekularen Gluteninen (high-molecular-weight glutenin subunits, HMW-GS) während der Anteil am niedermolekularen Gluteninen (low-molecular-weight glutenin subunits, LMW-GS) zunahm.

Die SDS-PBS unlöslichen Fraktionen der Backwaren zeigten keine konsistenten Veränderungen der HMW-GS, LMW-GS und alkoholunlöslichen ALGL-Gehalte. Die Veränderungen in der Zusammensetzung der Glutenproteintypen konnten nicht eindeutig mit der verminderten Nachweisbarkeit von Gliadin/Gluten mittels ELISA in Zusammenhang gebracht werden.

Im nächsten Schritt erfolgte eine eingehende Analyse des Proteoms der Osborne-Fraktionen. Es war die erste Studie, welche RP-HPLC, SDS-PAGE und markierungsfreie, explorative (nicht zielgerichtete) Flüssigkeitschromatographie gekoppelt mit Tandem-Massenspektrometrie (LC-MS/MS) einsetzte, um ALGL-, Gliadin- und Gluteninfraktionen von Mehl, Teig und verschiedenen Backwaren umfassend zu profilieren. Für diesen Ansatz mussten die Weizenproteinfraktionen in größeren Mengen isoliert werden. Die Charakterisierung der Fraktionen mittels RP-HPLC entsprach den Ergebnissen der vorherigen Untersuchungen. In Übereinstimmung der mittels HPLC bestimmten Proteinzusammensetzung machte eine SDS-PAGE die Veränderungen durch die Verarbeitung für die einzelnen Fraktionen und Extraktionsrückstände sichtbar. Die SDS-PAGE lieferte weitere Informationen über die Proteinzusammensetzung der ALGL-Fraktionen, die ebenfalls erheblich durch das Backen verändert wurde.

Die Analyse des Proteoms mittels LC-MS/MS führte zur Identifizierung von insgesamt 1488 Peptiden, die 93 Proteingruppen zugeordnet werden konnten. Die ALGL-Fraktionen enthielten 71, die Gliadinfraktionen 27 und die Gluteninfraktionen 47 verschiedene Proteingruppen. Die

Fraktionen enthielten ingesamt 16 gleiche Proteingruppen. Der Großteil der Proteine in den ALGL-Fraktionen waren Stoffwechsel- und Schutzproteine (60 %), während die Gliadin- und Gluteninfraktionen überwiegend aus Speicherproteinen bestanden (≥58 %).

Es wurde festgestellt, dass der übliche Ansatz zur Trennung von Weizenproteinen auf der Grundlage von Löslichkeit und Hydrophobie limitiert ist, was durch die strukturellen Ähnlichkeiten und die polymere Natur bestimmter Glutenproteintypen erklärt werden kann.

Die Beteiligung spezifischer Gliadine und Glutenine an der Glutenpolymerisation und -vernetzung wurde mit Hilfe markierungsfreier Quantifizierung (label-free quantitation, LFQ) untersucht. LFQ ermöglichte die relative Quantifizierung von Proteinen in den Fraktionen der Brotkrume und -kruste im Vergleich zum Mehl. Es wurden signifikante Veränderungen in der relativen Häufigkeit von 82 Proteinen festgestellt, darunter 25 Glutenproteine. Generell zeigten die Veränderungen der relativen Häufigkeit als Reaktion auf die Verarbeitung, dass Glutenine über Disulfidbrückenbindungen und anderen kovalenten Bindungen, und die meisten Gliadine eher über nichtkovalente Bindungen an der Bildung des Glutennetzwerks beteiligt sind.

Die Ergebnisse der HPLC-Analysen brachten die verminderte Nachweisbarkeit von Gliadin/Gluten mittels ELISA mit einer verminderten Extrahierbarkeit von Gluten, insbesondere der Gluteninfraktionen, aus den Backwaren in Verbindung. Die LC-MS/MS-Analysen zeigten das Vorhandensein einer beträchtlichen Menge an Gliadinen in den Gluteninfraktionen, auf die der ELISA empfindlich reagiert.

Die Erkenntnisse dieser Arbeit tragen dazu bei, das Verständnis über den Einfluss der Lebensmittelverarbeitung auf Gluten sowie den Nachweis von Gluten in verarbeiteten Lebensmitteln zu verbessern. Darüber hinaus wurden spezifische Glutenproteine identifiziert, die maßgeblich zur Bildung des Glutennetzwerks und damit zur Qualität des Brotbackens beitragen.