Rye and barley reference materials for the analysis of gluten

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Karlsruhe, den 14.06.2023 Majlinda Xhaferaj

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Abbreviations

Ab	antibody
AL/GL	albumin and globulin
ANOVA	analysis of variance
AOAC	Association of Official Agricultural Chemists
AUC	area under the curve
CAA	2-chloroacetamide
CD	celiac disease
DDA	data-dependent acquisition
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FA	formic acid
GFD	gluten-free diet
GP-HPLC	gel-permeation chromatography
GS	glutenin subunits
GPT	gluten protein types
HCA	hierarchical cluster analysis
HLA	human leukocyte antigen
HMW	high-molecular-weight
iBAQ	intensity based absolute quantification
IRMM	Institute for Reference Materials and Measurements of the European Commission
LC-MS/MS	liquid chromatography–tandem mass spectrometry
LMW	low-molecular-weight
mAb	monoclonal antibody
MOPS	3-(*N*-morpholino)propanesulfonic acid
M_w	relative molecular weight
MMW	medium-molecular-weight

OMA	official method of analysis
PWG	Prolamin Working Group
red.	reduced
RP-HPLC	reversed-phase high-performance liquid chromatography
RM	reference material
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
TFA	trichloroacetic acid
TRIS	tris(hydroxymethyl)aminomethane
TG2	tissue transglutaminase

List of amino acids and their one-letter codes

A	alanine
C	cysteine
D	aspartic acid
E	glutamic acid
F	phenylalanine
G	glycine
H	histidine
I	isoleucine
K	lysine
L	leucine
M	methionine
N	asparagine
P	proline
Q	glutamine
R	arginine
S	serine
T	threonine
V	valine
W	tryptophan
Y	tyrosine

Zusammenfassung

Glutenfreie Lebensmittel unterliegen den Vorschriften der Durchführungsverordnung (EU) Nr. 828/2014. Weitere Standards sind im Codex Alimentarius festgelegt. Lebensmittel dürfen nur als glutenfrei gekennzeichnet werden, wenn der Grenzwert von 20 mg/kg Gluten im Endprodukt nicht überschritten wird. Um die Lebensmittelsicherheit für Zöliakie-Betroffene zu gewährleisten, ist eine genaue Quantifizierung des Glutengehalts in glutenhaltigen und potenziell mit Gluten kontaminierten Lebensmitteln von entscheidender Bedeutung. Von lebensmittelherstellenden Betrieben werden zur Quantifizierung von Gluten hauptsächlich immunologische Methoden wie der R5 ELISA eingesetzt, da diese zertifiziert sind. Es gibt jedoch viele Nachteile der ELISA Methoden aufgrund von Unterschieden in den verwendeten Kalibrierungsstandards und der Spezifität der Antikörper. Die Unterschiede in der Proteinstruktur verschiedener glutenhaltiger Getreidearten (Roggen, Gerste und Weizen) führen zu einer Unter- oder Überschätzung des Glutengehalts in mit Roggen oder Gerste kontaminierten Lebensmitteln. Grund hierfür ist die Nutzung von ausschließlich weizenbasierten Kalibrierstandards. Zudem mangelt es an standardisierten Gluten-Referenzmaterialien und harmonisierten analytischen Methoden in der Glutenanalytik. Darüber hinaus gibt es nur wenige Untersuchungen zu Roggen- und Gerstenproteinen im Allgemeinen sowie zu Gluten-Referenzmaterialien aus Roggen und Gerste.

Das Ziel dieser Studie ist es, repräsentative Referenzmaterialien auf Basis von Roggen und Gerste für die Glutenanalyse zu etablieren. Dafür wurden geeignete Sorten ausgewählt und verschiedene Proteinisolate aus ihren Mehlmischungen hergestellt. Durch die Charakterisierung von 32 verschiedenen Roggen- und 35 verschiedenen Gerstensorten mittels RP-HPLC, GP-HPLC und zwei kommerziell erhältlichen ELISA-Kits (R5 und G12) konnten repräsentative Sorten für die Produktion neuer Gluten-Referenzmaterialien identifiziert werden. In Bezug auf die Proteinverteilung zeigten Roggensorten eine durchschnittliche Zusammensetzung von 40% Albumine und Globuline, 23% γ-75k-Secaline, 17% γ-40k-Secaline, 14% ω-Secaline und 6% HMW-Secaline. Die relative Proteinzusammensetzung der 35 Gerstensorten betrug durchschnittlich 25% Albumine und Globuline, 11% D-Hordeine, 19% C-Hordeine und 45% B/γ-Hordeine. Darüber hinaus wurde festgestellt, dass das verwendete Prolamin/Glutelin-

Verhältnis (1:1), welches üblicherweise zur Berechnung des Glutengehalts verwendet wird, sowohl für Roggen als auch für Gerste ungeeignet ist. Für Roggen wurde ein Verhältnis von 4,4:1 und für Gerste ein Verhältnis von 1,6:1 ermittelt, was einen höheren Anteil der Prolamine im Vergleich zu den Glutelinen aufzeigt. Bei Verwendung beider ELISA-Kits wurde der Glutengehalt für die Mehrheit der Proben überbestimmt. Zudem konnte festgestellt werden, dass bei Roggen- und Gerste im Gegensatz zum Weizen eine klare Unterscheidung zwischen den Osborne-Fraktionen Prolamine und Gluteline nicht eindeutig möglich ist.

Von den 32 Roggen- und 35 Gerstensorten wurden jeweils sieben bzw. acht Sorten mithilfe statistischer Werkzeuge wie der hierarchischen Clusteranalyse für die Produktion der Referenzmaterialien ausgewählt. Die Glutenzusammensetzung der ausgewählten Sorten wurde in zwei verschiedenen Erntejahren verglichen. Zudem wurden vier verschiedene Proteinisolate aus einer Mischung der ausgewählten Sorten hergestellt. Bei den Isolaten handelt es sich um Prolamine, Gluteline, das Gesamtgluten und einem Acetonitril-Wasser extrahierbaren Protein (AWEP). Die Isolate wurden mittels LC-MS/MS charakterisiert und ihre Reaktivität gegenüber dem R5-monoklonalen Antikörper im Sandwich-ELISA-System getestet. Bei den Roggen-Isolaten ergab sich folgende Reaktivitätsreihenfolge gegenüber dem R5-monoklonalen Antikörper: Prolamine $>$ AWEP $>$ Gluten $>$ Gluteline. Bei den Gerste-Isolaten war die Reaktivität gegenüber AWEP und den Prolaminen am höchsten, gefolgt von Gluten und Glutelinen. Die Ergebnisse zeigen, dass die Verwendung des Weizenprolamin Referenzmaterials (PWG-Gliadin) in ELISA-Testsystemen nicht zu einer optimalen Bestimmung des Glutengehalts in Roggen- und Gerstenmehlen führt. Demnach sind Roggen- und Gersten-Referenzmaterialien erforderlich, um die Glutenanalyse in mit Roggen und Gerste kontaminierten Lebensmitteln zu optimieren. Die in dieser Studie hergestellten Isolate stellen eine Möglichkeit für solche Referenzmaterialien dar, um die Quantifizierung des Glutengehalts von mit Gerste und Roggen kontaminierten Lebensmitteln zu verbessern und somit die Lebensmittelsicherheit für Zöliakie-Patienten zu gewährleisten.

Abstract

Gluten-free foods are subject to the Codex Alimentarius as well as the Commission Implementing Regulation (EU) No. 828/2014. It is stated that gluten-free labelled foods must not exceed 20 mg/kg of gluten in the final product. To ensure food safety for celiac disease patients, accurate quantification of gluten in gluten-containing and potentially contaminated foods is crucial. Immunological methods such as the R5 ELISA are the main methods for gluten quantification that are used by food producers, as they are certified. However, there are many disadvantages of the method, due to differences in materials used for calibration and specificity of antibodies. The differences in the protein structure of different grain species (wheat, rye and barley) leads to under- or overestimation of the gluten content of rye- or barley-contaminated foods. The reason for this is the use of wheat-based calibration standards. There is a lack of standardized gluten reference materials and harmonized analytical methods in gluten analysis. Moreover, there is only little research on rye and barley proteins in general but as well as gluten reference materials from rye and barley.

The objective of this study is to establish representative rye- and barley-based reference materials for gluten analysis. For this purpose, suitable cultivars were selected and various protein isolates were produced from their flour mixtures. By characterizing 32 different rye and 35 different barley cultivars using RP-HPLC, GP-HPLC and two commercially available ELISA kits (R5 and G12), we were able to identify representative cultivars for the lab-scale production of new reference materials. In terms of protein distribution, rye cultivars showed an average composition of 40% albumins/globulins, 23% γ-75k-secalins, 17% γ-40k-secalins, 14% ω-secalins and 6% high-molecular-weight-secalins. The relative protein composition of the 35 barley cultivars averaged 25% albumins and globulins, 11% D-hordeins, 19% C-hordeins and 45% B/γ-hordeins. Moreover, we discovered that the commonly used prolamin/glutelin ratio of 1:1 for calculating gluten content was unsuitable for both rye and barley. For rye, a ratio of 4.4:1 was determined, while for barley, a ratio of 1.6:1 was observed, indicating a higher proportion of prolamins compared to glutelins. The gluten content in the majority of samples was overestimated when using both ELISA kits. Additionally, we discovered that separating

rye and barley gluten protein types into prolamins and glutelins using the modified Osborne fractionation was not straightforward. Of the 32 rye and 35 barley varieties, seven and eight varieties suitable for the production of the reference materials were selected using statistical tools such as hierachical cluster analysis, respectively. The gluten composition of the chosen cultivars were compared in two different harvest years. Moreover, four different protein isolates were produced using a mixture of the selected cultivars: prolamin, glutelin, total gluten and an acetonitrile water extractable protein (AWEP). The isolates were characterized using LC-MS/MS and their reactivity towards the R5 monoclonal antibody in the sandwich ELISA system was tested. For rye isolates, the reactivity order towards the R5 monoclonal antibody was as follows: prolamins $>$ AWEP $>$ gluten $>$ glutelins. For barley isolates, the reactivity was highest for AWEP and prolamins, followed by gluten and glutelins. The results show that the use of the wheat-based PWG-gliadin standard in ELISA test systems does not lead to an optimal determination of the gluten content in rye and barley flours. Rye and barley reference materials are necessary to optimize gluten analysis in rye- and barley-contaminated foods. The isolates produced in this study represent one possibility for such reference materials to improve gluten quantification of barley- and rye-contaminated foods and to ensure food safety for celiac disease patients.

1 Introduction

Gluten is a complex protein mixture which occurs in the endosperm of grains such as wheat, rye and barley. The mixture is a combination of the glutelin fraction and the fraction named prolamin (Wieser, 2014). Gluten has received much attention, because of its ability to trigger hypersensitivity reactions including celiac disease (CD), non-celiac gluten sensitivity and wheat allergy in genetically predisposed individuals. For those affected, a strict and lifelong gluten-free diet (GFD) is the only treatment to date that shows an improvement of gastrointestinal symptoms (Jericho and Guandalini, 2018). In addition, the prevalence of CD is rising, leading to an increased demand for safe gluten-free products (Choung et al., 2017). In order to ensure food safety for CD patients, a proper quantification of gluten in gluten-containing and potentially contaminated foods is essential. According to the Codex Alimentarius Commission and the Commission Implementing Regulation (EU) No. 828/2014, gluten-free labelled foods must not exceed 20 mg/kg of gluten in the final product (Codex Alimentarius Commission, 2008; European Commission, 2014). Therefore, an accurate quantification of gluten traces is necessary.

Due to the complex structure and special properties of gluten, the correct quantification in food matrices faces numerous analytical challenges. The most common methods for detecting gluten in foods are immunoassays like enzyme-linked immunosorbent assay (ELISA). However, different ELISA test systems do not always achieve the same result, due to differences in materials used for calibration and specificity of antibody (Ab). Furthermore, the lack of a standardised reference material (RM) and harmonised analytical methods poses difficulties in the comparability of quantitative results. The Prolamin Working Group (PWG) succeeded in producing an RM by extracting gliadins (wheat prolamins) from a selection of the most common wheat cultivars in Europe at the time (Van Eckert et al., 2006). Although it has not been approved by the Institute for Reference Materials and Measurements of the European Commission (IRMM), it is still used for calibration as it is the best characterized RM for gliadin analysis. However, PWG-gliadin does not represent the total gluten content and is based on wheat gluten only. Therefore more appropriate RMs are required to improve method validation and verification of gluten in foods contaminated with gluten from wheat, rye and barley.

1.1 Cereals and cereal proteins

Cereals play a crucial role in human nutrition as they contain essential nutrients such as carbohydrates, proteins, dietary fiber, vitamins and minerals like iron and zinc. They are considered the most important staple foods worldwide, with corn, rice and wheat being the top three cereal crops. Approximately 41% of the grains are used for human consumption, while up to 35% are used for animal feed (Poutanen et al., 2022).

Cereals are monocotyledonous plants and members of the grass family *Poaceae* (Fig. 1.1). Wheat, rye, barley and oats are related members of the subfamily *Pooideae*. More closely related are the members of the tribe *Triticeae*: wheat (*Triticum*), rye (*Secale*) and barley (*Hordeum*) (Bouchenak-Khelladi et al., 2008). Wheat and barley were domesticated in the Middle East in the Fertile Crescent region about 10,000 years ago. The genomes of the *Triticeae* comprise seven sets of chromosomes in diploid and polyploid species. Rye and barley have diploid chromosome sets where a diploid set consists of two copies of chromosomes (2n=2x=14). Barley has the smallest genome (5.1 Gigabase pairs (Gbp)) followed by rye (7.9 Gbp) and wheat (16.9 Gbp) (Bartos et al., 2008). Genome constitutions are designated using capital letters of the Roman alphabet. For instance, rye has an RR genome constitution while barley has an HH genome constitution. Bread wheat (*Triticum aestivum*) possesses a hexaploid chromosome count (2n=6x=42) and is characterized by the chromosome constitution AABBDD. Durum wheat (*Triticum turgidum durum*) consists of an tetraploid chromosome set (AABB) with a chromosome count of 2n=4x=28 (Langridge et al., 2006; Feuillet et al., 2008; Martis et al., 2013). There is a clear homology between the chromosomes of wheat and rye. Rye is the product of hybridization events and split from wheat about 7,000 years ago (Martis et al., 2013). The different evolutionary hybridization, domestication and selection have resulted in modern wheat, barley and rye cultivars with different genotypes and phenotypes.

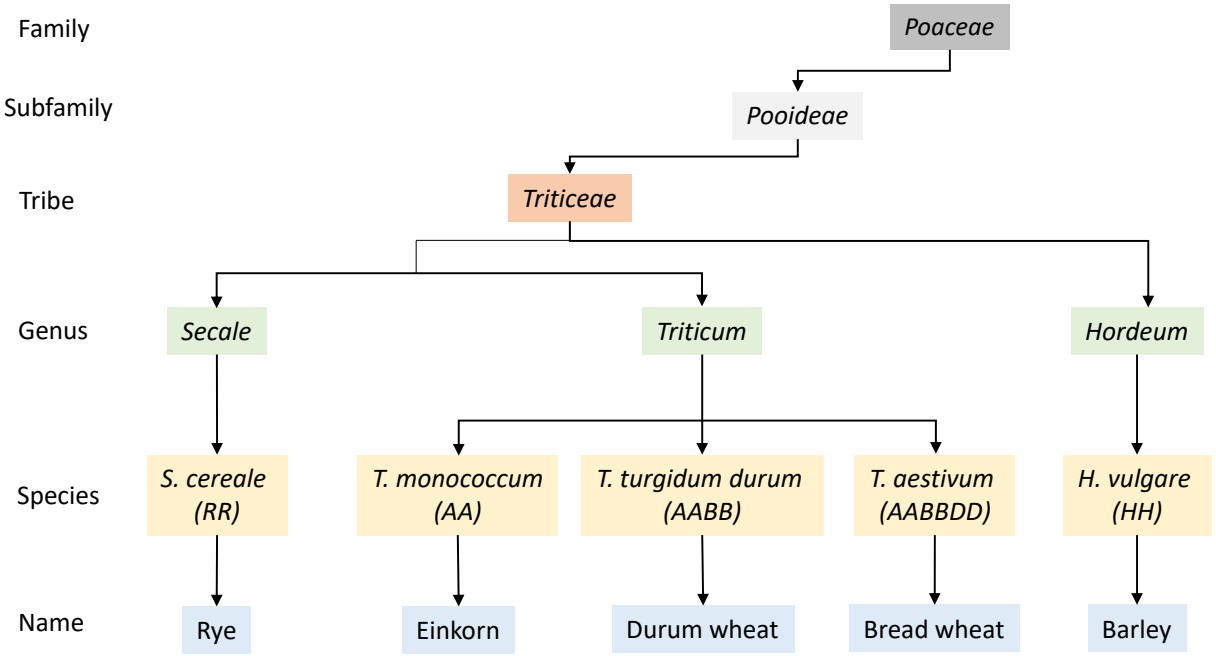

Figure 1.1: Polygeny of cereals. The evolution of rye and barley from a common ancestor; RR: genome constitution of rye; HH: genome constitution of barley, AABB: genome constitution of durum wheat; AABBDD: genome constitution of bread wheat. Adapted from Feuillet et al. (2008).

The grains of cereals are composed of three parts: the bran, the germ and the endosperm. The bran is the outer layer of the grain and rich in fibre, vitamins and minerals. The germ is a component of the grain and is a good source of protein, fats, vitamins and minerals. The endosperm is the largest part of the grain and is mainly composed of carbohydrates, including starch (Poutanen et al., 2022). Cereals comprise 55% to 69% of carbohydrates and are therefore a good source of calories for human and animal nutrition. However, a cereal grain also contains significant amounts of protein with about 8 to 15% which makes it a crucial protein source as well (Rimbach et al., 2015). Grain proteins can be classified in structural proteins, metabolic proteins and storage proteins. Structural proteins are membrane proteins and metabolic proteins are enzymes which are present in the aleurone layer and the embryo of the grains. Some of these enzyme inhibitors are known as amylase trypsin-inhibitors (Geisslitz et al., 2022). The storage proteins can be found in the starchy endosperm of the grains. Their function is to provide nitrogen and amino acids during germination of the grain embryo. About 50% of the total protein content in mature cereal grains are storage proteins (Kim et al., 2004).

The foundation for studies on grain proteins have been laid early in 1731 by J. B. Beccari (Beccari, 1731). In the following, systematic studies on plant proteins have been carried out by T. B. Osborne starting in 1895. He found that proteins from different plant sources

have different chemical properties and can be classified into different groups based on their solubility in various solvents. The foundation for the understanding of the chemistry of plant proteins was then known as the Osborne fractionation (Osborne, 1895). The proteins were classified into four groups: water-soluble albumins, salt-soluble globulins, alcohol-soluble prolamins and alkaline- or acid-soluble glutelins (Osborne, 1909). Due to more sophisticated analysis methods more detailed investigations into grain proteins were possible. The metabolic proteins can be found in the albumin and globulin (AL/GL) fraction. The grain storage proteins comprise prolamins and glutelins. Their name is based on the fact that they are particularly rich in the amino acids proline and glutamine. The combination of both fractions, the prolamins and glutelins, is called gluten. Gluten is known for its unique properties such as the ability to form a viscoelastic dough that can hold gas and produce bread with a good pore structure after baking, when using wheat flour (Wieser, 2007). The reason is the combination of the monomeric prolamins with the polymeric glutelins and their intra- and intermolecular interactions due to disulfide bonds (Belton, 1999).

Many descriptions of gluten from different grain species are presented in the literature. When referring to the cereal species, different names of prolamins and glutelins were attributed such as gliadins and glutenins for wheat. In rye or barley these fractions are called secalins and secalinins or hordeins and hordenins, respectively (Garcia-Calvo et al., 2020). However, for rye and barley, secalins and hordeins have been used more frequently for both fractions. There are similarities in the amino acid sequences when comparing the prolamins of the different species. The reason is the genetic homology because of the common ancestor. Since the protein separation techniques have evolved in the last decades the nomenclature of the different storage proteins has been changed as well. The classification is therefore more specific using the electrophoretic mobility and the differences in molecular weight. The storage proteins of wheat, rye and barley have been investigated extensively by Shewry et al. (1991). They classified the proteins in three main groups: high-molecular-weight (HMW), sulphur-rich and sulfur-poor. These studies have led to the determination of the subfractions of the proteins from wheat, rye and barley. Figure 1.2 illustrates the more usual classification of the proteins according to their molecular weight and their homology in amino acid sequences.

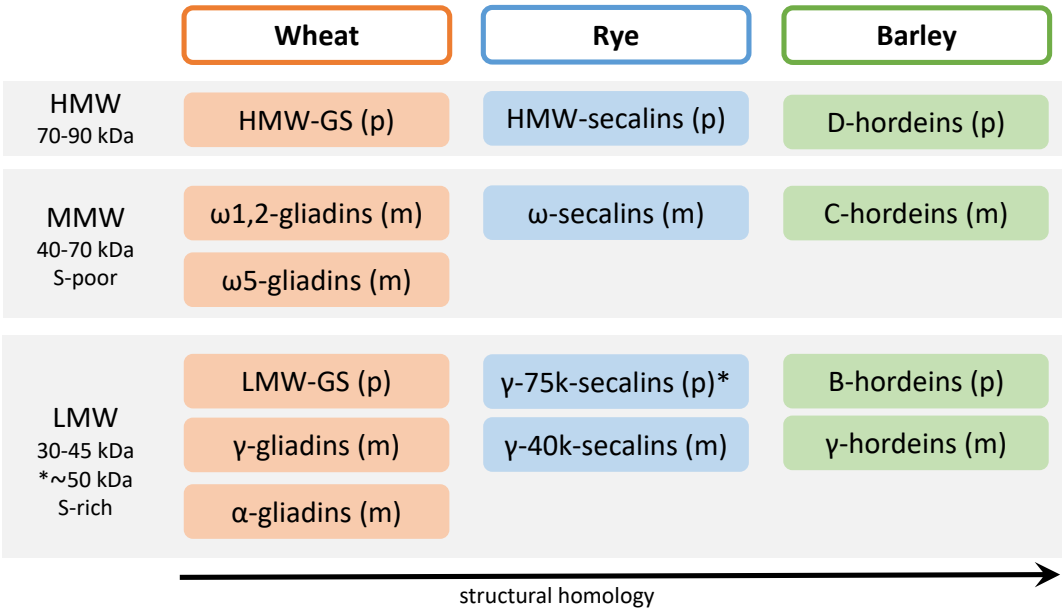

Figure 1.2: Classification of *Triticeae* gluten proteins according to their relative molecular weight and structural homology. HMW: high-molecular-weight; LMW: low-molecular-weight; MMW: medium-molecular-weight; m: monomeric; p: polymeric; GS: glutenin subunits.

1.1.1 High-molecular-weight proteins

The HMW-subunits are typically composed of 600-800 amino acid residues, with a relative molecular weight (M_w) between 70-90 kDa. The amino acid sequences in these proteins exhibit a three-sectional organization with two highly conserved end domains, N-terminal (100 residues) and C-terminal (40 residues) (Shewry and Tatham, 1990). These consist of amino acid residues with charged side chains as well as cysteine that can form interchain linkages via disulfide bonds. The central repetitive region (500-700 residues) consists of the central domain of QQPGQG as a backbone with repeats of tri- and hexapeptides such as YYPTSP, QQP and QPG (Shewry and Halford, 2002; Koehler and Wieser, 2014; Scherf et al., 2016a).

1.1.2 Medium-molecular-weight proteins

The medium-molecular-weight (MMW) proteins are monomeric storage proteins with a M_w of around 40-50 kDa. In wheat there are two types of MMW proteins such as ω1,2-gliadins and ω5-gliadins (Koehler and Wieser, 2014). In rye and barley these homologous protein types are the ω-secalins and C-hordeins, respectively. Similar to the HMW-subunits they are organized in three sections with the N-terminal, the central and the C-terminal domain (Scherf et al., 2016a). Typically, they consist of 300-400 amino acid residues. Glutamine, proline

and phenylalanine are most represented in the central domain with repetitive units such as QPQQPFP (Shewry and Halford, 2002).

1.1.3 Low-molecular-weight proteins

The low-molecular-weight (LMW) protein types are composed of roughly 300 amino acid residues and have a M_w of around 28-35 kDa. Both polymeric and monomeric protein types can be found within the LMW group. They consist of up to five protein domains that differ in their composition and are categorized into N-terminal domains section Ia, Ib and II and C-terminal domain section III-V. Domain Ia is comprised of non-repetitive unique sequences with a length of 32 residues (Scherf et al., 2016a). Domain Ib consists of repetitive units such as QPQPFPPQQPY (found in α-gliadins). Domain II is unique to α-gliadins and B-hordeins. Domain III shows a high degree of homology and length. Domain IV has partly unique and partly homologous sequences, while domain V consists of homologous sequences. The sequence length and composition is unique to each protein type within the LMW-subunits (Koehler and Wieser, 2014).

1.2 Rye and rye secalins

Rye is predominantly cultivated in Europe, Russia and North America. 7.6 million tons were produced by the European Union followed by 2 million tons by Russia in 2022 (Statista, 2023). It is mainly used for food production or animal feed. Rye grains are rich in carbohydrates, proteins, dietary fibre and minerals. They contain the highest amount of dietary fibre and the lowest amount of gluten compared to wheat and barley (Rani et al., 2021). Rye is commonly consumed as bread, breakfast cereals and flakes. The traditional use of rye flour originates in the Nordic and Baltic countries, where the sourdough method is used for bread making. The main ingredients are wholegrain rye flour, water and a starter culture. The starter culture contains a colony of microorganisms including wild yeast and lactobacilli. After mixing, the dough is fermented for 8-18 hours, which results in the formation of flavour compounds like lactic and acetic acid accompanied by the lowering of the pH. This creates the distinctive taste of rye bread, which is intense, sour and slightly bitter (Arora et al., 2021).

The main components of rye fibre are arabinoxylans, fructans, mixed linked β-glucans, cellulose and lignin. The fibre in rye has positive effects on the human health. Due to the increased viscosity and volume of the intestinal content, the cholesterol, glucose and insulin levels can be regulated by the slower absorption of these which prevents short- as well as long-term disease complications. Other important compounds in rye are the alkylresorcinols which are found

to possess anti-oxidant, anti-inflammatory and anti-mutagenic properties (Agil et al., 2016; Kruk et al., 2017; Xie et al., 2020). Although rye has beneficial characteristics, it cannot be used alone to produce bread resulting in the same texture as wheat bread. The proteins in rye lack the ability to form the known gluten network, which is required to create the structure as they do not have high amounts of polymeric proteins (HMW). Moreover, the formation of an arabinoxylan network is believed to hinder the formation of the gluten network by trapping the proteins. As a consequence, the proteins lose their capacity for mutual interaction (Döring et al., 2015). Thus, the structure of rye bread is very different from that of wheat bread.

1.2.1 High-molecular-weight-secalins

Rye is known for its genetic diversity and this is partly due to its tendency to outcross with other rye plants. The HMW-secalins, which are a type of gluten protein found in rye, are encoded by genes located on the long arm of the chromosome 1R at the Glu-R1 locus. This locus contains alleles that encode for two different types of HMW subunits, referred to as x- and y-type subunits (De Bustos and Jouve, 2003). The x- and y-type subunits differ in both their M_w and the number of repeat units present within. The HMW-secalin x-type protein Q94IK6 (UniProt accession) comprises 760 amino acids, with glutamine (33.5 mol%), glycine (20.1 mol%) and proline (14.7 mol%) being the most abundant amino acids. On the other hand, the HMW-secalin y-type protein Q94IL4 (UniProt accession) comprises 716 amino acids. In native state, these proteins occur aggregated and in a polymeric form, being cross-linked by interchain disulfide bonds (Koehler and Wieser, 2014; Scherf et al., 2016a). They are classified into the glutelins and make up to 10% of the rye secalins and can be extracted using reducing agents and high temperatures (60 °C) (Gellrich et al., 2003).

1.2.2 ω-secalins

ω-Secalins make up around 18% within the secalins and can be classified into the prolamins. Other than the HMW-secalins they are encoded on the short arm of the rye chromosome 1R by the Sec-1 locus (Martis et al., 2013). ω-Secalins are homologous with the ω1,2-gliadins of wheat and contain around 330 residues. For instance, the ω-secalin Q04365 (UniProt accession) contains 338 amino acid residues with 39.6 mol% being glutamine, 29.3 mol% proline and 7.4 mol% phenylalanine. These amino acids occur repetitively in the central domain of the ω-secalins such as QPQQPFP. The A domain is the N-terminus which consists of 12 residues followed by the B domain of the repetitive central domain and the C domain with four residues at the C-terminus (Koehler and Wieser, 2014; Scherf et al., 2016a).

1.2.3 γ-secalins

The LMW group of rye proteins consists of the monomeric γ-40k-secalins and the polymeric γ-75k-secalins. The γ-secalins are encoded on the Gli-R2 (Sec2) locus on the short arm of the chromosome 2R (Murray et al., 2001). The monomeric γ-40k-secalins consist of approximately 300 amino acids residues with a molecular weight of around 28-35 kDa. The γ-75k-secalin sequence consists of around 430 residues and therefore has a higher molecular weight of 50 kDa (Koehler and Wieser, 2014; Scherf et al., 2016a). The γ-secalins can be divided into four sections, with the N-terminal section containing high amounts of glutamine, proline and phenylalanine that create repeating units in subsection Ib. Section I of γ-75k-secalins contains 130 residues. Sections III, IV and V have a more balanced composition of amino acids, including charged amino acid residues and cysteines that create intra-chain disulfide bonds in monomeric proteins or both intra-chain and inter-chain disulfide bonds in polymeric proteins (Scherf et al., 2016a). The γ-75k-secalins are present in a major proportion (45-52%) in the secalins followed by the γ-40k-secalins (10-26%) (Gellrich et al., 2003).

1.3 Barley and barley hordeins

Barley, a highly adaptable cereal crop, is ranked fourth among the world's most important cereals. The leading countries (2021) in barley production are Russia (17.9 million tons), Australia (14.6 million tons), France (11.3 million tons), Germany (10.4 million tons) and Ukraine (9.4 million tons) (FAO, 2023). Compared to wheat, barley is more tolerant to unfavourable growing conditions such as drought, cold and poor soil. Barley is mainly used for animal feed and is the most important raw material for the malt and brewing industries (Celus et al., 2006). The proteins in barley have a large influence in the malting and beer brewing process. The consumption of barley is beneficial for general health due to its protein and fibre content, which promotes rapid food passage in the colon and has a positive effect on serum cholesterol and glucose levels. In the process of brewing beer, the β-glucans and arabinoxylans in barley can impact the viscosity of wort and beer filtration rates (Baik and Ullrich, 2008). When barley hordeins are extracted according to the modified Osborne fractionation (with reducing agents), its typical composition is 7-8% D-hordeins, 10-20% C-hordeins, 70-80% B-hordeins and a small amount of γ-hordeins (Shewry et al., 1985; Tatham and Shewry, 1995).

1.3.1 D-hordeins

The D-hordeins are encoded by the Hor3 locus on chromosome 5 (1H) and have similar structures to the HMW-GS of wheat (Pistón et al., 2007). Unlike the HMW-secalins or the

HMW-glutenin subunits they do not have x- and y-type proteins. However, they consist of three regions with the central region having repetitive motifs such as PFQGQQ and PHQGQQ (Qi et al., 2006). The D-hordein Q40054 (UniProt accession) consists of 686 sequences in total with glutamine (25.7 mol%), glycine (15.8 mol%), as well as proline (10.5 mol%) and serine (10.8 mol%) being the most abundant amino acids. D-hordeins can be classified into the glutelins and are minor components of the barley hordeins with around 5% (Shewry et al., 1986).

1.3.2 C-hordeins

The monomeric C-hordeins correspond to the prolamins (ω-secalins and ω-gliadins). The C-hordeins consist of three domains and are defined as sulphur-poor proteins. For example, the C-hordein Q40055 (UniProt accession) consists of 328 residues in total with the most abundant amino acids being glutamine (37.3 mol%), proline (29.1 mol%), as well as leucine and phenylalanine (8.6 mol% and 7.7 mol%) (Lange et al., 2007). In contrast to the other subgroups of hordeins, the C-hordeins are completely free of cysteine (Shewry et al., 1986).

1.3.3 B- and γ-hordeins

The genes responsible for encoding B-hordeins and γ-hordeins are Hor2 and Hor5, respectively. These genes are located on chromosome 5 (1H) in barley. B-hordeins and γ-hordeins are part of the LMW group and are characterized by repetitive octapeptide sequences rich in glutamine, proline, leucine and valine residues. The protein structure in γ-hordeins consists of four domains (I, III, IV and V) in which the domain I is the domain with the highest number of amino acid residues containing the repetitive units. Some γ-hordeins have additional cysteine and can exist in both monomeric and polymeric forms, such as γ1, γ2 and γ3-hordeins (Pistón et al., 2004). The protein structure of B-hordeins is unique because it lacks domain Ia and contains domain II instead, which contains sequences of 30 amino acid residues that are rich in glutamine and leucine. This domain shows a high degree of polymorphism (Shewry and Tatham, 1990). Disulfide-stabilized polymers are likely to be formed between B- and γ-hordeins with D-hordeins, through several cysteine residues (Gu et al., 2003).

1.4 Celiac Disease

CD is an autoimmune disease affecting 1.4% of the population worldwide (Singh et al., 2018). It can be triggered by the digestion of gluten in genetically predisposed individuals possessing the human leukocyte antigen (HLA)-DQ2 or -DQ8 genotype. The presence of the genotype

alone is not sufficient to develop CD and other factors are investigated which may act as possible triggers (Scherf et al., 2016a). Possible risk factors have been hypothesized to influence the risk of developing CD such as viral infections (e.g. rotavirus) or changes in the intestinal microbiota (Scherf et al., 2020). Specific peptide fragments of ingested gluten are recognized by immune cells and initiate the immune response in the enterocytes in the small intestine. CD manifests as chronic inflammation of the small intestine. The consequences include villous atrophy, the loss of the finger-like villi of the intestinal epithelium and a flattening of the mucosa, which prevents normal nutrient absorption (Sollid, 2002; Tye-Din and Anderson, 2008). Gastrointestinal symptoms such as diarrhea or malabsorption, as well as extraintestinal diseases such as dermatitis, ataxia and anemia, may occur (Tye-Din and Anderson, 2008).

1.4.1 Pathophysiology

Proline-rich proteins such as gluten proteins are inadequately hydrolyzed by the digestive enzymes of the upper gastrointestinal tract (e.g. pepsin, trypsin and chymotrypsin). This results in the formation of gluten peptides with a length of 10-50 amino acids. In the small intestine these peptides cause an increase in the permeability of the tight junctions between intestinal epithelial cells in the small intestine, resulting in a cascade of immune reactions from the innate and adaptive immune systems when entering the lamina propia (Fig. 1.3, (1)). The innate immune response involves an increase in intraepithelial lymphocytes triggered by interleukin-15 secretion from macrophages, enterocytes, or dendritic cells in response to gluten peptide stimulation (Fig. 1.3, (2)). The enzyme tissue transglutaminase (TG2) plays a paramount role in the adaptive immune response. TG2 catalyzes cross-linking (transamidation) or deamidation of neutral glutamine residues to produce glutamic acid residues (Fig. 1.3, (3)). The cross-linking leads to the formation of covalent isopeptide complexes between TG2 and gluten peptides (Lexhaller et al., 2019b). These complexes have an increased affinity towards HLA-DQ2/DQ8 heterodimers and immunostimulatory activity such as the activation of the CD4+ T cell adaptive immune response (Fig. 1.3, (4)) (Serena et al., 2020). This leads to the stimulation of two different pathways of the immune response. The release of cytokines and chemokines (interferon-γ, tumor necrosis factor-α, metalloproteinases) which is stimulated by the pro-inflammatory immune response T helper cells 1 leading to damage and inflammation of the small intestinal mucosa (Fig. 1.3, (5)). Similarly, the anti-inflammatory immune response T helper cells 2 is stimulated, leading to the release of antibodies. These are immunoglobulin A and immunoglobulin G directed against gliadin, deamidated gliadin and against TG2 (Fig. 1.3, (6)). Since the latter are directed against TG2 as an endogenous enzyme, they are also referred to as auto-Ab, which highlights the autoimmune nature of CD. The continuous ingestion of gluten leads to chronicity of the immune response (Serena et al., 2020).

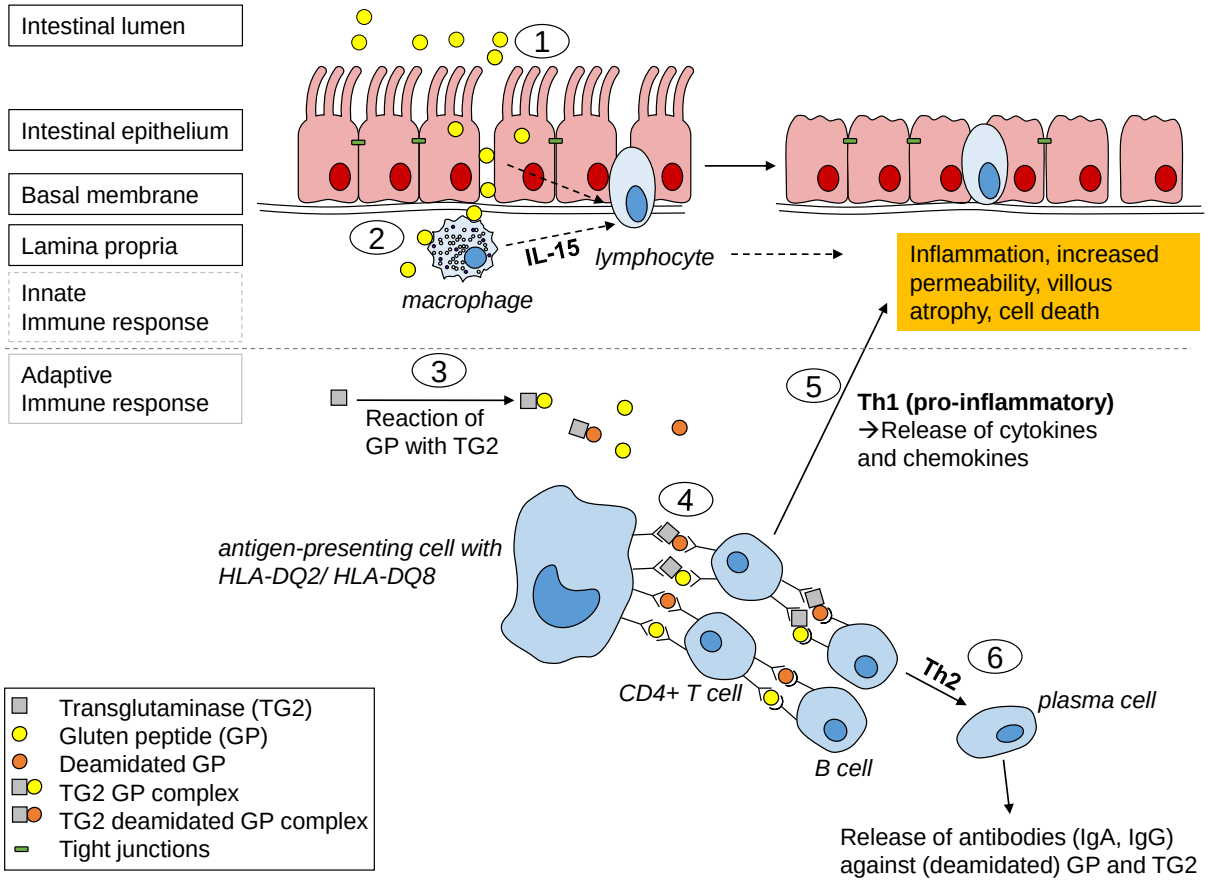

Figure 1.3: Schematic representation of the pathomechanism of celiac disease. IL-15: Interleukine-15, Th1: T helper cells 1, Th2: T helper cells 2, HLA: human leukocyte antigen, IFN-γ: Interferone-γ, TNF-α: Tumor necrosis factor-α, IgA: Immunoglobulin A, IgG: Immunoglobulin G. Adapted from Schuppan and Zimmer (2013), Koehler and Wieser (2014), Marić (2020) and Serena et al. (2020).

1.4.2 Celiac disease-active peptides

The immune reaction described above is triggered by certain immunoactive or toxic peptides that are formed by incomplete proteolytic digestion. These peptides show high proline and glutamine content, length of at least nine amino acids and the affinity towards HLA-DQ2/DQ8 molecules. Sollid et al. (2020) published a list of relevant epitopes (9-mer core region) recognized by CD4+ T cells. Due to the high degree of structural homology (section 1.2) there is a considerable cross-reactivity between gliadins, hordeins and secalins. The major disease-causing prolamins contain repetitive motifs based on PSQQ or SPQQ. In addition, variations with motifs such as PSDQ, PSEQ and PSSQ are also included and other series, such as the octapeptide PQQPFPQQ and the related pentapeptide PQQPY together with the variants QPFPQ, PQQP, or QQQP, appear to be important (Marsh, 1992).

The database of CD-active peptides by the Food Allergy Research and Resource Program (http://www.allergenonline.org) compiled 1,041 naturally occurring, mutated or deamidated peptides from wheat, rye and barley that have been shown to induce CD. The 33-mer peptide (LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF) derived from α2-gliadin has been described as the most immunoactive peptide. It leads to a strong immunoreaction as it contains three T cell epitopes including three overlapping T cell epitopes: PFPQPQLPY (DQ2.5-glia-α1a), PYPQPQLPY (DQ2.5-glia-α1b) and PQPQLPYPQ (DQ2.5-glia-α2). Several key features are shared by immunoactive peptides: motifs rich in proline and glutamine resistant to digestion by proteases, deamidation by TG2, specific peptide conformation binding to major histocompatibility complex class II and multiple HLA-DQ–binding epitopes have a greater T cell stimulatory activity (Kim et al., 2004; Shan et al., 2005). In terms of risk assessment a stepwise approach in evaluating newly expressed proteins/peptides with respect to its potential to cause CD is provided by European Food Safety Authority (EFSA) (Naegeli et al., 2017).

1.4.3 Diagnosis and treatment

The diagnosis of CD consists of the examination of the clinical history (symptomatology), serological testing, testing of small intestinal biopsies and the clinical and serological response to a GFD or gluten challenge after a GFD. The serological testing of CD includes the testing of anti-TG2, -IgA and -IgG as well as anti-endomysial IgA antibodies. Since the serum half-lives are 30-60 days patients should consume gluten-containing food for at least a few days before serological testing (Schuppan and Zimmer, 2013). The diagnosis is confirmed by histologic evaluation according to the Marsh classification, using biopsies of the descending duodenum and the duodenal bulb (Marsh, 1992; Klapp et al., 2013). CD can manifest at any point in a person's life, starting from their early years to old age. There are two primary stages of onset - one after weaning and introduction of gluten in the first two years of life and the other during the second or third decade of life. It can be difficult to diagnose CD because symptoms differ significantly from patient to patient (Alessio, 2003).

The most successful treatment for individuals suffering from CD up until now is a GFD for life. Following this diet allows a clinical and histological recovery in the span of one year and reduces the risk of long-term complications. However, there are several negative aspects of a lifelong GFD for those affected, such as exclusion from social events due to lack of gluten-free alternatives and concerns about food costs, eating outside the home and time limitations in food preparation (Demirkesen and Ozkaya, 2022). The development of additional non-dietary therapies has been a focus in recent years. The use of recombinant glutamine-specific endopeptidases such as Latiglutenase (combination of two peptidases) or prolyl-endopeptidase

derived from *Aspergillus niger* have been studied in clinical trials as oral drug to decrease the amount of immunogenic peptides in the digestive tract by enzymatic digestion (Siegel et al., 2006; Salden et al., 2015). Some of the other complementary therapies are designed to effect the specific steps within the CD pathomechanism such as tight junction modulators, TG2 inhibitors, HLA DQ2/DQ8 blockers and immune therapies targeting the immune response such as IL15 secretion by the enterocytes, just to name a few (Paterson et al., 2007; Kapoerchan et al., 2010; Sulic et al., 2015). The majority of the non-dietary treatments has shown major limitations due to safety issues and low effectiveness. Nevertheless, CD patients are dependent on gluten-free foods to lead a healthy life.

1.5 Legislation and gluten-free products

Gluten-free products are subject to international and national regulations with the aim of protecting consumer health and ensuring fair practices in the food trade. In the international food standard (Codex Standard 118–1979) the term gluten-free foods is specified as dietary foods "consisting of or made only from one or more ingredients which do not contain wheat (i.e. all *Triticum* species, such as durum wheat, spelt and khorasan wheat, which is also marketed under different trademarks such as KAMUT), rye, barley, oats or their crossbred varieties and the gluten level does not exceed 20 mg/kg in total, based on the food as sold or distributed to the consumer" (Codex Alimentarius Commission, 2008). In addition, the presence of gluten-containing grains, i.e., wheat, rye, barley, oats, spelt or their hybrids and foods made from them must be indicated on the packaging of pre-packaged foods. This requirement is consistent with the guidance provided in Codex Standard 1-1985 of 2018 and Codex Standard 146-1985 of 2009. The pre-packaged gluten-free products are usually labelled with certified logos by national celiac associations, organizations or societies (Xhaferaj et al., 2020). The regulations outlining the labelling requirements for gluten-free foods in the European Union, including both prepacked and non-prepacked products, can be found in the Regulation (EU) No. 1169/2011 and in Regulation (EU) No. 828/2014 (European Commission, 2014, 2011). One of the known symbols is the crossed grain symbol for gluten-free products certified within the framework of the European Licensing System. The threshold for gluten-free claims differs depending on the countries and regions due to different national regulations. However, most countries use the threshold stated in the Codex and the Regulation (EU) No. 828/2014 (20 mg/kg product). Some exceptions are for example, Chile with 5 mg/kg, Japan, Argentina and El Salvador with 10 mg/kg and New Zealand/Australia with the claim "not detectable" according to state-of-the-art techniques, i.e., below 3 mg/kg of gluten (Xhaferaj et al., 2020).

Since the prevalence and diagnosis of CD (1%) and related diseases such as non-celiac wheat sensitivity (6%) is increasing, the demand for safe gluten-free products is rising (Serena et al., 2020). It has been shown that the nutritional value of gluten-free products is not balanced, because they usually contain more fat, sugar and salt compared to their gluten-containing counterparts. This can lead to further health problems such as fibre, vitamin and mineral deficiencies (Martin et al., 2013; Vici et al., 2016). However, manufacturers try to balance the missing nutrients with the addition of flours containing higher fibre such as pseudocereals (buckwheat, quinoa and amaranth) (Demirkesen and Ozkaya, 2022). Certain gluten-free products may surpass the safe limit of 20 mg/kg due to possible cross-contamination with gluten. This cross-contamination poses a health risk not only to individuals with CD but also individuals with related disorders. Cross-contamination is one of the most difficult challenges faced in the production of gluten-free products since it can occur at any stage in the process, from field to milling, manufacturing, shared production areas, unsatisfactory sanitation, inappropriate practices by industry/restaurant staff and improper storage conditions (Koerner et al., 2011; Huang et al., 2022). For this reason, a reliable analysis of gluten in possibly contaminated gluten-free food is essential.

The method and the requirements for the quantification of gluten in foods are stated in the Codex Standard 118–1979. The method should be an immunologic method specifically detecting the immunogenic protein fractions and it should be validated and calibrated against a certified RM. Moreover, it should have a detection limit of 10 mg/kg of gluten or less. The ELISA R5 Mendez method is classified as a type I method for gluten determination, indicating that it is the only approved method for determining the accepted value (Codex Standard 234–1999, 2019).

1.6 Gluten detection in foods

Detecting gluten in foods is a complex task fraught with several challenges. The protein structure of gluten is complex and comprises multiple subunits with varying physical and chemical properties that make extraction difficult. Furthermore, gluten undergoes structural modifications during food processing, making analysis even more challenging (Liao et al., 2017). Additionally, the composition of gluten is highly influenced by genetic and environmental factors, rendering it a formidable obstacle for food analysis. Different extraction protocols can yield varying gluten compositions and, consequently, divergent results (Socha et al., 2016; Amnuaycheewa et al., 2022). Moreover, trace analysis of gluten relies on the use of differ-

ent antibodies, which react to different epitopes in gluten, thus generating diverse outcomes. Additionally, the lack of standardized RMs for identifying different types of gluten is a critical issue (Diaz-Amigo and Popping, 2012; Xhaferaj et al., 2023a). The following sections describe the most commonly used techniques for measuring and characterizing gluten from foods.

1.6.1 Protein extraction

One of the most critical parts in gluten analysis is the proper protein extraction from food matrices. The extraction protocols are usually designed on the basis of Osborne's principle (Osborne, 1895). Gluten extraction out of unprocessed food such as flours is usually performed using aqueous alcohols such as ethanol or propanol. However, the use of alcohol solutions mainly allows the extraction of the prolamin fraction, which is assumed to be 50% of the total gluten content (Codex Standard 118-1979, 2015). To extract the total gluten (prolamins and glutelins) in either processed or unprocessed foods, the addition of reducing and/or disaggregating agents such as 2-mercaptoethanol and sodium dodecyl sulfate (SDS), respectively, is required (García et al., 2005; Wagner et al., 2011; Fallahbaghery et al., 2017). However, different analytical methods use different extraction protocols which will be covered in the following sections.

1.6.2 Gluten reference materials

The use of RMs is crucial for validating analytical techniques, calibrating instruments, testing laboratory accuracy, estimating uncertainty and ensuring quality control. To be suitable as calibration standard, RMs should possess certain characteristics, including purity, solubility and stability. In gluten quantification, various types of RMs have been used in different analytical methods, such as flours, isolated gluten protein types (GPT) and recombinant proteins.

The best characterized gluten protein RM is the PWG-gliadin. Van Eckert et al. (2006) extracted and isolated the gliadin fraction from a selection of the most common European wheat cultivars. PWG-gliadin consists of the alcoholic extract (prolamins) of wheat flour and therefore does not correspond to the total gluten content. The typical assumption that prolamin makes up 50% of the gluten content is not the case for all cereals, as the distribution of prolamins and glutelins varies depending on the cereal species, cultivar and environmental factors (Hajas et al., 2018). PWG-gliadin is mainly employed in sandwich ELISA (Codex Type I method) assays to quantify intact gluten proteins or as a calibration standard in RP-HPLC methods to measure GPTs in wheat proteins (Méndez et al., 2005; Codex Alimentarius Commission, 2008). However, there are several disadvantages associated

with using PWG-gliadin in such assays. Since wheat, rye and barley proteins differ in structure, the Abs used in ELISA react differently to these proteins (Lexhaller et al., 2016). Calibration using only wheat-based RMs such as PWG-gliadin can lead to over- or underestimation of gluten levels in rye- or barley-contaminated samples (Rzychon et al., 2017). Moreover, the measurement is considered unreliable as the contamination source is unknown. When the contamination source is identified, using a more suitable RM can prevent the over- or underestimation of gluten levels (Huang et al., 2016). Hence, more appropriate RMs should be utilized.

Flours are suitable RM because they are easily accessible, derived from the natural sources and are therefore as similar as possible to the food samples to be analyzed. Nonetheless, the protein composition and quantity are greatly affected, by both genetic and environmental factors. This must be considered when preparing RMs from natural sources. Studies have been conducted to explore the impact of the RM type (flour or isolate) and the degree of genetic and environmental variability. Wheat flours and GPTs have been characterized, based on either single cultivars or blends of several cultivars (Schall et al., 2020). The influence of the environment and genetic effects on flours as well as flour mixtures have been investigated on wheat gluten. A set of five common wheat cultivars were found to be suitable for the production of a RM (Hajas et al., 2018). Moreover, flour mixtures seemed to be the preferable choice to reduce the genetic and environmental effects in comparison to flours of single cultivars (Schall et al., 2020).

Species-specific GPTs isolated from the flours have been proposed as well-defined RMs. Barley prolamins have been proven to be more suitable for R5 ELISA quantification of barley-based or -contaminated foods, compared to wheat-based RMs, due to the differences in protein composition and Ab specificity to different grain species (Huang et al., 2017). Several fully characterized GPTs with high purity derived from rye, barley and wheat flour mixtures were used for calibration of several quantitative methods, including targeted liquid chromatography–tandem mass spectrometry (LC-MS/MS) and ELISA (Schalk et al., 2017b; Lexhaller et al., 2019a).

Finding an appropriate RM for gluten analysis is a challenging task, due to the complexity and heterogeneity of gluten. Several factors must be taken into account to establish an appropriate RM for gluten analysis, such as genetic and environmental factors and the various forms of RMs such as flours and GPTs, either from single cultivars or a combination of several cultivars. Additionally, the solubility and stability of the RM are crucial in the production process and determining its suitability as an RM.

1.6.3 Enzyme-linked immunosorbent assay

The ELISA is an important immunochemical method for gluten detection in food. This method involves the interaction between a polyclonal or monoclonal antibody (mAb) and the corresponding antigen. Due to the high specificity of the mAb to the corresponding antigens, very low concentrated molecules like peptides and proteins can be analyzed. In general, two types of ELISA procedures are used for gluten quantification, competitive and sandwich ELISA (Fig. 1.4).

In sandwich ELISAs (Fig. 1.4, A) gluten-specific mAbs are captured on the plate surface. When the tested sample is applied, the corresponding antigen (gluten) binds to the mAb (Fig. 1.4, step 1). For the detection of the antigens, enzyme-conjugated mAbs (e.g. with peroxidase) are added which bind to a second site of the antigen (Fig. 1.4, step 2). The addition of the substrate activates the enzymes of the detection mAb, converting the substrate and producing a colour change that can be analyzed (Fig. 1.4, step 3). Between each step the plate is washed several times to ensure that there are no residues. The reaction is monitored by measuring the absorbance. In this case, the amount of antigen is correlated to the concentration of the antigen in the sample extract. The antigen amount is determined by using a gluten RM (e.g. PWG-gliadin) and the calibration curve (Scherf and Poms, 2016).

The R5 sandwich ELISA has been endorsed by the Association of Official Agricultural Chemists (AOAC) as the official method of analysis (OMA) of gluten in foods containing wheat, barley and rye (AOAC OMA 2012.01) (Lacorn et al., 2022). The R5 mAb is raised against ω-secalins and primarily recognizes the epitope QQPFP and the related sequences QQQFP, LQPFP and QLPFP that are present in many CD-toxic or -immunogenic peptides (Méndez et al., 2005; Kahlenberg et al., 2006; Koehler et al., 2013a). For the determination of gluten in rice flours and rice-based products the mAb G12 sandwich ELISA is suitable (AOAC OMA 2014.03) by detecting the 33mer-peptide (Halbmayr-Jech et al., 2012; Huang et al., 2022). However, the sandwich ELISA technique is only appropriate for larger antigens, such as intact gluten proteins, that have two separated binding sites for both the capture and detection mAbs.

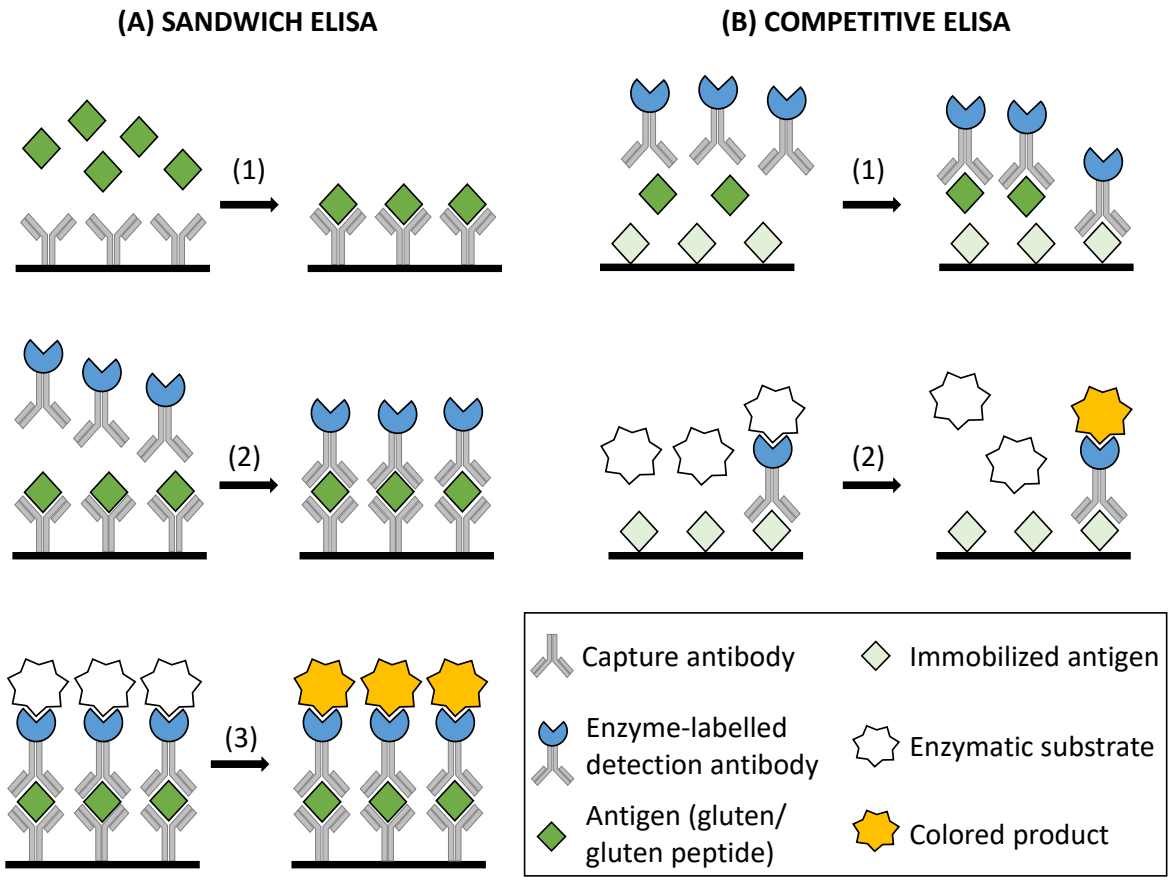

Figure 1.4: Schematic overview of the sandwich and competitive enzyme-linked immunosorbent assay (ELISA). Adapted from Scherf and Poms (2016) and Marić (2020).

The gluten contents of samples containing partially hydrolyzed gluten, such as beer, sourdough products, or malt extracts is measured with the competitive ELISA (Fig. 1.4, B), because it requires only one binding site for detection (Koehler et al., 2013b). A known quantity of antigen is immobilized on the surface. The sample containing the antigen and a fixed, limited amount of enzyme-labelled mAb are both introduced at the same time. During incubation, both the immobilized and free antigens compete for the mAb binding sites, resulting in fewer bound mAb, if more antigens are present in the sample (Fig. 1.4, step 1). Following washing to remove any unbound mAb, antigens and antigen-antibody complexes, a coloured product is generated by adding the enzymatic substrate (Fig. 1.4, step 2). In this case, the absorbance measured is inversely proportional to the antigen concentration in the sample extract. A calibration curve using a gluten peptide or mixture of peptides is used to determine the antigen concentration in the sample extract (Scherf and Poms, 2016).

1.6.4 Sodium dodecyl sulfate – polyacrylamide gel electrophoresis

The sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) technique is commonly used to separate various types of cereal proteins and is commonly employed in gluten characterization studies. This technique is particularly useful in determining protein molecular weights and differentiating between cultivars based on polymorphisms (Echart-Almeida and Cavalli-Molina, 2000). For instance the x- and y-type HMW-secalin subunits can be detected using SDS-PAGE. Due to the genetic variation, different banding patterns of HMW-secalin subunits can be detected using SDS-PAGE, showing distinct molecular weights (Salmanowicz et al., 2014). To prepare the gluten proteins for analysis, they are first extracted using an SDS buffer and then reduced with reducing agents like β-mercaptoethanol or dithiothreitol (DTT). SDS is an anionic surfactant that binds to the proteins and covers their intrinsic charges, resulting in a constant negative charge distribution among the proteins. During electrophoresis, the charged protein molecules are subjected to an electric field and move according to their electrophoretic mobility within a polyacrylamide gel. Larger proteins have reduced mobility due to their size and move more slowly within the gel matrix. In contrast, proteins with lower molecular weights move faster within the gel. Overall, SDS-PAGE is a highly useful tool for gluten characterization due to its ability to separate and identify proteins based on their molecular weight and charge distribution (Kurreck et al., 2022).

1.6.5 Chromatography

Chromatography is a technique used to separate and identify individual components in a mixture. It involves the separation of a sample mixture into its individual components based on their physical and chemical properties, such as size, charge and polarity, using a stationary phase (column) and a mobile phase. The chromatographic separation of gluten proteins has been used for the characterization and quantification of the proteins. The detection is mainly carried out by measuring UV absorbance in the range of 200–220 nm (Anthis and Clore, 2013).

1.6.5.1 Gel permeation chromatography

The differentiation of proteins based on their hydrodynamic volume, (which is proportional to the molecular weight) is achieved using gel-permeation chromatography (GP-HPLC). It is also known as size exclusion chromatography. The separation occurs using porous beads packed in a column (e.g. silica-based). Molecules with small hydrodynamic volumes enter the pores more easily and remain there for a longer time, which leads to an increase in their retention time. On the other hand, larger molecules do not spend much time in the pores and are eluted more quickly (Kurreck et al., 2022). In gluten analysis, the GP-HPLC

method is used for the characterization and investigation of protein M_w distributions. Some applications include the comparison of the molecular weights of different hydrolyzed wheat proteins, the investigation of gluten protein types from rye, or the general quantification and characterization of gluten proteins (Gellrich et al., 2003; Scherf et al., 2016b; Gabler and Scherf, 2020). The comparison of different extraction procedures and the changes in size distribution were investigated with GP-HPLC as well (Batey et al., 1991). For the investigation of differences in the M_w distribution, marker proteins with known molecular weights have been used for a more detailed investigation of the molecular weight ranges, such as bovine serum albumin (66 kDa), carbonic anhydrase from bovine erythrocytes (29 kDa) and cytochrome c from horse heart (12.4 kDa) (Gabler and Scherf, 2020).

1.6.5.2 Reversed-phase high-performance liquid chromatography

For the separation of the proteins according to different surface hydrophobicity, the reversed-phase high-performance liquid chromatography (RP-HPLC) is mainly used. It utilizes a hydrophobic stationary phase to separate analytes based on their hydrophobic interactions with the stationary phase. The elution is typically achieved using a gradient with increasing amounts of organic solvents. In gluten analysis, RP-HPLC is commonly used to characterize and quantify gluten or GPT in protein extracts of flours or RMs (Huang et al., 2017; Schalk et al., 2017a; Xhaferaj et al., 2023a). It plays a crucial role in gluten analysis especially for the characterization of the GPT of either the prolamin or glutelin extract of the Osborne fractions. The hydrophobicity series of wheat can be elucidated with RP-HPLC such as ω5-, ω1,2-, α- and γ-gliadins (gliadin extract) and glutenin ω-gliadins, HMW and LMW subunits.

One RP-HPLC method has been optimized to the extent that it can be used in conjunction with SDS-PAGE to confirm the composition of HMW-GS, specifically the y- and x-subunits, which reveals important information about the baking quality properties (Jang et al., 2017). To predict bread baking properties and quality of wheat flour the prolamin/glutelin ratio is important, which can only be determined with RP-HPLC (Thanhaeuser et al., 2014). In addition, the prolamin/glutelin ratio is a main factor for the calculation of gluten with the ELISA test kits. It is commonly assumed that the ratio is equal to 1, indicating an equal distribution of prolamins and glutelins. According to the Codex the gluten content is calculated by the duplication of the prolamin content. However, this hypothesis has been called into question by RP-HPLC analysis in combination with Osborne fractionation. The reason is, that the distribution of prolamins and glutelins is not uniform across different wheat cultivars, as well as in rye and barley (Wieser and Koehler, 2009; Xhaferaj et al., 2023a).

1.6.6 Proteomics-based methods

Proteomics-based approaches are used for the detection of gluten peptides or the identification of unique gluten peptides in foods. This involves utilizing a tandem mass analyzer such as a hybrid quadrupole-Orbitrap combined with the previous separation using HPLC (LC-MS/MS). The LC-MS/MS method is based on the measurement of the mass-to-charge ratio of the ionized analytes. To measure peptides, gluten proteins need to undergo reduction, alkylation and enzymatic digestion (trypsin, thermolysin, or chymotrypsin) (Alves et al., 2019). Protein digestion is crucial because gluten proteins have a large molecular weight and the focus is on measuring peptides rather than proteins. This approach is known as the bottom-up technique. Additionally, peptides exhibit predictable fragmentation patterns, enabling the determination of their primary amino acid sequence using protein databases.

In order to identify and characterize gluten proteins two proteomics approaches can be used: the targeted and the untargeted workflow. The targeted proteomics workflow can be used to identify and quantify specific peptides such as the immunodominant 33-mer (Schalk et al., 2017a). The untargeted workflow can be used for the identification of suitable and specific gluten marker peptides to generate and characterize a proteomic profile of the digested samples (Colgrave et al., 2017b). Databases and bioinformatics tools are used to identify unique gluten marker peptides, which include both sequences that are known to be immunogenic and sequences that are specific to the grain species (Colgrave et al., 2016; Martínez-Esteso et al., 2016).

There are two main untargeted approaches such as the data-dependent acquisition (DDA) and the data-independent acquisition. A schematic LC-MS/MS DDA workflow using an hybrid quadrupole-Orbitrap mass analyzer is shown in Figure 1.5. First the peptides are separated using HPLC (Fig. 1.5, I). Before mass analysis the ionization of the peptides is required. The electrospray ionisation technique is used for the initial ionization of liquid analytes, resulting in the formation of positively charged gaseous peptide ions (Fig. 1.5, II). The generated ions are directed into the first quadrupole mass filter (Q1). In Q1 the most abundant precursor ions are filtered and subsequently fragmented in Q2 using collision gas such as nitrogen (Fig. 1.5, III and IV). All the product ions are monitored in the Orbitrap for peptide identification (Fig. 1.5, VI). With the help of databases and software the mass spectra generated can be used to identify unique peptides and proteins (Li et al., 2021).

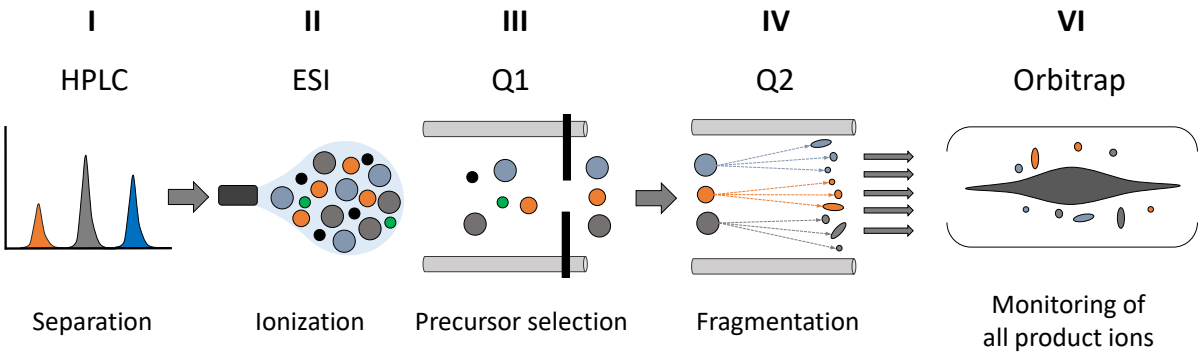

Figure 1.5: Schematic overview of the hybrid quadrupole-Orbitrap LC-MS/MS data dependent acquisition approach. The peptides separated by HPLC are ionized by electrospray ionisation (ESI) and directed to the first quadrupole (Q1) for precursor selection. The selected precursors are fragmented in (Q2) and all fragments are monitored in the Orbitrap mass analyzer. Adapted from Li et al. (2021).

To ensure food safety, accurate determination of the gluten content is necessary. While labelled internal peptide standards have been used in some studies, quantification of peptides alone is not sufficient to meet legal requirements of declaring gluten content in mg/kg. Due to lack of standardized RMs and the complexity of gluten, conversion of peptide content to total gluten content is challenging. External calibration procedures using spiked peptides or peptide mixtures into gluten-free or gluten-containing matrices have been proposed for quantification purposes (Manfredi et al., 2015; Van den Broeck et al., 2015).

Nonetheless, the ability of LC-MS/MS to detect grain-specific gluten marker peptides in either processed or non-processed foods has been demonstrated in several reports, making it a complementary method to ELISA. However, standardization of RMs and workflows is required for successful and responsible comparison of results. In addition this method requires significant effort in terms of instrumentation, time and expertise (Alves et al., 2019).

2 Aim of the study

The aim of this thesis was to provide rye- and barley-based RMs for the analysis of gluten from rye- and barley-contaminated foods. The focus was on the in-depth analytical characterization and investigation of gluten in different cultivars of rye and barley. The results were used to select suitable cultivars for the production of rye- and barley-specific isolates to optimize method validation and verification of gluten analysis.

The initial part of this work involved the analytical characterization and investigation of various commercially available wheat protein RMs from different production batches using different analytical methods including GP-HPLC, RP-HPLC and SDS-PAGE. The investigation intended to show a comparison of the best-characterized RM, PWG-gliadin, with different commercially available RMs. Moreover, it highlights the lack of high quality RMs available for the research on gluten.

The influence of different gluten compositions of various cultivars of common rye and barley species was investigated in the second part of this project. In cooperation with research groups, institutes and the industry, rye and barley samples of different species, varieties and harvest years were collected. The analytical characterization was carried out by combining Osborne/RP-HPLC, GP-HPLC, ELISA (R5 and G12) and SDS-PAGE with simultaneous data analysis to assess variability. The findings gave an understanding of how different species and cultivars influence the gluten composition of rye and barley. With the help of statistical tools such as hierarchical cluster analysis (HCA) and analysis of variance (ANOVA), a certain number of cultivars was selected showing the highest variability, which was used as a basis for RM production.

The last part of this work aimed to optimize the isolation of gluten from rye and barley flours for isolate production. Therefore, different extraction methods were investigated and compared using one flour each. The extraction resulted in the production of four different isolates each consisting of the prolamin, glutelin, gluten and an acetonitrile water extractable protein isolate.

After determination of the protein content, the protein isolates were characterized using RP-HPLC, GP-HPLC, SDS-PAGE and LC-MS/MS. Moreover, the produced isolates were tested on the R5 ELISA assay to investigate their reactivity towards the R5 monoclonal antibody.

3 Results and discussion

This chapter is a comprehensive evaluation of the characterization and production of gluten RMs, focusing on rye and barley isolates. Divided into three sections, this chapter provides a thorough examination of the entire process, from variety selection to characterization and production of the gluten isolates from rye and barley. The first section 3.1 lays the foundation for the importance of well-characterized isolated gluten RMs. The composition of existing wheat gluten RMs is highlighted and the need for well-characterized and more consistent RMs to improve gluten analysis is demonstrated. In sections 3.2 and 3.3, the characterization and preparation of rye and barley flours and gluten isolates are discussed in depth. Both chapters are similar in structure, as the characterization and production have been carried out in the same manner. A number of methods, including RP-HPLC, SDS-PAGE, LC-MS/MS and GP-HPLC, are used to characterize and evaluate the flours and isolates prepared. The suitability of the newly prepared rye and barley RMs for the R5 ELISA kit will also be demonstrated. Each chapter concludes with a comprehensive discussion of the results, summarizing the most important findings.

3.1 Wheat gluten reference materials

The gluten RM PWG-gliadin is used to estimate the gluten content in foods with ELISA kits and for the characterization and quantification of gluten or GPT using RP-HPLC. Due to its well-defined properties, the RP-HPLC elution profile can be used to identify and differentiate the protein types of wheat prolamins. Additionally, some wheat gluten and gliadin standards are available from different manufacturers. This section presents the findings of the investigation of two batches of commercially obtained RMs, which were compared to PWG-gliadin. Two samples each of "Gluten from wheat" ($Gluten_{sigma}$) and "Gliadin from wheat" ($Gliadin_{sigma}$) were acquired from the manufacturer Sigma-Aldrich and two samples of "Gluten, from wheat" ($Gluten_{abcr}$) were acquired from abcr GmbH. The investigation focused on the protein content and protein distribution measured by RP-HPLC, GP-HPLC and SDS-PAGE.

3.1.1 Characterization by RP-HPLC

The typical PWG-gliadin elution profile using RP-HPLC was compared to the prolamin elution profiles of the three commercially available wheat RMs. The wheat prolamin types were assigned according to the literature and marked as dotted lines in Figure 3.1 (Schalk et al., 2017b). Both the $Gluten_{sigma}$ and $Gluten_{abcr}$ had a comparable elution profile to PWG-gliadin. $Gliadin_{sigma}$, however, did not show a typical prolamin elution chromatogram. For example, the area assigned to the ω5-gliadins showed differences in the height of the peaks in the chromatograms. For PWG-gliadin and both gluten RMs ($Gluten_{sigma}$ and $Gluten_{abcr}$), two distinct peaks were visible, while for $Gliadin_{sigma}$ only small undefinable peaks were identified in the ω5-gliadin range. The ω1,2-gliadin fractions eluted within 10.0 to 11.0 min showed one higher peak in the PWG-gliadin chromatogram. In this retention time range small peaks were visible for $Gluten_{sigma}$ and $Gluten_{abcr}$ and no distinct peak was visible in the $Gliadin_{sigma}$ chromatogram. The retention time range starting from 11.0 to 13.8 min was assigned to α-gliadins and the part from 13.8 to 24.0 min to γ-gliadins. Again, here the sample with the most differences in the elution profile compared to the PWG-gliadin was $Gliadin_{sigma}$. The elution profiles of the extracted protein fractions such as the AL/GL as well as the glutelins were compared to wheat flour chromatograms (not shown). The chromatograms of the $Gluten_{sigma}$ and $Gluten_{abcr}$ showed similar elution profiles in the AL/GL as well as in the glutelin fraction compared to fractions from wheat flour. $Gliadin_{sigma}$ did not show any specific and comparable elution profiles in both the AL/GL and glutelin fractions.

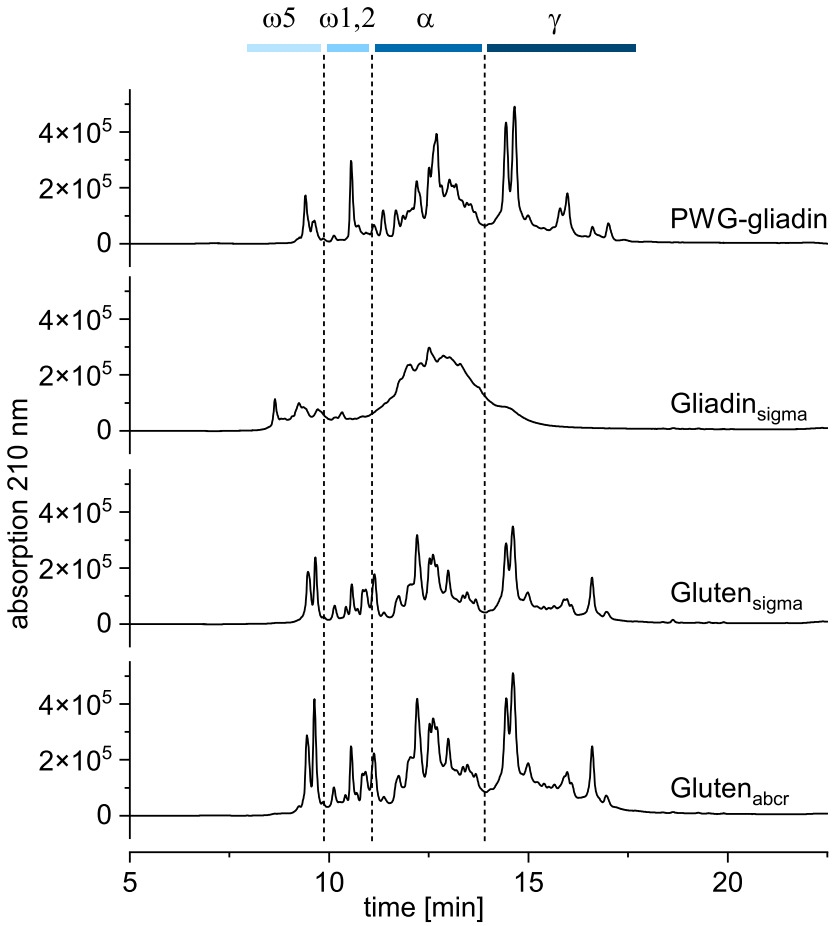

Figure 3.1: RP-HPLC profiles of PWG-gliadin and the prolamin fraction of three commercially available gluten and gliadin reference materials obtained by the manufacturers Sigma Aldrich (Sigma) and abcr GmbH (abcr). The wheat gliadin integration sections are marked and shown as dotted lines separating the wheat prolamin fractions ω5-gliadins (ω5), ω1,2-gliadins (ω1,2), α-gliadins (α) and γ-gliadins (γ).

The protein fractions (AL/GL, prolamins and glutelins) obtained after modified Osborne fractionation (section 5.3.5.1) were characterized quantitatively using RP-HPLC. Two batches were compared in their relative protein composition as well as the protein content considering the AL/GL as well as the prolamins and glutelins (Fig. 3.2). The relative protein composition of both $\text{Gluten}_{\text{sigma}}$ batches showed significantly different AL/GL percentages of 8.1% and 4.9% (T-test, $p < 0.05$, Tab. 5.12). The distribution of the glutelins and prolamins was similar when comparing both batches of $\text{Gluten}_{\text{sigma}}$, showing 34.2% and 35.9% prolamins and 57.7% and 59.3% glutelins. $\text{Gluten}_{\text{sigma}}$ showed a prolamin/glutelin ratio of 0.6 in both batches, whereas $\text{Gluten}_{\text{abcr}}$ showed a prolamin/glutelin ratio of 0.9 and 1.0. The protein distribution of $\text{Gluten}_{\text{abcr}}$ in both batches did not differ significantly and showed a higher prolamin percentage compared to $\text{Gluten}_{\text{sigma}}$ with 46.0% and 41.0% and a glutelin percentage of 42.3% and 43.7%. The AL/GL percentages with 11.7% and 15.7% differed

significantly and were higher compared to $Gluten_{sigma}$ (T-test, $p < 0.05$, Tab. 5.11). The same was observed for $Gliadin_{sigma}$, showing higher AL/GL percentages of 10.8% and 9.8% compared to $Gluten_{sigma}$. There was no significant difference between the AL/GL, prolamin and glutelin distribution within the two batches of $Gliadin_{sigma}$ (T-test, $p < 0.05$, Tab. 5.11). The prolamin percentages in both batches were the highest in $Gliadin_{sigma}$ with 68.3% and 71.8%.

The relative distribution of the gliadin types (wheat prolamins) was investigated and compared using one-way ANOVA (Tab. 5.10). The commercially available wheat RMs showed higher ω5-gliadin percentages, ranging from 8.3% to 11.2%, compared to PWG-gliadin (4.1%). Within the ω1,2-gliadin type the samples showed no significant differences except for PWG-gliadin and the second batch of $Gluten_{sigma}$ with the highest percentage measured of 13.4%. There were significant differences between the $Gliadin_{sigma}$ and the Gluten RMs (sigma and abcr) in the distribution of α-, γ- and ω5-gliadins. Within the gliadin types similarities were found for $Gluten_{sigma}$ and $Gluten_{abcr}$.

PWG-gliadin had a distribution order of prolamin subfractions in the following sequence: $\alpha > \gamma > \omega1,2 > \omega5$. The percentages of ω5-gliadins were higher than those of ω1,2-gliadins in both gliadin and gluten RMs, except for $Gluten_{sigma}$ 2 which had the same distribution order as the PWG-gliadin. According to literature, the proportions of ω1,2-gliadins are typically between 2% to 7% relative to the total gluten content. The α-gliadins are the most abundant type, ranging from 33 to 51%, followed by γ-gliadins (24–36%) and LMW-GS (10–20%). The minor types are the ω5-gliadins (3–12%) and ω1,2-gliadins (2–7%) and ωb-gliadins are found in trace amounts (0.4–1.6%) (Geisslitz et al., 2018). Regarding ω1,2-gliadins and ω5-gliadins, both fractions occur in small proportions (3-12%), which has also been proven in our experiments. Statistically, none of the commercially available RMs showed a similar relative composition of the gliadin types compared to PWG-gliadin.

The protein content of the two batches of each wheat RM were compared as well (Fig. 3.2, Tab. 5.14). There was a significant difference in protein content comparing the batches for $Gluten_{sigma}$ and $Gluten_{abcr}$. The second batch of both gluten RM showed smaller protein contents such as 60.5 g/100 g for $Gluten_{sigma}$ and 58.1 g/100 g for $Gluten_{abcr}$. The protein content in $Gliadin_{sigma}$ was the highest in both batches with 94.4 g/100 g and 93.9 g/100 g. There was no significant difference in the protein content and composition comparing both $Gliadin_{sigma}$ batches. The protein content of both batches was as high as the PWG-gliadin RM which is known to be at 93%. This protein content was used for the calculation of the content, based on the reference area under the curve (AUC) measured for the PWG-gliadin at 210 nm.

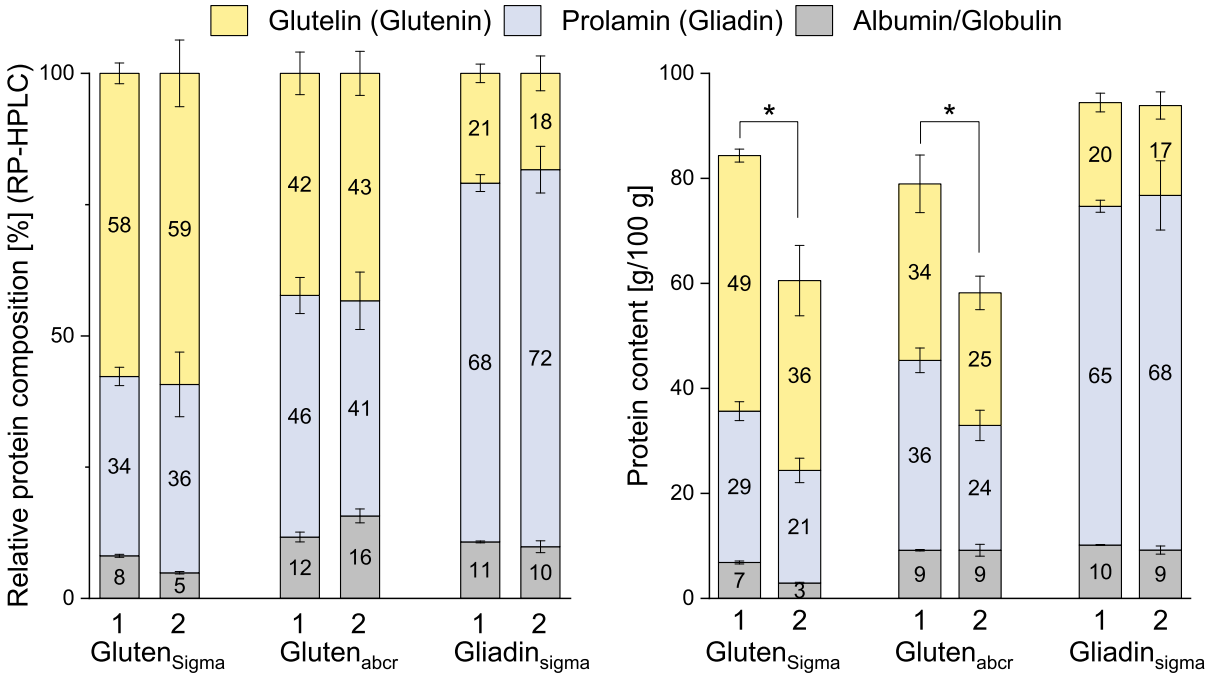

Figure 3.2: Relative and absolute protein composition of three commercially available wheat reference materials. Two batches each are shown here for comparison. The error bars indicate the standard deviations (n = 3). The * indicates the significant difference between the protein content (sum of all fractions) (Two sample T-test, $p < 0.05$).

The manufacturer claims a crude protein content of ≥75% for $Gluten_{sigma}$. The stated protein content measured by RP-HPLC was found only in the first batch (84.3 g/100 g), while the second batch had a lower protein content (60.5 g/100 g). For $Gliadin_{sigma}$ the manufacturer gives an nitrogen content of 15%. The calculation of the protein content (N x 5.8) resulted in 87.0%, which is lower than the contents measured by RP-HPLC. There is no information about the protein content of $Gluten_{abcr}$ provided by the manufacturer. The protein content seems to differ between batches, especially in the RMs marketed as "gluten from wheat".

3.1.2 Molecular weight distribution by GP-HPLC

The M_w distribution of the three wheat RMs was investigated by GP-HPLC. The samples were extracted based on the modified Osborne fractionation and three gluten extracts were evaluated, the prolamins, the reduced (red.) prolamins (reduction of the prolamins with DTT) and the glutelins. To compare the samples, four M_w ranges were evaluated using the M_w of reference substances: (1) > 66 kDa, (2) 66-29 kDa, (3) 29-12.4 kDa and (4) < 12.4 kDa (Fig. 3.3, Tab. 5.13).

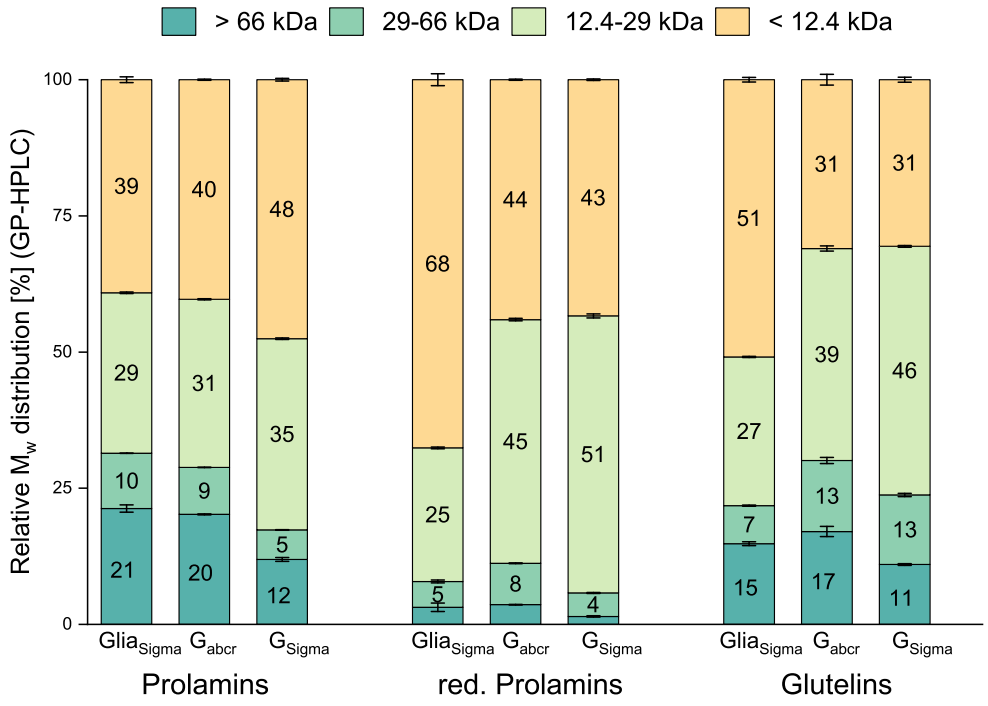

Figure 3.3: The relative molecular weight distribution of three commercially available gluten and gliadin reference materials obtained by the manufacturers Sigma Aldrich (Sigma) and abcr GmbH (abcr) measured by with GP-HPLC. The fractions have been categorized according to the following molecular weight ranges: (1) > 66 kDa; (2) 66-29 kDa; (3) 29-12.4 kDa; (4) < 12.4 kDa. Values are given as means and error bars indicate the standard deviations (n = 3). With the abbreviations G:Gluten, Glia:Gliadin and red.:reduced.

The results indicate that the M_w composition varied across different Osborne fractions. In the prolamin fraction the highest proportion was found in M_w range (4), followed by (3), (1) and (2) in all three samples. The distribution within the samples differed significantly between the samples and the fractions, except for the range (1) between $Gliadin_{sigma}$ (21.3%) and $Gluten_{abcr}$ (21.3%). The range (1) of all three samples within the red. prolamins were significantly lower compared to range (1) within the prolamins. This is expected since the reduction of the disulfide-bonds leads to a disconnection of disulfide-linked gluten proteins. Therefore, when comparing the distribution within the prolamins to the red. prolamins an increase in range (4) and a decrease in all other ranges was observed in the M_w of $Gliadin_{sigma}$. $Gluten_{abcr}$ and $Gluten_{sigma}$ showed higher percentages for range (3) compared to the prolamins. The glutelin fraction of $Gliadin_{sigma}$ had the highest proportion of M_w in range (4) with 67.6% followed by (3) for 24.5% and (2) for 4.8%. For $Gluten_{abcr}$ and $Gluten_{sigma}$, the proportions in range (4) and (2) were similar at 31.0% and 30.6% as well as 13.0% and 12.8%, respectively. However, there were significant differences in the percentages for fractions (1)

and (3). Overall, the M_w distribution in the samples varied from sample to sample. Slight similarities were found between $Gluten_{sigma}$ and $Gluten_{abcr}$. However, for a closer examination of the M_w, an SDS-PAGE gel was carried out and described in the next section.

3.1.3 Molecular weight distribution by SDS-PAGE

The M_w distribution of the RMs was examined with SDS-PAGE. The resulting SDS-PAGE gel of the commercially available RMs and their two batches as well as a chosen wheat flour is shown in Figure 3.4. The flour shows typical SDS-PAGE patterns of the wheat storage proteins (Lagrain et al., 2012; Schalk et al., 2017b). The HMW-glutenin subunits (GS) in flour samples are typically observed as bands in the 85-120 kDa range in SDS-PAGE gels. There are three bands located between 120 and 100 kDa and two well-separated bands between 100 and 85 kDa. Most of these bands associated with HMW-GS are visible in the gel of the RMs that were tested. These characteristic bands of HMW-GS in this range of 100–120 kDa are reported to be in the higher molecular weight range due to aggregation effects (Veraverbeke and Delcour, 2002; Lagrain et al., 2012). However, both batches of $Gliadin_{sigma}$ show only faint bands in this region. In addition, the wheat flour extract displays light bands in the 60-70 kDa range that are attributed to ω5-gliadins. These bands are almost not detectable or only slightly recognizable in the bands of all RMs tested. A more pronounced band can be recognized just below 60 kDa. This band is clearly visible in the flour, as well as in all batches of both $Gluten_{abcr}$ and $Gluten_{sigma}$ and it is associated to ω1,2-gliadins (range 45-60 kDa). Further bands at 45-55 kDa can be detected in all the RMs and the flour sample. The LMW-GS and the α- and γ-gliadins can be detected at 30-45 kDa. The bands in this range are the most prominent bands in the flour as well as in all RMs studied.

Generally, the bands observed in $Gliadin_{sigma}$ are minimal, indicating lower gluten concentrations when compared to $Gluten_{sigma}$ and $Gluten_{abcr}$. The RP-HPLC results however showed opposite results in protein content with higher values for $Gliadin_{sigma}$. However, the gliadin contents measured by RP-HPLC in $Gluten_{abcr}$ and $Gluten_{sigma}$ were significantly higher which is visible in the SDS-PAGE, due to the more prominent bands. It is clear that $Gliadin_{sigma}$ contains fewer HMW proteins such as HMW-GS and ω5-gliadins. Moreover, there is a noticeable difference in the consistency of the banding pattern between the two batches. In addition, bands below 30 kDa and specifically at 15 kDa can be detected, which indicates the presence of AL/GL residues. For $Gluten_{abcr}$ and $Gluten_{sigma}$ both batches seem to have similar band patterns. Here, too, bands in the range of 15 kDa can be seen which contain AL/GL (Scherf et al., 2016a; Lagrain et al., 2012; Schalk et al., 2017b). The appearance of AL/GL in all RMs has been show in the RP-HPLC results as well.

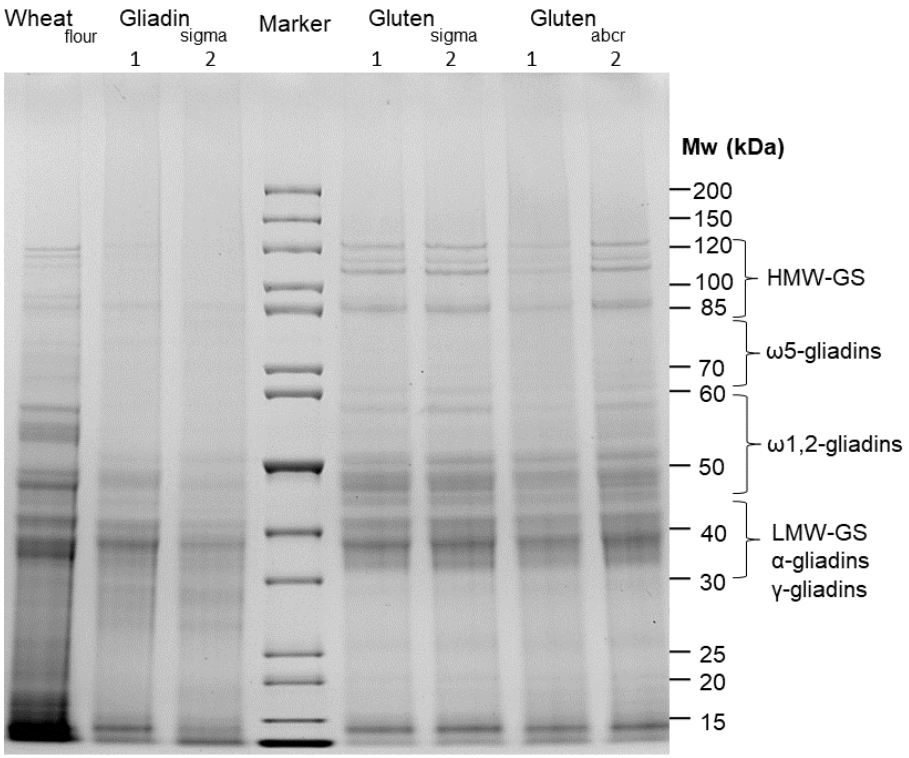

Figure 3.4: SDS-PAGE of three commercially available gluten and gliadin reference materials obtained by the manufacturers Sigma Aldrich (Sigma) and abcr GmbH (abcr). Two batches of the reference materials each were applied to the gel and the protein extract of the wheat cultivar Tommy.

3.1.4 Discussion of the wheat gluten reference materials

PWG-gliadin is the most used RM for calibration and validation of gluten detection methods, especially in ELISA test systems (Van Eckert et al., 2006; Bugyi et al., 2013; Amnuaycheewa et al., 2022). The use of PWG-gliadin guarantees a high degree of repeatability, reproducibility and stability. It is also used as a calibration standard for RP-HPLC measurements to analyse protein contents and distribution of protein types (Pronin et al., 2020b; Schall et al., 2020; Xhaferaj et al., 2023a).

Some manufacturers offer RMs containing gluten or gliadin for various applications. In one study, gliadin from wheat was used to assess the expression levels of two immune-related genes in biopsies from CD patients on a GFD, employing an *in vitro* gliadin challenge using digested $\text{Gliadin}_{\text{sigma}}$ (Bondar et al., 2014). Another use of gliadin is to test novel Abs for potential use in gluten quantification with ELISA test systems (Shatalova et al., 2020). Additionally, gliadin is used as a calibration standard for measuring gliadin levels as low as picograms by flow cytometry or for creating sandwich ELISA tests to quantify low levels of wheat prolamins (Valdés et al., 2003; Capparelli et al., 2005). Gliadin from wheat is also used for the production of isotype-specific gliadin antibodies using phage display (Rhyner et al., 2003). Gluten from wheat was used for example as the nitrogen source for the determination of the gluten hydrolysis ability and probiotic potential of *Lactobacillus brevis* KT16-2 (Kunduhoglu and Hacioglu, 2021). It was also used for the characterization of grain-specific peptide markers for the detection of gluten by mass spectrometry (Fiedler et al., 2014). These are only some examples of the applications where gliadin and gluten from wheat are used. In these applications, the gliadin and gluten is either diluted in 60% ethanol or diluted after the extraction of the AL/GL fraction. In some studies the protein content of the RMs is determined before use.

In this study we investigated the composition of three commercially available RMs ($\text{Gluten}_{\text{sigma}}$, $\text{Gluten}_{\text{abcr}}$, $\text{Gliadin}_{\text{sigma}}$) and compared them with the PWG-gliadin. We found that the protein content especially in $\text{Gluten}_{\text{sigma}}$ and $\text{Gluten}_{\text{abcr}}$ was significantly different when comparing two batches. $\text{Gliadin}_{\text{sigma}}$ is expected to show similar distributions as PWG-gliadin since both consists of wheat prolamins (gliadins). The protein content of $\text{Gliadin}_{\text{sigma}}$ was similar to that of PWG-gliadin. However, $\text{Gliadin}_{\text{sigma}}$ also contained residues of AL/GL and glutelins, which contributed to its high protein content. All three RMs tested showed residues of AL/GL which was confirmed in SDS-PAGE as well. Both the prolamin and glutelin distribution and the distribution of the prolamin types within the prolamin fraction showed differences in the RMs in comparison. $\text{Gliadin}_{\text{sigma}}$ showed

significant differences in the prolamin type distribution compared to PWG-gliadin. This might be due to the difficulty in assigning the integration ranges of the individual types because of the insufficient differentiation in the $Gliadin_{sigma}$ RP-HPLC chromatogram. $Gluten_{sigma}$ and $Gluten_{abcr}$ showed more distinct elution profiles in the prolamin chromatogram. The identification of the wheat gliadin types was therefore possible. The gliadin type distribution was different in the two batches. Significant differences in the M_w distribution in the Osborne fractions were found for all RMs.

In general, the RMs show differences in composition. The gluten RMs, particularly $Gluten_{sigma}$ and $Gluten_{abcr}$, show considerable variations in protein content and composition between batches, making them unreliable. According to the manufacturer, these RMs are also only to be used for research purposes and the exact protein fraction breakdown is not specified. All three RMs contain proportions of water-soluble AL/GL. In many studies, $Gliadin_{sigma}$ is dissolved exclusively in 60% ethanol, which may dissolve some of the AL/GL components and leads to inaccurate protein content (gluten content) measurements. Therefore, a prior separation of the water- and salt-soluble components is necessary, if these RMs are to be used for gluten quantification or research purposes. To ensure accurate concentration calculations a prior characterization of the RMs is necessary. The usage of PWG-gliadin is different, as it is directly dissolved with 60% ethanol, it can be used immediately and provides reproducible results. It is a well-characterized isolate produced from a mixture of 28 different European wheat cultivars (Van Eckert et al., 2006). It is used for the investigation of various scientific questions and improvement of gluten quantification. Unfortunately, there are no gluten RMs consisting of rye and barley on the market. The next chapters show the production of such RMs for the extension of research and closer studies of rye and barley proteins.

3.2 Rye reference materials

The selection of rye cultivars for RM production is based on an in-depth analytical characterization of the grain components, with a specific focus on the gluten proteins. This chapter presents the findings from the analytical characterization of 32 rye flours collected from different countries. It provides an overview of the analysis methods used, which includes SDS-PAGE, RP-HPLC, GP-HPLC, ELISA and the Dumas combustion method (ICC standard 167) for analysing the crude protein content. Both qualitative and quantitative criteria were taken into consideration in selecting the cultivars, with the goal of identifying those with the greatest variability in gluten composition. The second part of this chapter includes the results gained in the production of four different rye RMs and their comparison in gluten composition. Lastly, the suitability of these RMs for use in the R5 ELISA kit was investigated and compared to the PWG-gliadin responses. The results presented in section 3.2.1 and 3.2.2 have already been published in the Journal *Food Chemistry* (Xhaferaj et al., 2023a).

3.2.1 Rye flour characterization

Flours of 32 rye cultivars were first examined qualitatively, which included the visual examination and comparison of the RP-HPLC and GP-HPLC elution profiles of each sample (Tab. 5.2). The elution profiles consisted of three chromatograms each, including the prolamins, the red. prolamins and the glutelins. Figure 3.5 provides an example of the RP-HPLC elution profiles and the integration ranges used to determine the amounts of secalins within the prolamin and glutelin fraction, respectively. Visual examination of the individual RP-HPLC elution profiles revealed minor differences among the samples. The reason for these minor differences is the variability between the samples, leading to differences in the peak heights in certain retention time ranges. However, when compared to profiles from earlier literature, the RP-HPLC elution profiles of all 32 rye flour samples examined were similar (Gellrich et al., 2003; Gessendorfer et al., 2009; Schalk et al., 2017b). As a result, all 32 samples were considered as important for further investigation and were included in the statistical investigation for the cultivar selection.

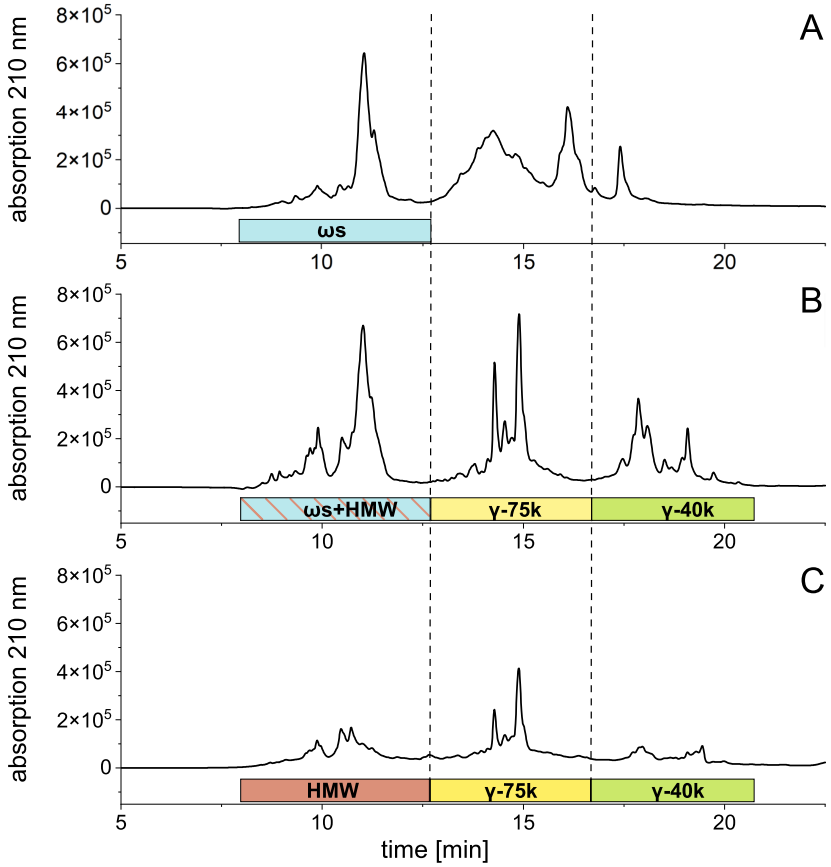

Figure 3.5: RP-HPLC profiles of the rye cultivar Elias. A: Unreduced prolamins, B: Reduced prolamins, C: Glutelins. With the rye protein fractions ωs: ω-secalins; HMW: high molecular weight secalins; γ-75k: γ-75k-secalins and γ-40k: γ-40k–secalins. Modified from Xhaferaj et al. (2023a).

The results obtained based on quantitative criteria were important for the selection of relevant rye cultivars for the production of the gluten/secalin RM. The selection of a small number of rye cultivars with the highest variability in secalin composition was the aim of this study. To investigate the differences between the samples, a specific selection procedure was employed to evaluate quantitative data including moisture, crude fat, protein content and composition, gluten content, prolamin/glutelin ratio and ELISA response. The results of the in-depth characterization of the 32 rye cultivars are presented in Figure 3.6 and in the Tables 5.16-5.18.

3.2.1.1 Moisture, fat and crude protein content

The moisture, fat and crude protein content of the flour samples were determined by the Research Group of Cereal Science and Food Quality (Budapest, Hungary).

The moisture content of the 32 rye samples ranged from 8.2% to 13.0% with a mean of 10.4 ± 1.0%. The average fat content of the samples was 1.2 ± 0.5% (Tab. 5.15 and 5.16).

The existing literature on fat (1.8%) and moisture content (6.0-9.8%) for rye flours shows similar results (Aprodu and Banu, 2017; Drakos et al., 2017).

The protein content varied between 5.2% and 13.2% by Dumas and between 4.2% and 11.2% by RP-HPLC. Overall, the results showed a positive correlation ($r = 0.98$) between the two methods. The protein content determined by Dumas was, on average, 1.9 g/100 g higher than the total protein content evaluated by RP-HPLC (sum of AL/GL, red. prolamins and glutelins) (Fig. 3.6 A). The main causes of the differences in the protein content between the two procedures is the loss due to multi-step protein extraction procedure and the presence of unextractable proteins in the residue after the third extraction step (section 5.3.5.1). Regarding the Dumas method, the released nitrogen is measured with a thermal conductivity detector after combustion of the sample. A species-specific conversion factor is used (5.8 for flour) to convert nitrogen to protein in order to determine the crude protein content. Non-protein nitrogen such as nitrate, ammonia, urea, nucleic acids, or free amino acids can also be found in food. This may be another factor contributing to the crude protein content being higher than the RP-HPLC method, as it has been reported before (Schalk et al., 2017b). Earlier studies have reported similar results to ours, regarding the crude protein content of various rye samples, which ranged from 5.1% to 13.6% total protein (Gellrich et al., 2003; Schalk et al., 2018). More recently, Rani et al. (2021) published research on the protein content of rye, wheat and barley flours measured using the Kjeldahl method, which found that the protein content of five rye cultivars ranged from 8% to 11.4%. These differences in content and composition are influenced by several factors, including genetic variation, environmental factors such as fertilization, harvest year and country of origin, in addition to the method of determination. Therefore, variations in protein content and composition are also expected.

3.2.1.2 Gluten quantification with RP-HPLC

The gluten content and distribution within each sample was analyzed using RP-HPLC. The separation of the proteins is based on the hydrophobic interaction between amino acid residues and a non-polar stationary phase (C18). Thus, proteins elute more slowly when the proportion of hydrophobic amino acids is higher, resulting in a chromatogram with protein sections at different retention times (Fig. 3.5) (Gellrich et al., 2003; Schalk et al., 2017b).

Rye secalins were extracted according to their solubility (Osborne fractionation) and then analyzed (section 5.3.5.1). However, compared to wheat, secalins cannot be clearly divided according to their solubility (prolamins and glutelins) (Schalk et al., 2017a; Lexhaller et al., 2019a). Since the γ-75k-secalins and minor parts of HMW-secalis are present in both fractions,

a different peak separation must be considered in order to determine the fractions (Fig. 3.5). The HMW-secalin content, for example, is only determined after reduction of the prolamin fraction (Fig. 3.5 B). The reduction with DTT leads to the cleavage of disulfide bonds from the remaining HMW-secalins within the prolamin fraction. This results in higher peak areas within the ω-secalin retention time window (7.6-12.5 min, Fig. 3.5 B). By subtracting the amount of ω-secalins from the prolamin fraction with the unreduced prolamins, the HMW-secalin amount within the prolamins is calculated (Fig. 3.5 A). In combination with the HMW-secalins within the glutelin fraction, the total content of HMW-secalins can be determined. Furthermore, the separation between the γ-45k- and γ-75k-secalins has improved due to the reduction with DTT. The gluten composition in the present study was determined based on the calculation described above and on previous studies on rye (Gellrich et al., 2003; Schalk et al., 2018).

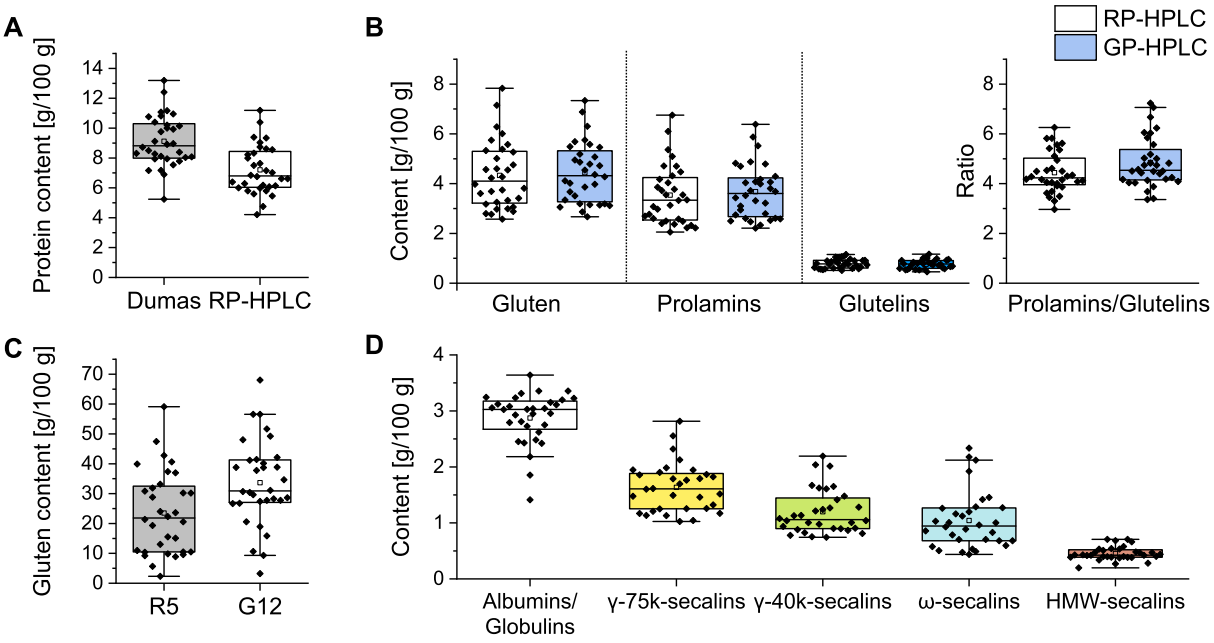

Figure 3.6: Boxplots showing the protein characterization of 32 rye cultivars. The box represents the 25th and 75th percentiles. The diamonds represent the data points for each cultivar (n = 32). The small square in the box indicates the mean, the line the median and the whiskers indicate the upper (75th percentile) and lower (25th percentile) inner fence with a 1.5 interquartile range (whisker length determined by the outermost data point that falls within upper and lower inner fence). A: Comparison of the protein content measured with Dumas and RP-HPLC. B: Gluten, prolamins, glutelins and the prolamin/glutelin ratio measured with RP-HPLC and GP-HPLC. C: Gluten content measured with R5 and G12 ELISA in comparison. D: Protein fractions measured with RP-HPLC. Modified from Xhaferaj et al. (2023a).

The gluten content of the 32 rye cultivars ranged from 2.6 (SAN_EST19) to 7.8 g/100 g (ELI_AUS20) of flour (Tab. 5.15), which is consistent with earlier research, reporting that the

gluten content of rye flour is usually lower than that of wheat flour (Schalk et al., 2017a). The protein fractions present in the samples were also measured and their content varied between 1.4–3.6 g/100 g for ALGL, 1.0–2.8 g/100 g for γ-75k-secalins, 0.7–2.2 g/100 g for γ-40k-secalins, 0.4–2.3 g/100 g for ω-secalins and 0.2–0.7 g/100 g for HMW-secalins (Fig. 3.6 D, Tables 5.15 and 5.17). On average, the relative protein distribution among the 32 rye cultivars was 40% for AL/GL, 23% for γ-75k-secalins, 17% for γ-40k-secalins, 14% for ω-secalins and 6% for HMW-secalins. There are only few studies investigating the protein distribution of rye protein fractions (Gellrich et al., 2003; Schalk et al., 2018; Rani et al., 2021). Nonetheless, the distribution pattern of rye gluten fractions observed (γ-75k-secalins > γ-40k-secalins > ω-secalins > HMW-secalins) was confirmed by our results for most of the samples (Fig. 3.6 D).

3.2.1.3 Molecular weight distribution by GP-HPLC

To complete the protein characterization, GP-HPLC was use to investigate the M_w and gluten content of the 32 samples. The M_w is used to compare the samples with each other. These results are crucial for the selection procedure for the cultivars to be used in the production of the RM.

The GP-HPLC analysis revealed that the gluten content of the rye flour samples ranged from 2.7 to 7.3 g/100 g (Tab. 5.15) and the results were very similar with a strong positive correlation ($r = 0.98$) to the RP-HPLC results, as expected. The M_w distribution was determined by dividing the chromatograms into four ranges, namely (1) $>$66 kDa, (2) 66-29 kDa, (3) 29-12.4 kDa and (4) $<$12.4 kDa (Tab. 5.18). On average, the prolamins showed a distribution of 29.6% in range (1), 10.6% in range (2), 25.1% in range (3) and 34.6% in range (4). The sample RET_FIN20 stood out significantly among the prolamins, with lower percentages of 19.9% (1) and 15.6% (3) and the highest percentage of (4) with 54.2%. The average distribution changed to 5.7% (1), 11.3% (2), 52.4% (3) and 30.6% (4) after prolamin reduction. Since the added DTT reduced the disulfide linkages in the proteins, the higher M_w fraction (1) was reduced and fraction (3) increased as expected. Fraction (2) of the red. prolamins showed the highest variation amongst the cultivars, ranging from 6.8% (DAC_CAN17) to 22.1% (DR_HUN19). The Canadian samples had a low proportion of fraction (2), ranging from 6.8% to 7.7% as shown in Table 5.18. However, the glutelin fraction had a more homogeneous distribution within the samples and M_w categories, with an average distribution of 12.6% (1), 12.7% (2), 33.5% (3) and 41.3% (4). These variations in M_w distribution were used as an additional criterion for selection as described in section 3.2.2.

3.2.1.4 Gluten quantification with ELISA

The ELISA measurements were conducted by the Research Group of Cereal Science and Food Quality (Budapest, Hungary).

The R5 sandwich ELISA and the G12 sandwich ELISA, which are both frequently used for gluten quantification (Méndez et al., 2005; Morón et al., 2008a), were used in the present study. For most samples, the gluten content was overestimated in both kits compared to RP-HPLC results (Tab. 5.16), except for two samples (WIB_HUN17 and DA_HUN17) which had RP-HPLC recovery rates of 76.9% (R5) and 96.1% (G12). Additionally, on average, the G12 ELISA resulted in a 26.7% higher gluten content than the R5 ELISA results. There was a strong correlation ($r = 0.82$) between the results obtained from the two ELISA kits for gluten content. However, when comparing the results from both ELISA kits to RP-HPLC, a moderate correlation was found for the R5 ELISA ($r = 0.76$) and the G12 ELISA ($r = 0.71$). The findings demonstrate that, in accordance to what has previously been reported, different ELISA methods do not always show the same results due to the different specificities of the Abs used (Scherf, 2017; Yu et al., 2021; Amnuaycheewa et al., 2022). Further reasons for the differences in results are the sample preparation, the type of grain and the conversion factor used to calculate gluten.

The sample preparation can significantly impact the measurement of gluten in ELISA. The ELISA methods are specifically designed to detect gluten traces. Therefore, highly concentrated samples, like flours, need a very large dilution (10,000-fold) before analysis (Muskovics et al., 2023). The usual extraction method for extracting gluten from flours involves using 60% ethanol, which has been found to produce acceptable results for wheat flours. However, when applied to rye and barley flours, this method has resulted in greater variability and overestimation of the gluten content (Lexhaller et al., 2016). The effects of sample preparation on gluten quantification in rye and barley flours have been investigated (Muskovics et al., 2023). Rye and barley flours were diluted before the extraction with a gluten-free matrix in solid phase. For rye this dilution lead to higher gluten quantification with ELISA. This effect was attributed to the formation of neopeptides, which are caused by structural changes in the gluten proteins during the dilution process (Muskovics et al., 2023). These findings demonstrate that sample preparation can have a greater impact on gluten quantification in rye and barley flours compared to wheat flours when using ELISA methods.

The prolamin/glutelin ratio is the basis for determining the gluten content in ELISA methods. This is because the alcohol-soluble prolamin fraction is primarily extracted by the R5 and

G12 ELISA sample preparation procedure. According to the Codex, the gluten content is subsequently calculated by duplication of the prolamin content. The prolamin/glutelin ratio is assumed to be 1 in this approach, but this is often not the case (Wieser and Koehler, 2009). As a result, gluten is either underestimated or overestimated. Furthermore, the PWG-gliadin standard is used to calibrate the R5 ELISA kit. Because wheat and rye prolamins differ, a rye-based RM is required for more accurate calibration. Our findings support the overestimation of rye gluten (Lacorn et al., 2019), as a higher content was detected using both ELISA kits when compared to RP-HPLC results (Fig. 3.62C, Tab. 5.15 and 5.17). The higher sensitivity of the R5 and G12 antibodies against rye prolamins compared to wheat is the main reason for overestimation (Lexhaller et al., 2016).

The 32 rye flours had a prolamin/glutelin ratio ranging from 3.0 to 6.3, with an average of 4.4 (Tab. 5.17). Compared to wheat, which has a ratio between 1.4 to 3.6, rye has a considerably higher ratio (Pronin et al., 2020a). This difference is due to the presence of γ-40k- and γ-75k-secalins in both the prolamin and glutelin fractions, which can also be observed in the RP-HPLC chromatograms (Fig. 3.5). Thus, the clear distinction of rye proteins based solely on the solubility of the Osborne fractionation (prolamins and glutelins) is less applicable for secalins, resulting in high prolamin/glutelin ratios and overestimation of gluten in ELISA test kits when calibrated with gliadins or wheat gluten. The conversion factor from rye prolamins to gluten is estimated to be 1.2, instead of 2, based on the average prolamin/glutelin ratio of 4.4 ($\pm$0.8). Using this rye-specific factor will already reduce overestimation of rye gluten. The mean values for both ELISA kits are lower (R5: 13.9 g/100 g of gluten; G12: 19.7 g/100 g of gluten) when 1.2 is applied to the mean ELISA values for R5 (23.1 g/100 g of gluten) and G12 (32.8 g/100 g of gluten). The conversion factor alone does not completely adjust the high reactivity of the R5 and G12 Abs to rye gluten, therefore the values are still higher than those of RP-HPLC.

Basically, various factors such as sample preparation, calibration, conversion factors and reactivity of the antibodies together contribute to the overestimation of the gluten content in rye samples. The results obtained can still be used for the comparison of different samples. However, the use of a suitable rye-based RM would significantly reduce the influence of the factors on the gluten determination in rye-contaminated samples.

3.2.2 Selection of representative rye cultivars

The qualitative and quantitative criteria were used to select representative rye cultivars from the whole collection of 32 rye samples. The selected cultivars will be used to produce the potential rye RMs. The aim of the selection procedure was to identify similarities and differences in cultivar characteristics and cover the range of variability in protein composition using quantitative criteria. To achieve this, a HCA was performed on all quantitative data (Tab. 5.16-5.17) to create clusters of samples with similar characteristics. The HCA identified five clusters (Tab. 5.2), with at least one cultivar from each cluster chosen to represent the highest rye variability.

Cluster C1 included 14 cultivars from different countries, with all seven Canadian samples being in this cluster due to their similar protein distributions, especially the highest amounts of AL/GL from 48% to 52% and the lowest amounts of ω-secalins (7–10%) and γ-75k-secalins (18–20%). Furthermore, varieties from the following countries were assigned to C1: Germany (2), Estonia (2), Latvia (1), Poland (1) and Hungary (1). C2 contained four cultivars from Hungary, only, while C3, C4 and C5 had seven (Hungary (2), Poland (2), Austria (2), Estonia (1)), six (Hungary (4), Austria (1), Finland (1)) and one Hungarian cultivar, respectively. At least one sample was selected from each cluster (Tab. 3.1), with the focus on differences between the samples, country of origin, differences in M_w distributions and availability. Two samples were selected from C1, because this cluster contained the highest number of cultivars. Since C1 contained all Canadian and both German cultivars, one was selected from each country (WHE_CAN17, DAN_GER19). Sample WIB_HUN17 was chosen from C2, because of the significant difference in the M_w distribution of the prolamin fraction compared to the other cultivars in C2 (Tab 5.18). One Polish (DAT_POL20) cultivar was selected from C3 and two samples were selected from C4 (Austrian ELI_AUS20 and Finnish RET_FIN20). The final selection covered a high variability of rye and represented the diversity of geographical origins, with cultivars from Canada, Germany, Hungary, Poland, Austria and Finland. This sample set is considered to be representative since the European Union is the leading region in terms of rye production.

Table 3.1: Content of protein and gluten fractions of the selected rye cultivars measured by RP-HPLC. With the rye protein fractions AL/GL: albumins and globulins; ωs: ω-secalins; HMW: high molecular weight secalins; γ-75k: γ-75k-secalins and γ-40k: γ-40k-secalins. The values are given as means (n = 3), (g/100 g) and different capital letters indicate significant differences between the samples in each column (one-way ANOVA, Tukey's post hoc test, $p < 0.05$). Data already published in Xhaferaj et al. (2023a).

Sample	Protein[a]	Gluten[b]	Prolamins	Glutelins	AL/GL	ωs	γ-75k	γ-40k	HMW	P/G[c]
					g/100 g					
RYEF	8.62 C	5.57 C	4.70 C	0.87 B	3.04 B	2.18 A	1.79 C	0.90 D	0.71 A	5.4
WIB	5.80 E	2.99 F	2.40 F	0.59 C	2.81 C	0.79 D	1.04 G	0.82 E	0.34 C	4.1
WHE	6.14 D	3.06 F	2.49 F	0.57 C	3.08 B	0.50 F	1.21 D	0.88 F	0.47 B	4.3
DAN	5.46 F	3.60 E	2.69 E	0.91 B	1.85 E	0.68 E	1.60 E	1.04 C	0.28 C	3.0
ELI	11.19 A	7.83 A	6.75 A	1.08 A	3.36 A	2.12 A	2.82 A	2.19 A	0.71 A	6.3
DAT	6.40 D	3.98 D	3.35 D	0.62 C	2.42 D	1.03 C	1.52 F	1.04 C	0.39 BC	5.4
RET	10.39 B	7.15 B	6.10 B	1.05 A	3.24 AB	1.92 B	2.55 B	2.01 B	0.66 A	5.8
Mean	7.71	4.88	4.07	0.81	2.83	1.32	1.79	1.27	0.51	4.9

[a] Sum of reduced prolamins, glutelins, albumins and globulins measured by RP-HPLC

[b] Sum of reduced prolamins and glutelins measured by RP-HPLC

[c] Ratio of reduced prolamins and glutelins measured by RP-HPLC

3.2.2.1 Gluten composition of the selected cultivars

The protein content of the seven selected cultivars measured with RP-HPLC ranged from 5.5 g/100 g (DAN_GER19) to 11.2 g/100 g (ELI_AUS20) (Tab. 3.1). The lowest and the highest gluten content was 3.0 g/100 g for WIB_HUN17 and 7.8 g/100 g for ELI_AUS20. The gluten as well as the protein content corresponded well to the previously measured content of the 32 samples with 2.6 to 7.8 g/100 g of gluten and 4.2 to 11.2 g/100 g of protein (Tab. 3.1, Tab. 5.15). Based on the protein content the proportion of AL/GL ranged from 30% (ELI_AUS20) to 50.2% (WHE_CAN17). The relative gluten protein composition of the selected cultivars and the mixture is shown in Figure 3.7. The RP-HPLC measurements resulted in an average secalin distribution pattern of 37% γ-75k-secalins, 27% ω-secalins, 26% γ-40k-secalins and 10% HMW-secalins (Fig. 3.7, mixture calc.). Lab-scale mixing of the selected flours in equal proportions showed slight differences in secalin distribution compared to the calculated mean (35% γ-75k-secalins, 25% ω-secalins, 25% γ-40k-secalins and 15% HMW-secalins). There was a difference in the HMW-secalin proportion between the calculated mean and the flour mixture with 10% for the calculated mean and 15%, respectively, leading to lower proportions for the other secalin fractions. The difference shown is possibly due to insufficient homogenization of comparatively small aliquots of the rye flours. To ensure good homogenization for the further lab-scale isolation, the grains of different cultivars are mixed prior to milling as reported before (Schall et al., 2020).

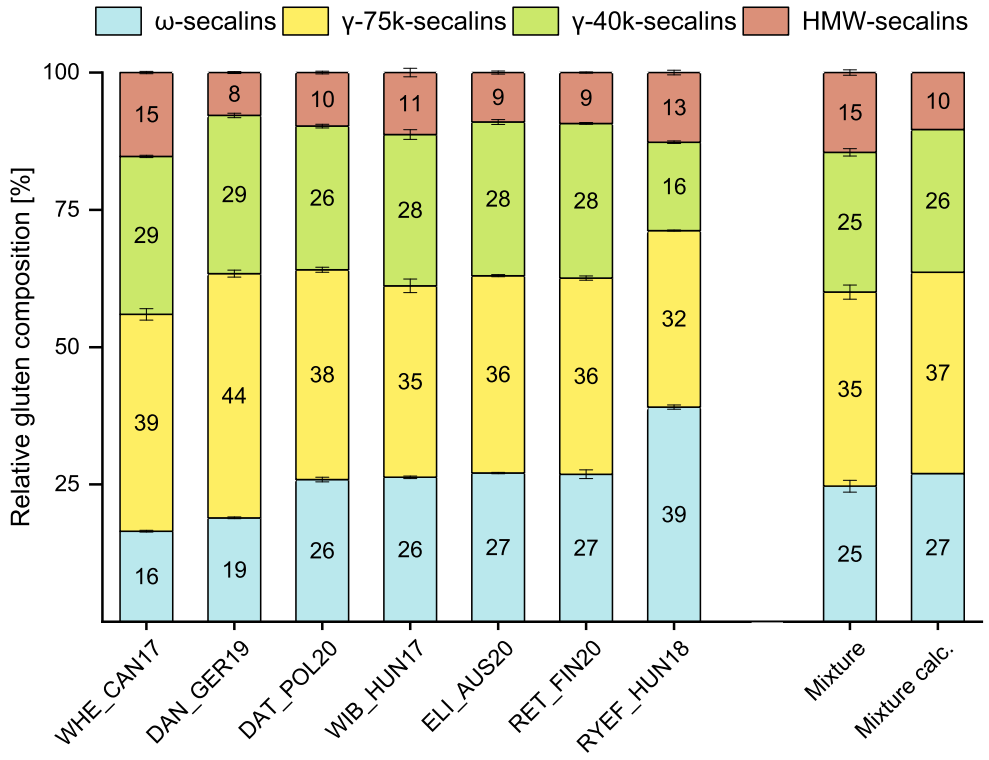

Figure 3.7: Relative gluten composition of selected rye cultivars and their mixture. The mixture consists of the flours of the selected 7 varieties in equal proportions. Mixture calc. is the calculated composition resulting from the mean values. Error bars indicate the standard deviations (n = 3). Modified from Xhaferaj et al. (2023a).

3.2.2.2 Molecular weight distribution by SDS-PAGE

The M_w of the selected flours and their flour mixture was investigated by SDS-PAGE, for further assessment (Fig. 3.8). The secalins were divided according to their experimental M_w based on previous studies on rye secalins (Shewry et al., 1982; Gellrich et al., 2003; Schalk et al., 2017b). Secalin specific bands are found between 85 kDa and 120 kDa for HMW-secalins, 70–85 kDa for γ-75k-secalins, 45–55 kDa for ω-secalins and 35–45 for γ-40k-secalins. Bands with lower M_w (< 30 kDa) ranges belong to salt-soluble AL/GL. Overall, secalin bands were observed between the range of 20-120 kDa. When comparing the samples, the bands show similar patterns as expected and reported previously for rye (Fig. 3.8). However, the bands are different in intensity depending on the protein content. Overall, more prominent bands were observed for ELI_AUS20 and RET_FIN20 in the ω-, γ-40k-, γ-75k- and HMW-secalin ranges, due to their higher protein content (Tab. 3.1). Lighter bands were visible for WHE_CAN17 and WIB_HUN17. The sample DAN_GER19 stood out the most, due to relatively pronounced bands between 70k Da and 85 kDa as well as the two distinct bands between 100 kDa and 120 kDa. This indicates a higher content of HMW-secalins and γ-75k-secalins. The assessment

of the secalins with SDS-PAGE highlights the intended variability in M_w distributions between the samples.

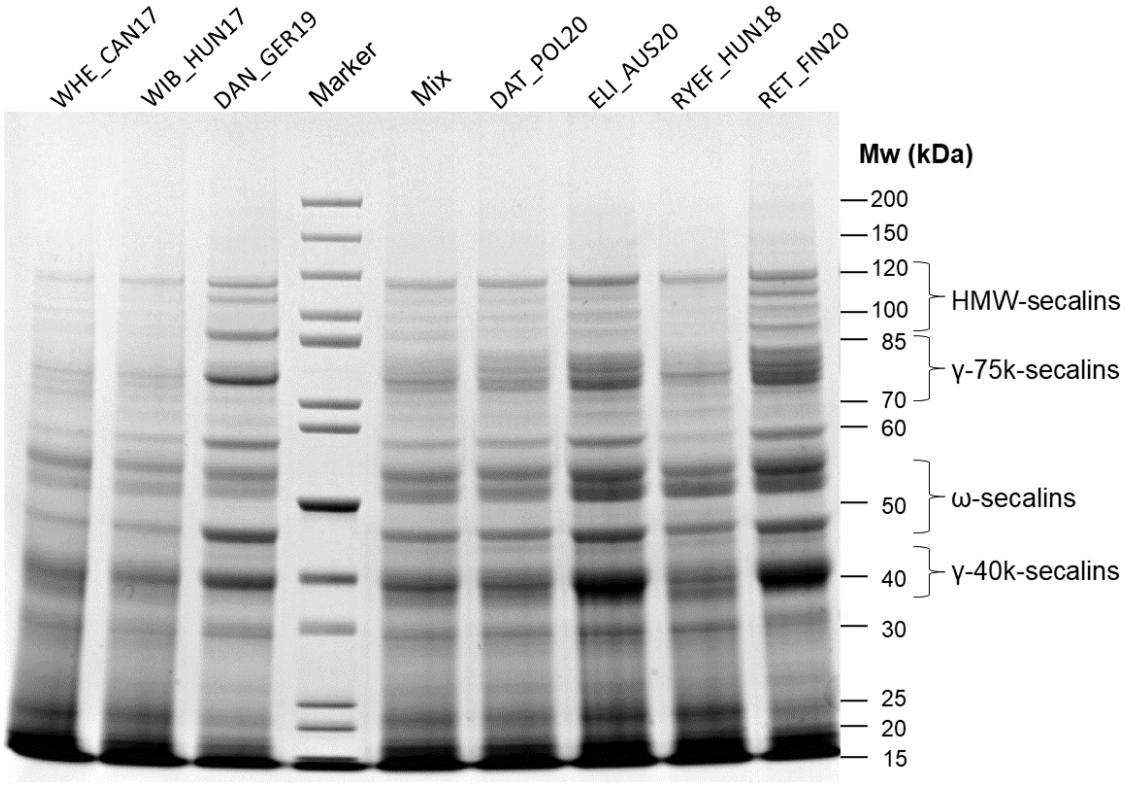

Figure 3.8: SDS-PAGE of 7 selected rye flours and their mixture. Mw: molecular weight. Modified from Xhaferaj et al. (2023a).

3.2.3 Isolation of rye secalins

This chapter focuses on the production of isolates using a mixture of the seven chosen cultivars from the harvest year 2022. The isolates were created by extracting secalins from a flour mixture using different extraction procedures. The chapter provides a comparison of the cultivars from the recent harvest year (2022) to the same cultivars from a earlier harvest years (2017-2020). In addition, the results of the production and characterization of four unique secalin isolates are described. The results presented in this chapter are prepared for publication.

3.2.3.1 Influence of different harvest years

The gluten composition is highly influenced by environmental effects such as the harvest year. The cultivars selected in chapter 3.2.2 were grown in different harvest years (Tab. 3.2) and the protein composition and the M_w distribution were investigated. The secalin content measured by RP-HPLC of the seven samples of the first and second batch is shown in Figure 3.9. Six of the seven cultivars generally showed a significant difference in gluten content depending on the harvest year (T-test, $p < 0.05$). There was no significant difference in gluten content for the different harvest years of the DAT_POL samples. The greatest difference in gluten content was observed for the cultivar ELI_AUS. The gluten content in ELI_AUS20 and ELI_AUS22 was 7.8 and 3.7 g/100 g (Tab. 5.19), respectively. The relative distribution of secalin fractions within ELI_AUS20 (27% ω-secalins, 36% γ-75k secalins, 28% γ-40k secalins and 9% HMW-secalins) and ELI_AUS22 (23% ω-secalins, 39% γ-75k-secalins, 27% γ-40k-secalins and 11% HMW-secalins) did not differ largely. The largest difference in composition was found in WIB_HUN. A significant difference was seen between each fraction (T-test, p-value > 0.05). When considering the mixtures, it is noticeable that both the protein content and the composition match well. This was also apparent in the comparison of the secalin composition, in which no significant difference could be seen between the secalin fractions except for the γ-40k-secalins (T-test, p-value > 0.05). The secalin distribution pattern of γ-75k-secalins $>$ γ-40k-secalins $>$ ω-secalins $>$ HMW-secalins determined in chapter 3.2.1.2 could not be observed for all samples from the second batch. The γ-75k-secalins had the highest percentage and the HMW-secalins the lowest percentage in all samples. Since no clear order for ω-secalins and γ-40k-secalins could be found, the following order resulted for samples harvested in 2022: γ-75k-secalins $>$ γ-40k-secalins $=$ ω-secalins $>$ HMW-secalins.

Table 3.2: Sample information of the first and the second collection of rye cultivars.

Cultivar	Sample code	Geographical origin	Year of collection
Daniello	DAN_GER19	Germany	2019
	DAN_GER22		2022
Dankowskie-Turkus	DAT_POL20	Poland	2020
	DAT_POL22		2022
Elias	ELI_AUS20	Austria	2020
	ELI_AUS22		2022
Rettaa	RET_FIN20	Finland	2020
	RET_FIN22		2022
Rye Food	RYEF_HUN18	Hungary	2018
	RYEF_HUN22		2022
Wheeler	WHE_CAN17	Canada	2017
	WHE_CAN22		2022
Wibro	WIB_HUN17	Hungary	2017
	WIB_HUN22		2022

The M_w for the prolamins, red. prolamins and glutelins of each sample were analyzed and compared. The analysis was conducted within four M_w ranges for each fraction: (1) >66 kDa, (2) 66-29 kDa, (3) 29-12.4 kDa and (4) <12.4 kDa. The results showed that range (2) within the prolamins, fraction had a consistent percentage of 10.1-12.0% in all samples tested (Tab. 5.21). When comparing the harvest years, the samples DAT_POL, DAN_GER and ELI_AUS of 2022 had higher percentages of M_w <12.4 kDa (4), whereas RET_FIN, RYEF_HUN, WHE_CAN and WIB_HUN showed lower percentages of (4). The reduction of the prolamins with DTT led to a decrease of higher molecular weight proteins in range (1), as expected. The percentage of (1) in all samples ranged from 2.1% to 9.5%. All samples from the second collection had a higher percentage in (3) compared to the first collection within the red. prolamins, resulting in an increase of (4). Both Hungarian samples WIB_HUN17 and RYEF_HUN18 showed a significantly higher percentage in (2) compared to their complementary sample in the second collection. Within the glutelin faction, the highest percentages of range (2) could be observed, when compared to the same range of the prolamin fractions. When comparing the different harvest years significant differences within the M_w ranges were observed by ANOVA but no trends were apparent (Tab. 5.21).

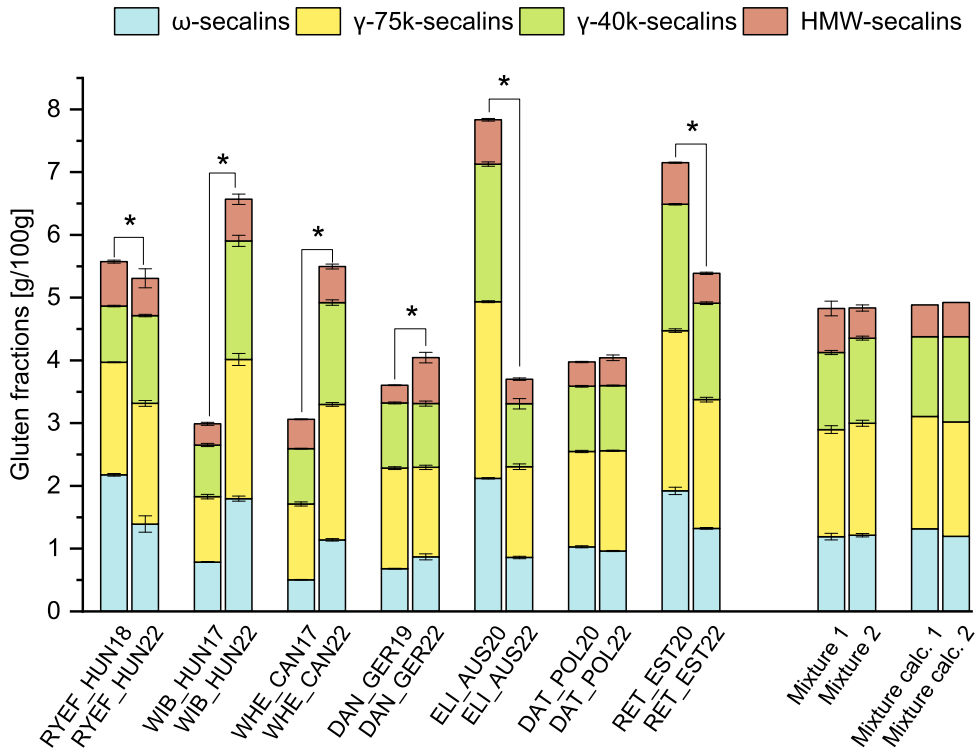

Figure 3.9: Secalin content of the seven selected rye cultivars and their mixture of different harvest years. The mixture consists of the flours of the selected 7 varieties in equal proportions. Mixture calc. is the calculated composition resulting from the mean values. Error bars indicate the standard deviations ($n = 3$). The * indicates the significant difference between the gluten content (Two sample T-test, $p < 0.05$).

Overall, the results show significant differences in gluten composition and M_w between the samples of different harvest years. The harvest year is one out of various factors to influence the gluten composition in grains. The gluten composition can be affected by environmental conditions during the growing season, such as temperature, rainfall and sunlight, as well as the agronomic practices used during cultivation, such as irrigation, fertilization and pest management (Johansson et al., 2003). So far there has been very little research directly investigating the contribution of genotype and environment to the gluten composition of rye. Hansen et al. (2004) determined the relative contributions of genotype and harvest year (3 years) to the total variation in grain characteristics, chemical composition and functional properties related to the baking quality of rye. They showed that the genotype (cultivar) had the highest influence on the protein content, compared with a significantly smaller effect of the harvest year (Hansen et al., 2004). The results support the influence of the genotype but did not support the finding on the smaller effect of the harvest year. This rather contradictory result may be due to greater unknown differences in weather conditions between the harvest years investigated in this study compared to Hansen et al. (2004).

Schall et al. (2020) investigated the genetic and environmental influence on flours of multiple harvest years as well as a mixture on gluten variability and ELISA response. The study found that in most cases the ELISA kits used showed higher gliadin recovery when using blended flour compared to individual cultivars. The harvest year did not have a significant effect on recovery values, but there were significant interactions between ELISA kit, protein source and harvest year. A reduction in variability was achieved by mixing the flours. Accordingly, the use of flour blends as a basis for the production of RMs is advantageous (Schall et al., 2020). The results indicate that mixing the flours reduces the influence of the harvest year and protein composition. Consequently, the flour mixture of the second cultivar collection can be used as a basis for RM production.

3.2.3.2 Yield and protein content

The flour mixture of the seven selected samples from the second cultivar collection were used for protein isolation. The isolation procedure was in many parts adopted from Schalk et al. (2017b) and Batey et al. (1991) with few changes (section 5.3.4). The isolation was carried out in three batches using the flour mixture and it resulted in four different isolates including prolamins ($PROL_{iso}$) and glutelins ($GLUT_{iso}$), which were extracted out of one batch, as well as gluten (G_{iso}) and acetonitrile water extractable proteins ($AWEP_{iso}$).

Around 100 g of flour mixture was used prior to defatting, which resulted in a weight reduction of 5%. The rye flours contained 1-2% fat (see section 3.2.1.1). The higher reduction in weight may be attributed to losses that occurred during the defatting process, such as losses because of the filtering. The protein isolation of the flour mixture yielded 2.2% for $AWEP_{iso}$, 1.8% for $GLUT_{iso}$, 5.2% for G_{iso} and 8.1% for $PROL_{iso}$ (Tab. 3.3). RP-HPLC analysis of the flour mixture indicated a prolamin, glutelin and gluten content of 4.2 g/100 g, 0.7 g/100 g and 4.8 g/100 g, respectively (Tab.5.19). The yields of the homogenized isolates were slightly higher than the RP-HPLC results for $PROL_{iso}$, $GLUT_{iso}$ and G_{iso}. The protein content was determined and compared using RP-HPLC and the Dumas method, respectively. The crude protein content was highest for $AWEP_{iso}$ and $PROL_{iso}$, with 84.6% and 83.7%, respectively. G_{iso} and $GLUT_{iso}$ showed crude protein contents of 77.3% and 52.3%. The protein content measured by RP-HPLC differed from those measured by Dumas. Apart for $GLUT_{iso}$, higher contents were determined by RP-HPLC (Tab. 3.3). The $AWEP_{iso}$ protein content of 105.4% was too high. This overestimation might be caused by the calibration by using the PWG-gliadin in RP-HPLC and by the different measurement principles. However, the crude protein content measured by Dumas was considered as more acceptable for further

calculations of the concentration, especially for the ELISA measurements of the isolates.

The production of hydrolyzed prolamins from rye was published by Gessendorfer et al. (2009). The extraction yielded in 2.2 g of secalins out of 100 g of flour with a protein percentage of 83.8%. Compared to our study, the yield was lower, but the protein content corresponds well to the $PROL_{iso}$ (84.6%). Little is known about yields of glutelins out of rye flours in general. The production of PWG-gliadin RM resulted in a protein content of 89.4%, which is higher than the protein content obtained for the secalins $PROL_{iso}$ (84.6%) in our study. The initial quantity of mixed grains used by Van Eckert et al. (2006) was 30 kg and the defatting process produced 18 kg of defatted flour. Milling and defatting led to a weight loss of 40% from the original grain. The differences in yields can be explained by the difference in the total amount used for isolation. The large-scale production of the PWG-gliadin resulted in higher losses in yield compared to the lab-scale isolation of rye in the present study. Additionally, little is known about the changes in yield and protein content of rye flour isolates in large-scale applications. These factors should be considered when planning a large-scale rye isolate production.

Table 3.3: Yield and protein content of the four rye isolates measured by Dumas and RP-HPLC. Values are given as means ($n = 3$). Yields are given as % and indicates g isolate/100g flour. The crude protein and protein content measured by RP-HPLC are given as g protein/100 g isolate. The different capital letters indicate significant differences between the samples in each column (one-way ANOVA, Tukey's post hoc test, $p < 0.05$). SD: standard deviation, PC: protein content.

Sample	Yield	Crude protein		RP-HPLC (g/100 g)							
	%	g/100 g	SD	PC		ω/HMW		γ-75k		γ-40k	
Gluten	5.2	77.3	0.4	96.0	AB	30.4	A	37.6	B	27.9	A
AWEP	2.2	84.6	0.3	105.4	A	30.2	A	56.3	A	18.8	B
Prolamins	8.1	83.7	0.1	88.7	B	28.6	A	31.6	C	28.5	A
Glutelins	1.8	52.3	0.1	48.2	C	14.6	B	22.3	D	11.4	C

3.2.4 Secalin isolate characterization

The isolates $PROL_{iso}$, $GLUT_{iso}$, G_{iso} and $AWEP_{iso}$ were characterized further by SDS-PAGE, RP-HPLC, GP-HPLC and LC-MS/MS. The characterization focused on the amount and distribution of the secalin fractions within the different isolates. An epitope search was performed and the reactivity of the isolates was tested against the R5 mAb. The results presented in this chapter are prepared for publication.

3.2.4.1 Molecular weight distribution by SDS-PAGE

For prior qualitative estimation the M_w distributions of the isolates were determined by SDS-PAGE (Fig. 3.10). The isolates had been obtained in high purity and showed similar band patterns in the ranges of 60 to 120 kDa (γ-75k, HMW) and 30 to 40 kDa (γ-40k), but only relatively light bands or none for ω-secalins in the range 45 to 55 kDa. This result is surprising since the amount (in the flour mixture) of ω-secalins is in the range of the γ-40k-secalins. Additionally, it is visible that the flour mixture shows four bands in this M_w range (45-55 kDa). $GLUT_{iso}$, G_{iso} and $AWEP_{iso}$ show relatively light bands for ω-secalins. Regarding the HMW- and γ-75k-secalins, two slight bands were observed right below the 100 kDa and 85 kDa for all samples, respectively.

Overall, two prominent bands were visible at 70 to 80 kDa indicating the γ-75k-secalins. In comparison with $PROL_{iso}$, G_{iso} and $AWEP_{iso}$, $GLUT_{iso}$ showed a different band pattern, showing a significantly weaker band at 30-40 kDa (γ-40k). The band intensities indicate a relatively similar secalin composition for $PROL_{iso}$, G_{iso} and $AWEP_{iso}$. In addition, the composition of $GLUT_{iso}$ differed as expected with higher amounts for the HMW-secalins. The ω-secalins appear to make up a lower proportion in the isolates compared to the flours. This finding is contrary to previous studies which showed one intense band at 45 kDa indicating the ω-secalins in prolamin fraction of rye flours (Gellrich et al., 2003; Gessendorfer et al., 2009; Schalk et al., 2017b).

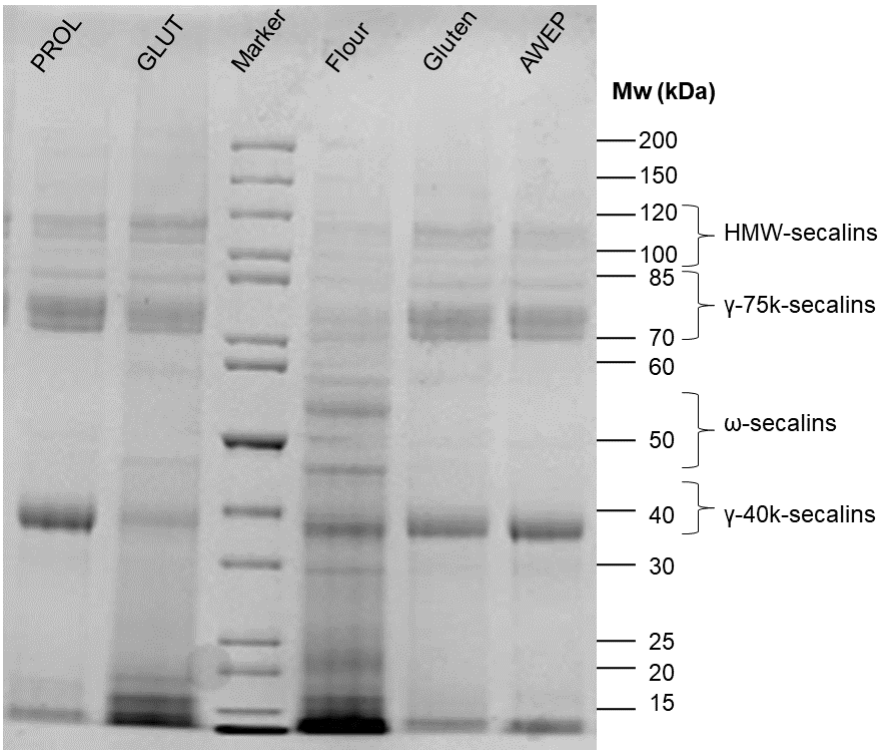

Figure 3.10: SDS-PAGE gel of the four isolates produced from the rye flour mixture. M_w: molecular weight; PROL: prolamins; GLUT: glutelins; AWEP: acetonitrile water extractable protein.

3.2.4.2 Molecular weight distribution with GP-HPLC

The M_w distribution of the four different isolates was measured with GP-HPLC and the patterns were compared to the prolamins, red. prolamins and glutelin fractions of the flour mixture (Tab. 3.4). The M_w of each range (>66 kDa, 29-66 kDa, 12.4-29 kDa and <12.4 kDa) differed significantly for most of the values. The different capital letters indicate significant differences between the samples in each column (one-way ANOVA, Tukey's post hoc test, $p<0.05$). Within the isolates the $AWEP_{iso}$ and the $PROL_{iso}$ showed similar results in the 29-66 kDa and <12.4 kDa M_w ranges. The most similarities were found for the <12.4 kDa M_w range between $AWEP_{iso}$ and $PROL_{iso}$ and between G_{iso}, $GLUT_{iso}$ and red. $PROL_{iso}$. When comparing the isolates with the flour mixture, similar M_w values were found between the glutelins and the red. prolamins as well as between $AWEP_{iso}$ and $PROL_{flour}$. $PROL_{flour}$ showed similar percentages in the 29-66 kDa range with $AWEP_{iso}$ (10.6% and 10.7%). The data showed the most similarities between $PROL_{flour}$ and the composition of $AWEP_{iso}$.

Table 3.4: Relative molecular weight distribution determined with GP-HPLC for the four rye isolates and the flour mixture according to four different molecular weight ranges. Values are given as means (n = 3), (%) and different capital letters indicate significant differences between the samples in each column (one-way ANOVA, Tukey's post hoc test, $p < 0.05$).

Samples		> 66 kDa		29-66 kDa		12.4-29 kDa		< 12.4 kDa	
		%							
Isolates	Gluten	7.0	D	9.3	E	51.7	B	32.0	DE
	AWEP	26.4	A	10.7	CD	27.6	F	35.3	C
	Prolamins	23.1	B	11.4	BC	30.2	E	35.3	C
	red. Prolamins	2.3	E	5.8	G	60.8	A	31.1	E
	Glutelins	10.7	C	13.4	A	44.1	C	31.8	DE
Flour mixture	Prolamins	28.2	A	10.6	D	28.6	EF	32.8	D
	red. Prolamins	3.6	E	7.9	F	44.1	C	44.5	A
	Glutelins	11.5	C	11.8	B	38.8	D	37.9	B

When comparing the results using ANOVA various factors such as the sample size and the standard deviations should be considered as well as the assumptions being made about the data. In relation to our data, the ANOVA easily reveals differences in composition because of small standard deviations. The differences between the isolates were to be expected. Although statistical differences between the flours and the complementary isolates were shown, the distributions of the M_w ranges are comparable. The M_w changes slightly after protein isolation. The flour and isolates were extracted at different scales, which is one reason for the change in M_w distribution. It was reported that the monomeric and polymeric protein ratio within flour blends changed, depending on the production scale. The change can be influenced by the milling method and differences in up-scaling equipment (Schall et al., 2020). For more detailed information on the secalin fraction distribution, the RP-HPLC measurements presented in the following chapter can be used.

3.2.4.3 Characterization by RP-HPLC

The secalin distribution of the four isolates was characterized by RP-HPLC and compared with the distributions of the flour secalins, which were extracted with the same extraction methods (Fig. 3.11). The HMW- and the ω-secalin results were merged for comparison. The relative values were compared between the flours and the respective isolates and resulted in significant differences for all fractions except between the γ-75k-secalins for $PROL_{iso}$ and $PROL_{flour}$ (T-test, $p < 0.05$, p-value = 0.62, Tab. 5.23). Comparing the gluten distribution of the G_{iso} and G_{flour}, a similar distribution resulted (ω/HMW: 32% and 33%, γ-75k: 39% and 37%, γ-40k: 29% and 30%). These results are in accordance with the faction distribution

of the flour mixture extracted in several steps using the modified Osborne fractionation (ω/HMW: 35%, γ-75k: 37% and γ-40k: 28%). More obvious differences were found for the comparative distribution of the other extractions (Fig. 3.11). Between the AWEP fraction the highest difference was observed for the γ-75k-secalins with 53% for $AWEP_{iso}$ and 48% for $AWEP_{flour}$. The percentages of the isolate and the flour for γ-40k- and ω/HMW-secalins were 18% and 22% as well as 29% and 30%, respectively. The prolamin extraction resulted in a distribution of 32% ω/HMW-, 36% γ-75k- and 32% γ-40k-secalins for $PROL_{iso}$ and 37% ω/HMW-, 35% γ-75k- and 37% γ-40k-secalins for $PROL_{flour}$.

The comparison of individual secalin fractions within the isolates and the flour mixture was performed using ANOVA (Tab. 5.20). The ω/HMW-secalin fraction ranged from 29 to 32% for the isolates. Within the flours the glutelin fraction stood out with a lower percentage of 23% for the ω/HMW-secalins which is surprising since the HMW-secalins are a main part of the glutelin fraction. For the γ-75k-secalin fraction in both isolates and flours the values showed significant differences. However, $AWEP_{iso}$ and $GLUT_{iso}$ and $GLUT_{flour}$ had higher percentages ($>46\%$) compared to G_{iso} and G_{flour} and $PROL_{iso}$ and $PROL_{flour}$ ($<39\%$) for the γ-75k-secalins. $AWEP_{iso}$ showed the highest γ-75k-secalin percentage (53%) of all isolates and flours. The highest γ-40k-secalin percentage within the isolates was found in $PROL_{iso}$ (32%) and the lowest in $AWEP_{iso}$ (18%). In the flours, the lowest γ-40k-secalin percentage was measured in $AWEP_{flour}$ (22%) as well.

In general, the results show that the isolates differed in composition from the flour mixture. In addition, the calculated composition of the flour mixture extracted by the modified Osborne fractionation was similar to the isolate and the flour extracted exclusively with the buffer solution (G_{iso} and G_{flour}). Similar compositions were found when extracting with 60% EtOH solution. The AWEP and the glutelin fractions showed higher percentages in γ-75k-secalins and lower ones in ω/HMW-secalins. Losses or changes in composition may result from the many steps of isolation, such as dialysis and freeze-drying. In addition, mixing and laboratory-scale production could have an impact on the composition (Schall et al., 2020). In the production of PWG-gliadin for instance, no major differences in the composition of the protein fractions compared to the flour were observed (Van Eckert et al., 2006). The isolates produced in our study correspond to a large extent to the composition of their source flour but show differences in composition. This was expected, since the small standard deviations ($<10\%$) have an impact on ANOVA resulting in significant differences in composition more easily. Extraction on lab-scale may have an influence in protein distribution as well.

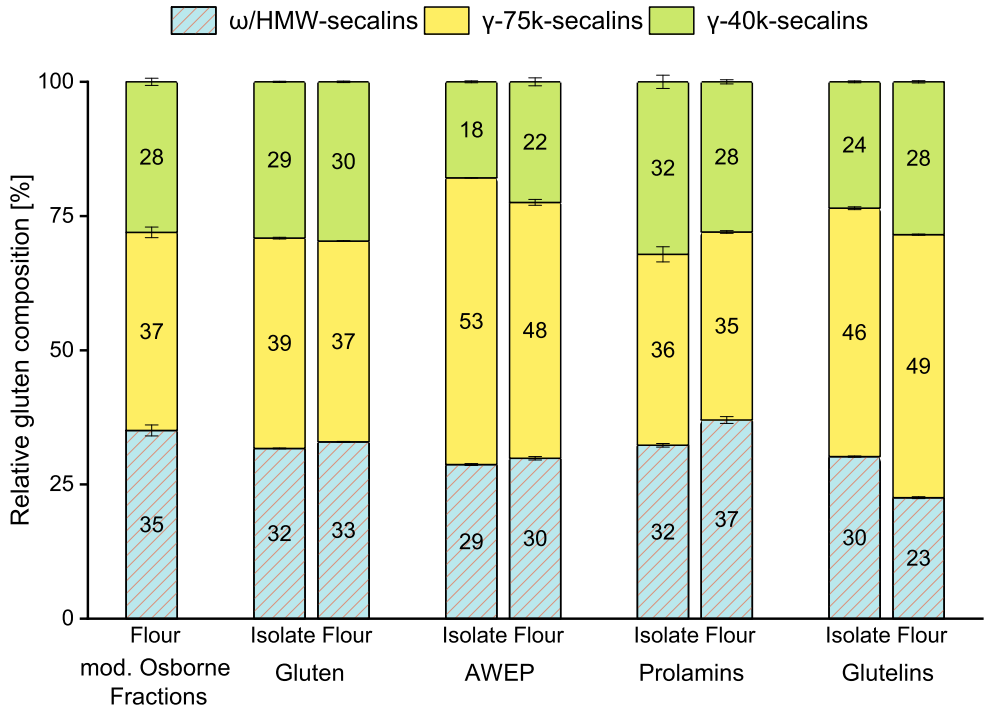

Figure 3.11: Comparison of the relative secalin distribution within the isolates and the flours extracted and isolated with respective buffers. Error bars indicate the standard deviations (n = 3). AWEP: acetonitrile water extractable proteins.

3.2.4.4 Characterization by untargeted LC-MS/MS

To identify the proteins and peptides showing the highest response in the LC-MS/MS system, the isolates were reduced, alkylated and digested with trypsin prior to measurement (section 5.3.8.1). Untargeted proteomics was used to characterize the secalins within the isolates using the MaxQuant software and the UniprotKB database. The identification of peptides relied on a reference database of the tribe *Triticeae* containing 557477 protein entries (Uniprot FASTA-file 23.02.2023). More information on the MaxQuant settings can be found in chapter 5.3.8.3.

The search led to the identification of 314 protein groups, of which 27 were labelled as gluten proteins based on a fasta header search for keywords such as "gluten," "gliadin," "glutenin," "prolamin", "glutelin", "hordein" and "secalin". Among all protein groups, there were 21 fragments, 13 uncharacterized proteins and 847 peptides identified across all samples. The assignment of the protein groups to the secalin fractions (ω-, γ-75k-, γ-40k- and HMW-secalins) was performed using the fasta header and the homology of the amino acid sequence. Only protein groups derived from *Secale cereale*, *Secale strictum* and *Triticum aestivum* species were considered as appropriate for selection. Since the entries

in the databases do not always have the correct designation and the proteins are similar in their sequence, the proteins derived from *Triticum aestivum* were compared using the BLAST tool available from the UniProtKB webpage to find comparable protein groups. The identification of the protein groups within MaxQuant is carried out using the Andromeda algorithm (probability-based), which calculates scores to each protein group. The higher the score is the higher is the probability of the protein group being in the sample. Therefore, protein groups with a score >20 were considered for relative quantification. The relative quantification of the protein groups of the isolates was performed using the intensity based absolute quantification (iBAQ) algorithm. It estimates the relative abundance of the proteins within each sample. For comparison, a total sum normalization of iBAQ protein group intensities between samples was performed. The resulted protein groups can be observed in Table 3.5.

Table 3.5: List of the selected protein groups identified in the secalin isolates after tryptic digestion and the untargeted LC-MS/MS measurement. iBAQ: Intensity Based Quantification.

UniProt KB accession	Species	UniProt Name	Score	iBAQ	Peptides
		ω-secalins			
C4NFP2	*Secale cereale* x *Triticum turgidum subsp. Durum*	Omega secalin	44.94	5.6×10^{7}	2
		γ-75k-secalins			
K7WF86	*Secale cereale*	75k gamma secalin	179.38	2.6×10^{9}	6
H6ULI9	*Secale strictum*	75k gamma secalin	76.16	4.4×10^{9}	3
		γ-40k-secalins			
H8Y0N7	*Secale cereale subsp. Afghanicum*	Gamma prolamin (Fragment)	108.93	1.7×10^{9}	1
A0A5B9Y471	*Triticum aestivum*	Gamma-gliadin	39.55	2.7×10^{9}	5
F4ZL28	*Secale strictum subsp. Africanum*	Alpha-gliadin storage protein	38.07	2.3×10^{8}	6
		HMW-Secalins			
W6AW92	*Triticum aestivum*	HMW glutenin subunit	151.56	1.7×10^{9}	10
W6AW98	*Triticum aestivum*	HMW glutenin subunit x	114.06	1.4×10^{9}	9
Q94IJ7	*Triticum aestivum*	HMW glutenin subunit x	22.57	7.7×10^{7}	7

Based on the selection criteria only one protein group could be assigned to the ω-secalins (C4NFP2) with two identified peptides. For the γ-75k-secalins, two protein groups were assigned (K7WF86, H6ULI9). The protein group K7WF86 had the highest score (179.4) and six peptides identified and H6ULI9 had three identified peptides and the highest iBAQ value. Three protein groups (H8Y0N7, F4ZL28 and A0A5B9Y471) were assigned for γ-40k-secalins

and three for HMW-secalins (Q94IJ7, W6AW92 and W6AW98) (Tab. 3.5). Within the HMW-secalins all selected protein groups were found from the *Triticum aestivum* species. The protein group W6AW92 showed the highest number of identified peptides (10).

The selected protein groups were used to calculate the relative secalin distributions in the isolates with the use of the iBAQ value. Figure 3.12 shows the relative secalin distribution in each isolate. It is apparent that the ω-secalins percentage was less than 0.01% in all samples and therefore they were not considered in the following order of distribution. The secalin distribution within G_{iso} was as follows: 63% γ-75k-, 24% γ-40k- and 13% HMW-secalins. $AWEP_{iso}$ consisted of 39% γ-75k-, 45% γ-40k- and 15% HMW-secalins. The composition of $PROL_{iso}$ included 43% γ-75k-, 35% γ-40k- and 21% HMW-secalins. $GLUT_{iso}$ showed the smallest percentage of 8% for γ-40k- and the highest percentage of 36% for HMW-secalins compared to all isolates and a percentage of 56% for γ-75k-secalins. The highest HMW-secalin percentage in $GLUT_{iso}$ compared to the other isolates was expected since the HMW proteins make up a higher percentage in glutelins in general. There were differences in the secalin distribution between all isolates. Because of high standard deviations (6-40%) of the MS measurements the differences cannot be stated statistically. High standard deviations are common in untargeted approaches. It should be noted that the focus of this study was on qualitatively assessing the distribution rather than conducting quantitative analysis. However, compared with the analytical characterization with RP-HPLC the ω-secalins and the HMW-secalins could not be examined separately and the ω-secalins measured with LC-MS/MS were relatively low, which is in accordance with the SDS-PAGE results shown in section 3.2.4.1. The following order emerges from the analytical characterization: $\gamma\text{-}75k > \gamma\text{-}40k = \omega > HMW$. This sequence could not be confirmed for the LC-MS/MS measurements of the isolates.

Lexhaller et al. (2019) conducted a study on rye GPTs using LC-MS/MS. Since they investigated GPTs purified by preperative RP-HPLC, they found more gluten protein groups for each protein type (HMW, ω, γ-40k and γ-75k) compared to our study. When comparing the gluten protein groups found in our study with the study conducted by Lexhaller et al. (2019) the same protein groups W6AW92 (HMW) and F4ZL28 (γ-40k) were identified by our measurements. Their investigation resulted in more identified protein groups for HMW- and ω-secalins compared to the other protein types. In our study, however, most proteins were identified for the HMW-secalins. The number of protein groups is not comparable since our isolates were not separated into secalin fractions and purified. Additionally, the enzyme they used compared to our study (trypsin) for digestion was chymotrypsin which has significant impact on the peptides identified in general. The impact of the chosen digestive enzyme on the results will be discussed in the next chapter more specifically.

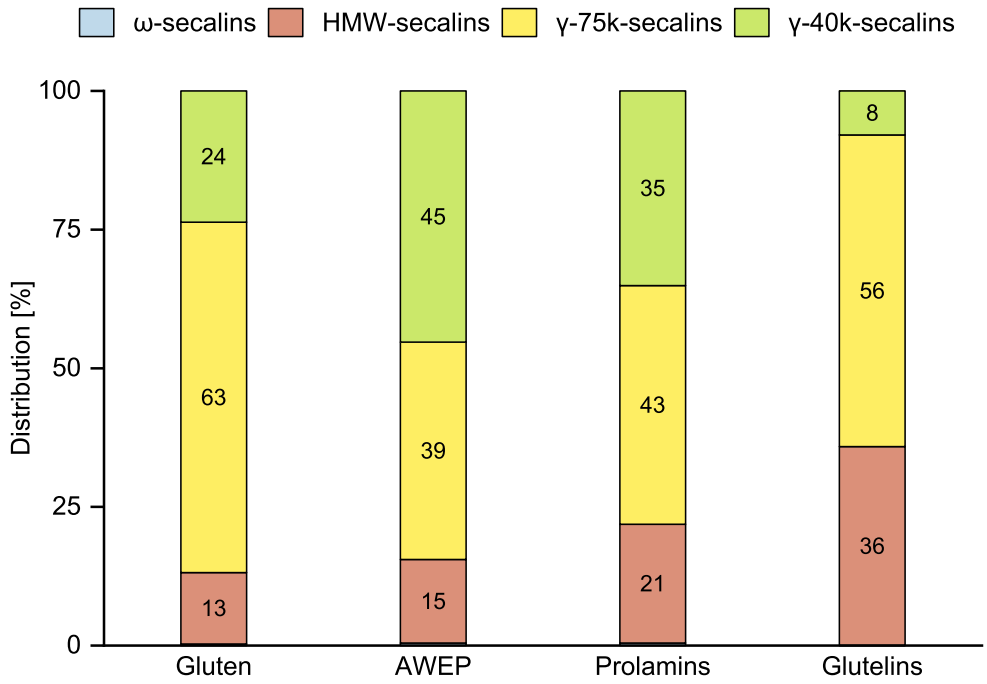

Figure 3.12: The relative secalin distribution in each isolate measured by untargeted LC-MS/MS. AWEP: acetonitrile water extractable proteins.

The data presented in this study is highly dependent on the reliability of the reference sequences in the databases used. We found 29 and 145 UniProtKB entries for "secalin" in *Secale cereale* and *Triticeae*, respectively, none of which have been reviewed (March, 2023). For a number of reasons, the analysis of rye proteins using the UniProtKB database may be insufficient. It does not completely cover all rye proteins, since certain secalins have not yet been characterized or have not been included in the database. The inaccurate naming of protein entries that are very similar in sequence is another reason for incorrect designations. Since the results are based on the quality of the database, the interpretation of the data must be viewed critically in general.

3.2.4.5 Identification of celiac disease-active peptides

The list of peptides present in the isolates generated by MaxQuant was searched for immunoactive peptides. Three search procedures were performed for different purposes. The first procedure contained the search for epitopes recognized by the mAb R5 listed in Table 5.25 (Osman et al., 2001; Kahlenberg et al., 2006). Thus, the specific protein fraction carrying these epitopes can be identified by R5 ELISA. The second search consisted of using the list of CD-relevant epitopes recognized by CD4+ T cells (Sollid et al., 2020). In addition, the available peptide sequences were searched for complete and partial matching sequence overlaps with previously known epitopes following the search strategy of the European Food

Safety Authority (EFSA) search strategy (Naegeli et al., 2017). For this purpose, the peptide sequences were explored according to the sequence Q-X1-P-X2, where X1 may correspond to the amino acids leucine (L), glutamine (Q), phenylalanine (F), serine (S), or glutamic acid (E) and X2 may correspond to tyrosine (Y), phenylalanine, alanine (A), valine (V), or glutamine. The peptide sequences thus obtained are considered to be potentially immunoactive peptides.

The search resulted in the identification of 14 peptides of which six peptides were found within the selected protein groups (section 3.2.4.4). Four sequences were found to be potentially harmful, one was found to be recognized by the mAb R5 and one was found to be recognized by CD4+ T cells (Tab. 3.6). The K7WF86 protein group (γ-75k) contains three tetrapeptides that are potentially harmful using the strategy described above. The other four protein groups (C4NFP2, A0A5B9Y471, W6AW92 and W6AW98) contained one tetrapeptides within the sequence identified. The sequence found within the protein group Q94IJ7 is QGYYPTSPQ. According to Sollid et al. (2020), this sequence is recognized by CD4+ T cells, because it contains the 9-mer core region QGYYPTSPQ and is part of the DQ8.5-glut-H1 restricted epitopes. This epitope was identified by testing T cell clones from HLA-DQ2/8 CD patients and found to be specific for the HMW-glutenins (Kooy-Winkelaar et al., 2011). The protein group Q94IJ7 was selected to be assigned to the HMW-secalin fraction in chapter 3.2.4.4. Since the HMW-secalins and HMW-glutenins have homologous amino acid compositions the protein group could be assigned to the HMW-secalins. A BLAST search within UniProt on the sequence resulted in 9 protein groups (5 of them reviewed, March 2023) with 100% coverage of the sequence which are part of the glutenin subunits of *Triticum aestivum* and *Triticum uratu*. However, peptide sequences found within the isolates with our search strategies do not show a high number of peptides in general. The number of identified immunoreactive peptides is highly dependent on the sample preparation, more specifically on the enzyme chosen for digestion (Manfredi et al., 2015).

The most commonly used enzymes for digesting gluten proteins in proteomics are trypsin and chymotrypsin (Martínez-Esteso et al., 2017; Schalk et al., 2017a; Pasquali et al., 2019). Trypsin cleaves at the C-terminal side of arginine and lysine residues, while chymotrypsin cleaves at the C-terminal side of hydrophobic amino acids, such as tyrosine, phenylalanine and tryptophan (Colgrave et al., 2017a). Gluten proteins in general are rich in proline and glutamine residues, which are resistant to digestion by many enzymes. Because gluten proteins have few tryptic cleavage sites, the resulting peptides are longer and less abundant. In our study a total of 14 peptides in which six peptides were identified within the selected protein groups using all three search strategies described above. Other enzymes, such as chymotrypsin, thermolysin and pepsin, have been used to enhance the sequence coverage

of proteins and to enable a more precise identification of closely-related isoforms (Manfredi et al., 2015; Colgrave et al., 2017a). The examination of the isolates with different enzymes is therefore very important to make more precise statements about the potential immunoreactivity and should be considered in future experiments.

Table 3.6: List of immunoactive peptides found based on the epitope search. The search procedure to identify immunactive epitopes within peptides identified by LC-MS/MS uses the following strategies: R5 mAb: The epitopes recognized by the R5 mAb ELISA (Osman et al., 2001; Kahlenberg et al., 2006; Amnuaycheewa et al., 2022), immunoactive peptides recognized by CD4+ T cells (Sollid et al., 2020) and potentially harmful epitopes selected based on the EFSA search strategy (Q-X1-P-X2; X1 = L, Q, F, S, E X2 = Y, F, A, V, Q) (Naegeli et al., 2017). The relevant epitopes are written in bold.

	Protein groups	**Sequence**	**Characteristics**
ω	C4NFP2	QLNPSEQEL**QSPQ**QPVPK	Potentially immunoreactive
γ-75k	K7WF86	S**QEPF**PQVHQP**QQPS**PQQ**QQPS**IQLSLQQQLNPCK	Potentially immunoreactive
γ-40k	A0A5B9Y471	PLFQIVQGQSIIQ**QQPA**QLEVIR	Potentially immunoreactive
	F4ZL28	LQVQP**QQSFP**HQPQQQTLQSFLEQQLISCR	R5 ELISA epitope
HMW	W6AW92 W6AW98	AQQLAA**QLPA**MCR	Potentially immunoreactive
	Q94IJ7	QPGQGQ**QGYYPTSPQ**HPGQGQQPGQGQQPGQGK	Recognized by $CD4^+$ T cells

3.2.5 New rye reference material and ELISA responses

This chapter aims to compare the sensitivity and specificity of the R5 mAb to different rye isolates using the R5 sandwich ELISA. The methodological approach used in the study was based on a previous publication by Lexhaller et al. (2016). The results presented in this chapter are prepared for publication.

The concentrations of the freshly prepared stock solutions of the isolates were analyzed by RP-HPLC and their protein content calculated by the Dumas method was taken into account. The R5 ELISA investigations were performed using the stock solutions of the isolates. The standards were diluted appropriately to fall within the R5 ELISA calibration ranges (4 to 5 serial dilutions). The last dilution step was done according to the manufacturer's instructions with

the sample dilution buffer provided in the R5 ELISA kit (1:12.5). The resulting absorbances of the isolates and the test kit standard were plotted in the same graph to compare their reactivity (Fig. 3.13). The concentrations determined by ELISA were calculated using the respective kit standard calibration and the cubic spline function of the Rida®Soft Win Software (R-Biopharm, Darmstadt, Germany). Linear approximations were used to calculate the estimated prolamin, glutelin and gluten contents (Tab. 3.7). The slope of the linear equation provides information about the strength of the sensitivities between the isolates to the R5 mAb.

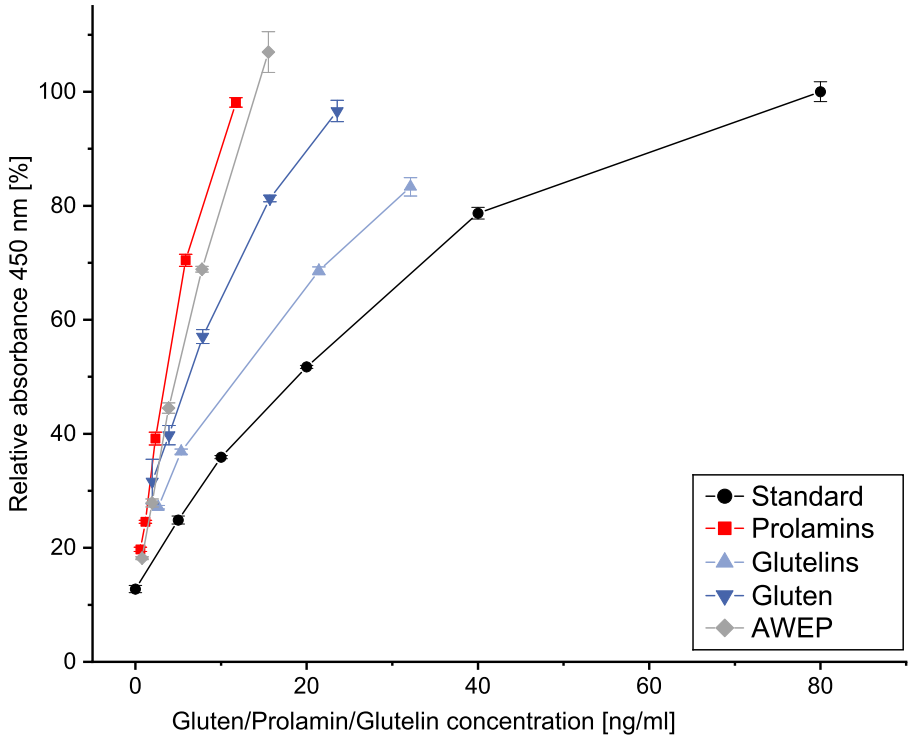

Figure 3.13: R5 sandwich ELISA absorbances ($\lambda = 450$ nm) of the test kit standard, the prolamin, glutelin, gluten and acetonitrile water extractable proteins fractions of rye as a function of the concentrations quantified by RP-HPLC. Error bars indicate the minimum and the maximum value measured.

The R5 mAb showed the highest sensitivity towards $PROL_{iso}$, followed by $AWEP_{iso}$, G_{iso} and $GLUT_{iso}$. Rye prolamins (slope 6.7186, $R^2 = 0.9971$) were more reactive to the R5 mAb than glutelins (slope 1.3309, $R^2 = 0.9992$), which is consistent with previous research (Lexhaller et al., 2016). The main epitope detected by the R5 mAb is QQPFP, which is repetitively present in several gluten proteins including secalins (Méndez et al., 2005). The R5 mAb was developed using rye secalins as its antigen. Therefore, it was expected that the mAb would have a high affinity towards rye prolamins, which has been reported in previous studies (Wieser and Koehler, 2009; Lexhaller et al., 2016). The rye glutelins showed a high sensitivity too,

which can be explained by the fact that the epitope QQPFP is also strongly represented in the γ-75k-secalins. The sequences of the secalins resulting from the untargeted LC-MS/MS experiment were investigated in terms of appearance of the QQPFP epitope using UniProtKB. C4NFP2 contains the epitope 13 times (ω-secalins), while K7WF86 contains the epitope ten times (γ-75k), H6ULI9 12 times (γ-75k), H8Y0N7 once (γ-40k) and A0A5B9Y471 three times (γ-40k). The other protein groups (γ-40k (F4ZL28), HMW (W6AW92, W6AW98, Q94IJ7) do not contain the QQPFP epitope. In both the RP-HPLC (section 3.2.4.3) and LC-MS/MS (section 3.2.4.4) experiment conducted on the $GLUT_{iso}$ the γ-75k-secalins were present in the highest percentage compared to the other fractions.

The sensitivity of the R5 mAb to $PROL_{iso}$ (slope 6.7186, $R^2 = 0.9971$) and $AWEP_{iso}$ (slope 4.3065, $R^2 = 0.9988$) were comparable which could be due to the two extraction methods mainly extracting the prolamins and thus having a similar protein composition. Since G_{iso} (slope 2.8716, $R^2 = 0.9946$) is composed of the prolamins and glutelins it showed a reactivity between $PROL_{iso}$ and $GLUT_{iso}$. The sensitivity studies on the isolates are in agreement with those obtained by Lexhaller et al. (2016). In addition, our data shows the reactivity of $AWEP_{iso}$ to the R5 ELISA, which has not yet been investigated in other studies. Further studies in relation to validation parameters such as accuracy, recovery and repeatability should be carried out.

Table 3.7: Comparison of rye gluten reactivity between R5 ELISA and RP-HPLC. Linear fit parameters indicate the reactivity of the R5 mAb with different isolates.

Calibration	Prolamins		Glutelins		Gluten		AWEP	
	ng/ml							
	RP-HPLC	ELISA	RP-HPLC	ELISA	RP-HPLC	ELISA	RP-HPLC	ELISA
1	0.59	<5	2.68	5.85	1.96	7.81	0.78	<5
2	1.17	<5	5.36	10.58	3.93	12.36	1.94	6.16
3	2.35	11.98	21.42	31.02	7.86	23.14	3.88	15.36
4	5.87	32.47	32.13	45.67	15.71	43.08	7.77	31.42
5	11.74	74.61	-	-	23.6	70.6	15.54	>80
Linear fit	$y = 6.7186x - 5.0228$ $R^2 = 0.9971$		$y = 1.3309x + 2.7886$ $R^2 = 0.9992$		$y = 2.8716x + 0.9462$ $R^2 = 0.9946$		$y = 4.3065x - 1.87$ $R^2 = 0.9988$	

3.2.6 Discussion of the rye reference materials

Due to the complex structure and special properties, the correct quantification of gluten in food matrices faces numerous analytical challenges. The commonly used method to detect gluten in food is ELISA, but different ELISA tests may produce different results due to variations in calibration materials and Ab specificity. Additionally, most analytical methods only target the alcohol-soluble prolamin fraction of gluten, which is assumed to represent 50% of the total gluten content (Rzychon et al., 2017). However, the proportions of prolamin and glutelin fractions as well as the sub-fractions in cereals vary depending on the type and cultivar of the cereal. Studies have shown that rye secalins show different reactivities than wheat or barley gluten proteins when tested with various ELISA kits (Lexhaller et al., 2016). This leads to under- or overestimation of gluten using a RM not fit for the purpose. The PWG-gliadin, which is a gliadin isolate of 28 European common wheat cultivars, is used as an RM in sandwich ELISA for intact proteins (Van Eckert et al., 2006). So far, no RMs have been produced on the basis of rye. There is a need for a rye-based RM to improve food safety for CD patients. As there is not much research on rye proteins in terms of RM production, this work aimed to fill this research gap.

Within the framework of this thesis, 32 rye cultivars were investigated in order to select a range of varieties that are suitable for the production of rye RMs. For this purpose, the cultivars were first analyzed using various methods and compared with each other with regard to their composition. The results were statistically analyzed using a hierarchical cluster analysis to identify differences between the samples. The aim was to select a number of cultivars to comprehensively cover the diversity of rye proteins. The selected varieties were used to produce different protein isolates and to analyse their composition. In addition, these isolates were tested for their reactivity to the R5 mAb and thus for their suitability as potential rye-based RM in gluten detection with ELISA.

The in-depth protein characterization of 32 rye varieties revealed a mean protein composition of 40% ALGL, 23% γ-75k-secalins, 17% γ-40k-secalins, 14% ω-secalins and 6% HMW-secalins measured by RP-HPLC. This distribution of the proteins has already been reported in studies for rye (Gellrich et al., 2003; Schalk et al., 2018; Rani et al., 2021). However, there is no publication studying the gluten composition of such a large number of cultivars from seven different countries. With regard to the protein distribution within the samples, we also found that the separation of gluten proteins into prolamins and glutelins is not applicable for rye secalins. The reason for this is that the HMW-secalins and γ-75k-secalins are present in the prolamin and glutelin fractions after extraction. For this reason, the measurement

of the prolamins and the reduced prolamins with DTT is important for the calculation of the individual subfractions. The coelution of the highly aggregated fractions (HMW- and γ-75k-secalins) was reported in earlier studies on rye proteins (Gellrich et al., 2003; Schalk et al., 2018). There are structural differences in rye storage proteins compared to the wheat proteins. Wheat consists of two additional types (ω5- and α-gliadins) which are not present in rye. Additionally, Gellrich et al. (2003) reported that the γ-75k-secalins tend to form intermolecular disulfide bonds to other protein chains because of their unique cysteine residue in position 12 of the N-terminal sequence found in the two rye cultivars studied. This ability to form disulfide bonds and thus aggregates is similar to LMW subunits of wheat glutenins.

The analysis of 32 rye flours with RP-HPLC and ELISA showed the over-determination of the gluten content measured by two ELISA kits (R5 and G12) for all rye flours studied. The over-determination of gluten out of flours in ELISA is dependent on three important factors: the Ab reactivity, the conversion factor for calculating the content and the extraction method used. The mAb R5 was raised against rye secalins and is most specific towards the pentapeptide motifs QQPFP and QLPFP. It also reacts to the motifs LQPFP, QLPYP, QLPTF, QQSFP, QQTFP, PQPFP, QQPYP, QQQFP and QVQWP (Sorell et al., 1998; Valdés et al., 2003; Kahlenberg et al., 2006). It is reported that the detection of epitopes from gliadins, hordeins and secalins using the R5 is equally effective in the determination of wheat, barley and rye gluten (Valdés et al., 2003; Méndez et al., 2005). The reason is that the epitopes are repetitively present in many peptides derived from rye, barley and wheat storage proteins that are toxic or immunogenic for CD patients. However, recent studies have found differences in reactivity when comparing various protein types, revealing that rye displays a higher reactivity towards the R5 compared to wheat (Lexhaller et al., 2016, 2017). Consequently, when using PWG-gliadin as RM for rye samples, the R5 ELISA kit tends to overestimate the results.

The G12 mAb is raised against the highly immunotoxic 33-mer peptide of the α-gliadin protein and it recognizes the hexameric epitope QPQLPY within the 33-mer (Morón et al., 2008b). Other immunogenic peptides of wheat, rye and barley can be detected by G12 such as QPQQPY, QPQQPF, QPQLPF, QPQLPL and QPELPY (Real et al., 2014). Compared to the R5 target (QQPFP) the G12 hexamer targets occur much less frequently in gluten proteins (Morón et al., 2008b). A higher reactivity for rye prolamins compared to wheat gliadins has been observed for the G12 (Lexhaller et al., 2016). Since different secalin subfractions also have different compositions, differences in the reactivity of individual subfractions can be observed in both ELISA kits. The G12 mAb has the highest affinity towards ω- and γ-40k-secalins and the R5 towards ω- and γ-75k-secalins (Lexhaller et al., 2017). Thus, the

over-determination of rye proteins in R5 and G12 ELISA kits can be explained by the higher sensitivity of the Abs towards the secalins compared to wheat prolamins. For both Abs, the overestimation of rye secalins is therefore to be expected and it is in accordance to our findings. This conclusion strongly questions the use of only PWG-gliadin as RM.

The second major influential part for the over-determination is the prolamin/glutelin ratio. Since PWG-gliadin only covers the prolamin faction, the gluten content is estimated using a conversion factor of 2 assuming the prolamin/glutelin ratio to be one. This conversion factor is questioned to be valid (Wieser and Koehler, 2009). Our study shows an average prolamin/glutelin ratio of 4.4 ($\pm$0.8). Since rye contains relatively more prolamins compared to glutelins, the conversion factor cannot be used for rye-contaminated samples and is therefore not valid. The conversion factor has also been found to be inadequate for various types of wheat and other cereals. According to Wieser and Koehler (2009), the proportion of prolamins to glutelins in common wheat varied from 1.5 to 3.1. In barley the ratio ranged from 1.4 to 5, in rye from 6.3 to 8.2 and in einkorn from 4.9 to 13.9. The conversion factor is thus a source of error in the analysis of different cereals, not only rye. However, rye seems to show one of the highest prolamin to glutelin ratios among the grains. When using a rye-specific conversion factor on the basis of our study (1.2) the results may show lower values but still the gluten content is higher compared with the RP-HPLC results.

The extraction procedure of the samples prior to testing has a high influence on the results. The sandwich ELISA test systems use different extraction solutions. In R5 and G12 ELISA the extraction is based on the following chemicals: β-mercaptoethanol, guanidine hydrochloride and 80% ethanol (Amnuaycheewa et al., 2022). Using these extraction solutions leads to the extraction of prolamins and glutelins as well because of the reducing properties of β-mercaptoethanol. In our study we showed that rye glutelins are more reactive compared to PWG-gliadin in R5 ELISA. This was supported by other studies on reactivity (Lexhaller et al., 2016, 2017). The extraction with reducing agents will lead to the extraction of prolamins and glutelins of rye. This means that more peptides may react to the R5 mAb and in turn lead to the overestimation of gluten.

However, the reliability of the ELISA data is also impacted by the sample preparation of the rye flours. The determination of gluten out of rye flours needs a 10,000-fold dilution of the flours. It was shown that the sample preparation of rye flours seems to have a greater effect on the results compared to wheat. The differences were tested in the dilution (10,000-fold) of the flour with either liquid or solid dilution using a gluten-free matrix. The results indicate that for rye the liquid dilution resulted in lower gluten contents compared to the solid

dilution (Muskovics et al., 2023).

Furthermore, the protein composition varies from cultivar to cultivar. With the help of the HCA we have found that cultivars from the same harvest country are most likely in the same cluster, showing similarities in protein composition. The protein composition is highly influenced by genetic and environmental factors. The environmental factor contains not only the weather conditions but also the soil and growing conditions as well as fertilization (Wieser and Seilmeier, 1998; Daniel and Triboi, 2000; Johansson et al., 2003; Dubois et al., 2018). We found significant differences in protein content when comparing two harvest years of the same cultivar. To date there has been little research on the genetic and environmental effects on the secalin distribution in rye. One study showed that the variations in the protein content in rye cultivars were mainly due to genotype differences and to a lesser extent to differences among harvest years according to Hansen et al. (2004). However, the genetic factors seem to have the highest effect on the composition compared to the environmental factors, such as N fertilization regarding the expression of CD-active epitopes found in spelt cultivars, as shown by Dubois et al. (2018).

Seven rye cultivars were selected based on their high variability in gluten composition. We found that when these cultivars were mixed, the resulting mixture had a secalin distribution similar to the calculated mean. This similarity in secalin distribution was also observed when mixing the same cultivars from different harvest years. This means that both mixtures show very similar secalin compositions. The use of flour mixtures in producing RMs for ELISA test kits was investigated by Schall et al. (2020), who found that mixing flours reduced variability, a finding which was confirmed in the current study. However, due to incomplete information about the growing conditions and genetic characteristics of the rye cultivars, no definitive conclusions can be drawn regarding the effects of environmental and genetic factors on secalin distribution.

The isolation of gluten proteins for use as RM in ELISA has proven to be appropriate for PWG-gliadin when measuring wheat gluten. The modified Osborne fractionation was used as basis for the isolation of the gliadins (Van Eckert et al., 2006). In another major study, Schalk et al. (2017b) produced well characterized GPTs of rye, barley and wheat suitable as RMs for ELISAs, clinical assays or other studies on gluten proteins. Here again the modified Osborne fractionation was used for isolation. The present study aimed to produce four different secalin isolates. One isolate was produced aiming at the extraction of total gluten which includes both prolamin and glutelin fractions. Another isolate similar to the PWG-gliadin protocol, was designed specifically to extract the prolamin fraction of rye.

Additionally, two other isolates were produced, one isolating only the glutelin fraction and the other using acetonitrile/water with formic acid as the extraction solution. The four isolates differed significantly in yields and protein content. The highest yield was observed for the $PROL_{iso}$ (8.1%) followed by G_{iso} (5.2%) $AWEP_{iso}$ (2.2%). $AWEP_{iso}$ and $PROL_{iso}$ showed high crude protein contents of 84.6 and 83.7 g/100 g, respectively, followed by G_{iso} with 77.3 g/100 g, while $GLUT_{iso}$ showed the lowest crude protein content with 52.3 g/100 g. Only little is known about the yields and protein content when it comes to rye isolates using the described methods. PWG-gliadin showed a similar crude protein content (83.4 g/100 g) compared to the rye prolamins isolated in the current study (Van Eckert et al., 2006).

When comparing the flour mixture and the isolates slight changes can be observed for the M_w in SDS-PAGE and GP-HPLC. The change in composition may be due to losses during isolation in e.g. dialysis tubing and the extraction of large amounts of flours. The milling of the grains and the up-scaling of the laboratory scale are other reasons (Schall et al., 2020). In the SDS-PAGE gel, only very few ω-secalins were found in all isolates compared to the flour. This does not correspond to the results obtained by RP-HPLC. Overall, only small differences in the secalin composition were detected by RP-HPLC for all isolates compared with the corresponding flours. The isolation of total gluten showed the greatest similarity to the results of the analysis of the flour mixture. However, it has been shown that the isolation process can lead to changes in the composition. Nevertheless, no major differences in the composition of the flours and isolates could be detected in comparison.

The untargeted LC-MS/MS analysis of the isolates revealed different secalin distribution patterns for each isolate. From the protein groups found, gluten-specific ones were selected to determine the composition. Two of nine gluten protein groups were identified, W6AW92 and F4ZL28, which were also found in a previous study by Lexhaller et al. (2019a). One notable finding was the high presence of HMW-secalins in the GLUT isolate, compared to other isolates. Furthermore, $AWEP_{iso}$ and $PROL_{iso}$ showed similarities in their secalin distributions. Additionally, only very small amounts of ω-secalins were found similar to the SDS-PAGE findings. In terms of the SDS-PAGE, this could be due to the staining with Coomassie, which stains differently depending on the amino acid composition in the proteins. The Coomassie dye binds to the amino acids with basic groups via its sulfonylanions (Tal et al., 1985). Since ω-secalins contain fewer basic amino acids that are important for Coomassie colouring (lysine, arginine and histidine), the bands may be less intense in comparison. In contrast, the reduction of ω-secalins could not be shown in RP-HPLC analysis of the isolates. Possible reasons why hardly any ω-secalins were found in the LC-MS/MS analysis are, on the one hand, the quality of the databases and the use of specific digestive enzymes.

The number of protein groups identified by LC-MS/MS and the designation into gluten proteins in each study is not directly comparable due to differences in protein extraction protocols as well as enzymes used for digestion (Martínez-Esteso et al., 2017; Schalk et al., 2017a; Pasquali et al., 2019). The reliability of the results is dependent on the accuracy of the reference sequences in the databases used. In terms of rye proteins some of the entries are incomplete or inaccurately named and not reviewed. Therefore, the interpretation of the data should be viewed critically.

Within the identified protein groups immunoactive peptides in isolates generated by MaxQuant were searched for immunoactive peptides using three search procedures. The first procedure aimed to identify the protein fraction carrying epitopes recognized by the mAb R5, while the second search used the list of CD-relevant epitopes recognized by CD4+ T cells (Osman et al., 2001; Kahlenberg et al., 2006; Sollid et al., 2020). The third search looked for complete and partial matching sequence overlaps with previously known epitopes following the EFSA search strategy (Naegeli et al., 2017). The investigation of the peptides can help to better elucidate the reactivity in the R5 ELISA.

Four peptides were found to be potentially harmful, one was found to be recognized by the mAb R5 and one by CD4+ T cells. Compared to other studies, the number of the protein groups as well as the peptides found is lower. To date, not many studies have been performed to investigate the epitopes found in rye isolates. The number of protein groups assigned to gluten proteins in this study is also lower than those found in the literature for isolated rye protein types (Lexhaller et al., 2019a). As mentioned before the choice of enzyme used for proteomics studies on gluten can significantly impact the identification of immunoactive peptides. Therefore, different digestive enzymes such as chymotrypsin and thermolysin is crucial to make more precise statements about potential immunoreactivity.

The isolates were subjected to an assessment of their suitability for use in the R5 ELISA. The responsiveness of the isolates towards the R5 mAb was evaluated and the results showed the following order of reactivity: $PROL_{iso} > AWEP_{iso} > G_{iso} > GLUT_{iso}$. These findings were consistent with the existing literature on the topic (Lexhaller et al., 2016). Interestingly, $AWEP_{iso}$ exhibited similar M_w distributions and relative secalin distribution (as assessed using LC-MS/MS) to $PROL_{iso}$. This observation may be partly explained by the nature of the extraction solution used in both cases, which lacked reducing agents. As a result, mostly monomeric secalins were extracted. A more detailed investigation was conducted on the reactivity of rye secalin protein types towards the R5 mAb (Lexhaller et al., 2017). The ω-

type gliadins were found to exhibit the highest reactivity towards the R5 mAb (Valdés et al., 2003; Van Eckert et al., 2010; Lexhaller et al., 2017). The ω- and γ-75k-secalins showed the highest reactivity followed by γ-40- and HMW-secalins. Interestingly, the peptide to be potentially reactive towards the R5 ELISA found in the LC-MS/MS studies originates form the γ-40k-secalins. However, the outcomes obtained from the LC-MS/MS measurements must be used carefully for data interpretation. This is due to the inadequacy of databases, which lack revised, complete, or correctly named entries, especially for rye proteins.

3.3 Barley reference materials

The selection, characterization and production of the new barley RMs was done in a similar manner as explained for rye in chapter 3.2. In this chapter the flours of 35 barley cultivars were collected from different countries and characterized utilizing different analytical methods such as SDS-PAGE, RP-HPLC, GP-HPLC, ELISA, LC-MS/MS and nitrogen determination with the Dumas method. Based on the analytical data the aim was to identify the cultivars with the highest variability in gluten composition. The selected culitvars were used to produce barley RMs using different extraction and isolation methods. The barley gluten RMs were characterized and were compared in their gluten composition. Lastly, the R5 mAb were tested for its reactivity against the isolates. The results presented in section 3.3.1 and 3.3.2 have already been published in the Journal *Food Chemistry* (Xhaferaj et al., 2023b).

3.3.1 Barley flour characterization

The flours of 35 different barley cultivars were examined qualitatively and quantitatively regarding their protein content and composition. The methods used for the qualitative examination of the proteins were RP-HPLC and GP-HPLC. The chromatograms were examined visually in all 35 samples. The protein content and distribution of the samples was evaluated using four chromatograms including AL/GL, prolamins, red. prolamins and glutelins. The prolamin and glutelin fractions were separated into the hordein subfractions. An example of the RP-HPLC elution profiles with the integration ranges indicating different hordein fractions is provided in Figure 3.14. As expected, the visual examination of the individual elution profiles revealed differences in peak heights at different retention time ranges, indicating different protein distributions (see section 3.3.1.2). The elution profiles of the RP-HPLC measurements were compared to literature, showing similar elution profiles (Šimić et al., 2007; Gessendorfer et al., 2009; Schalk et al., 2017b; Huang et al., 2017). For the cultivar selection in this study all 35 barley cultivars from seven different countries of origin were considered.

A specific procedure was employed to select a smaller number of suitable cultivars for RM production. The selection was based on the differences in the results using statistical tools such as HCA and ANOVA as well as the differences in the countries of origin of the cultivars. Therefore, a set of quantitative data including moisture, crude fat, protein content and composition, gluten content, prolamin/glutelin ratio and ELISA response were evaluated statistically. The results of the in-depth characterization of the 35 barley flours are presented in Figure 3.14 an in the Tables 5.27-5.29.

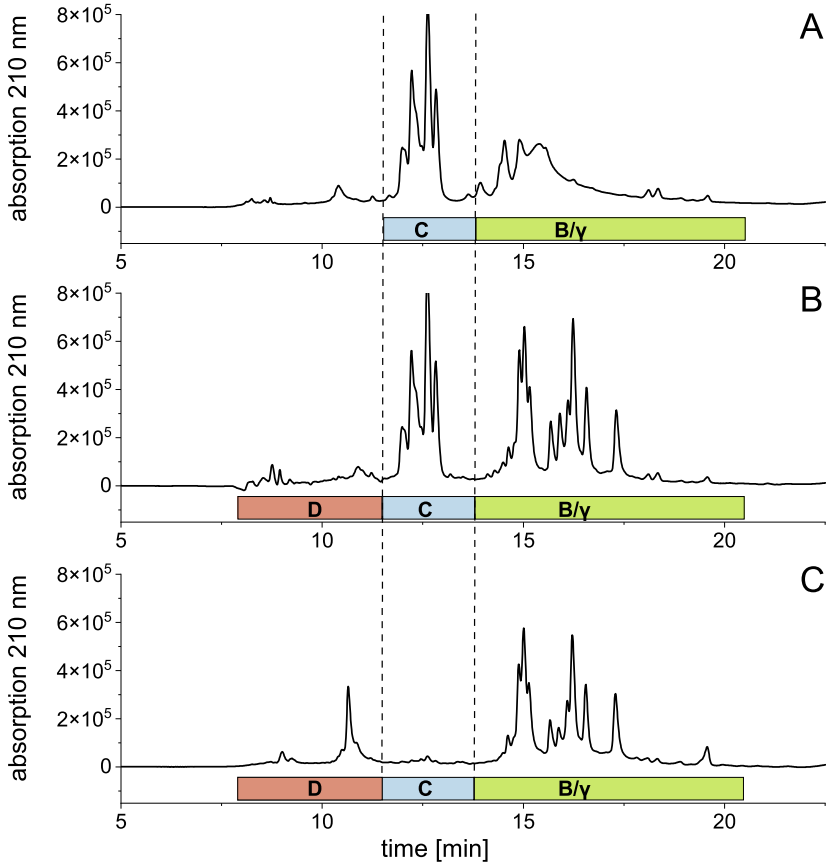

Figure 3.14: RP-HPLC profiles of the barley cultivar Pixel. A: Unreduced prolamins, B: Reduced prolamins, C: Glutelins. With the barley protein fractions C: C-hordeins; D: D-hordeins and B/γ: B/γ-hordeins. Modified from Xhaferaj et al. (2023b).

3.3.1.1 Moisture, fat and crude protein content

The moisture, fat and crude protein content of the flour samples were determined by the Research Group of Cereal Science and Food Quality (Budapest, Hungary).

The fat content of the barley flours ranged from 1.3 to 2.2 g/100 g with a mean fat content of 1.7 g/100 g, which is lower compared to literature with 3.3 to 6.1 g/100 g (Aprodu and Banu, 2017; Drakos et al., 2017). The flours had an average moisture content of 10.2 g/100 g (Tab.5.27). It was shown by Drakos et al. (2017) that the moisture content of the grains depends on the flour preparation such as milling. The 35 barley samples were milled all in the same manner (section 5.3.1). Compared to literature, commercially available barley flours had comparable moisture content such as 11.5 g/100 g and a barley flour mixture of three different cultivars resulted in 12.9 g/100 g moisture (Drakos et al., 2017; Schalk et al., 2018).

The protein content determined by Dumas ranged from 8.8 to 19.9 g/100 g with an average

value of 11.6 ± 2.0 g/100 g. The protein content measured by RP-HPLC (sum of ALGL, red. prolamins and glutelins) was lower and resulted in a mean of 9.6 ± 2.0 g/100 g (Fig. 3.15, Tab. 5.16). The difference in the protein content is due to the difference in the method used for the determination. The resulting protein contents of both methods correlated positively ($r = 0.98$). The findings are similar to earlier reports on barley, which showed a crude protein content ranging from 7.7 g/100 g to 15.1 g/100 g (Yu et al., 2017; Schalk et al., 2017b). The sample KOR_LAT19 had a significantly higher protein content of 18.3 g/100 g compared to the other samples (which ranged from 7.1 to 12.1 g/100 g). Therefore, the results for KOR_LAT19 are presented separately in the following. The high protein content is most likely influenced by the growing location and genetic potential since the same sample from the current harvest year 2022 (KOR_LAT22) showed comparably high protein content (Tab. 5.27). However, differences in protein content and composition are expected since the protein content is dependent on several factors including genetic variation, fertilization, harvest year and country of origin, in addition to the method of determination.

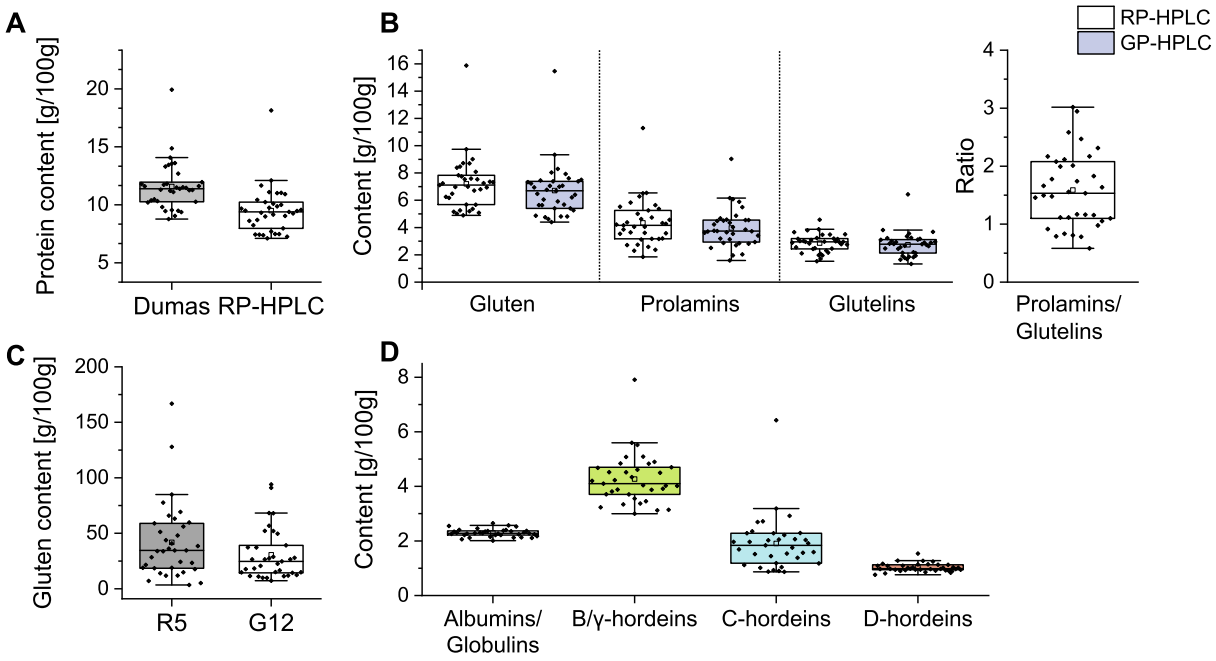

Figure 3.15: Boxplots showing the protein characterization of 35 barley cultivars. The box represents the 25th and 75th percentiles. The diamonds are the data points for each cultivar ($n = 35$). The small square in the box indicates the mean, the line the median. The whiskers indicate the upper (75th percentile) and lower (25th percentile) inner fence with a 1.5 interquartile range (whisker length determined by the outermost data point that falls within upper and lower inner fence). A: Protein content measured with Dumas and RP-HPLC. B: Gluten, prolamins, glutelins and the prolamin/glutelin ratio measured with RP-HPLC and GP-HPLC. C: Gluten content measured with R5 and G12 ELISA. D: Protein fractions measured with RP-HPLC. Modified from Xhaferaj et al. (2023b).

3.3.1.2 Gluten quantification with RP-HPLC

For gluten quantification the barley flours were extracted according to the modified Osborne fractionation (section 5.3.5.1). The resulting fractions (prolamins and glutelins) were analyzed with RP-HPLC for gluten quantification using PWG-gliadin as calibration standard. The gluten content of the 35 barley samples ranged from 4.7 to 9.3 g/100 g (15.9 g/100 g for KOR_LAT19) with a mean content of 7.2 $\pm$ 2.0 g/100 g (Fig. 3.15, Tab. 5.27). The chromatograms were then used to divide the different hordein fractions within the prolamins and glutelins according to the literature into the C-hordeins, D-hordeins and B/γ-hordeins (Šimić et al., 2007; Gessendorfer et al., 2009; Schalk et al., 2017b). The hordein distribution was determined by integration of the corresponding fractions as shown in Figure 3.14. The methods used for measuring the prolamin and glutelin fractions, as well as the hordein types, were similar to those used in a previous study by Schalk et al. (2017b). The elution profile did not clearly distinguish between the B- and γ-hordeins in the prolamin fraction, as shown in Figure 3.14, A. However, when the prolamins were reduced, a clearer separation of the B- and γ-hordeins was observed, as shown in Figures 3.14, B and C. Since the exact composition of each peak was unknown, the B- and γ-hordein fractions were treated as a single fraction (B/γ-hordeins). The main difference between the prolamin and the red. prolamin fraction is the clearer separation in B/γ-hordeins and an apparent peak representing the D-hordein fraction. Comparing the sum of both no significant difference in protein content was evident. Therefore, the red. prolamins were summed with the glutelins to determine the total hordein (gluten) content. The protein content of 35 barley flours was analyzed based on their integration ranges (Fig. 3.14) and found to vary within specific ranges for different hordein types. The examination resulted in 2.0-2.7 g/100 g for AL/GL, 0.8-1.5 g/100 g for D-hordeins, 0.4-2.3 g/100 g for C-hordeins and 3.0-7.9 g/100 g for B/γ-hordeins. The proportions of these hordeins in the overall protein content of the cultivars were found to be approximately 25% AL/GL, 11% D-hordeins, 19% C-hordeins and 45% B/γ-hordeins, with the B/γ-hordeins being the most abundant type, which is consistent with previous studies on hordeins (Gessendorfer et al., 2009; Schalk et al., 2017b).

3.3.1.3 Molecular weight distribution by GP-HPLC

The M_w distribution of the 35 flours measured by GP-HPLC was used to identify differences between the samples. The gluten content ranged from 4.4 to 9.3 g/100 g of flour (15.5 g/100 g for KOR_LAT19) (Tab. 5.27). A strong positive correlation ($r = 0.98$) was found between the GP-HPLC and RP-HPLC results in terms of gluten content. To characterize the samples, the GP-HPLC chromatograms were subdivided into four ranges to determine the M_w distribution within the prolamin, the red. prolamin and the glutelin fraction: (1) $>$ 66 kDa; (2) 66–29 kDa;

(3) 29–12.4 kDa; (4) $<$12.4 kDa (Tab. 5.29). On average, the prolamin fractions were distributed as 29.6% (1), 7.5% (2), 24.5% (3) and 38.4% (4), with one sample (PIX_FRA20) showing a significantly lower percentage of fraction (1) and the highest percentage of fraction (4) within the prolamin group. Compared to the prolamins, the distribution of the red. prolamin fractions differed due to the reduction with DTT, with fraction (1) being lower and fractions (3) and (4) being higher. The glutelin fractions had an average distribution of 11.4% (1), 8.3% (2), 29.7% (3) and 50.6% (4), with samples from Denmark having higher percentages in fraction (4) and lower percentages of the fractions (1), (2) and (3) compared to the other samples.

3.3.1.4 Gluten quantification with ELISA

The ELISA measurements were conducted by the Research Group of Cereal Science and Food Quality (Budapest, Hungary).

The R5 sandwich ELISA and the G12 sandwich ELISA, which are often used to quantify gluten, were used on the 35 barley samples (Tab. 5.27) (Méndez et al. 2005; Morón et al. 2008). With the exception of GKJ_HUN17, MVI_HUN17 and MOR_HUN17, which had R5 ELISA recoveries of 59.7%, 93.5% and 68.2% compared to RP-HPLC, respectively, the gluten content measured with the R5 kit was overestimated for the majority of the samples (up to a recovery of 1747.8%) compared to the RP-HPLC values. The gluten content assessed by the G12 and R5 ELISA was in a range from 7.3 to 94.0 g/100 g and 3.4 to 166.8 g/100 g, respectively (Tab. 5.27). The gluten content measured by the G12 kit was overestimated for all 35 samples showing RP-HPLC recovery ranges from 140.7 to 1043.7%. The correlations between the kits ($r = 0.52$) and the correlation between each kit to RP-HPLC (R5, $r = 0.53$; G12, $r = 0.53$) showed no correlation for both. The ELISA kits differ in their mAb specificity which is the main reason why the results do not correlate with each other. Additionally, both methods use different standards for calibration, which differs in reactivity depending on the grain (Lexhaller et al., 2016; Scherf, 2017; Yu et al., 2021; Amnuaycheewa et al., 2022; Xhaferaj et al., 2023a).

Both the variation in reactivity towards various grain proteins and the distribution of prolamins and glutelins are significant contributors to the overestimation of gluten by ELISA. Earlier studies have indicated that different grain species and cultivars have different ratios of prolamins to glutelins in their protein distribution (Wieser and Koehler, 2009). The duplication of prolamin content in gluten calculation using ELISA kits is based on the assumption that the prolamin/glutelin ratio is always one, as established by the Codex Alimentarius Commission (conversion factor = 2). However, this assumption should be viewed critically due to the varia-

tions not only between different grain species but also among cultivars within the same species.

The prolamin/glutelin ratio of the 35 barley samples ranged from 0.6 to 3.0 depending on the cultivar, with an average of 1.6 $\pm$ 0.6 (Table 5.26). The results show a clear difference in prolamin/glutelin ratio compared to the assumed value of one, which explains the overestimation of the gluten content. For instance, using a conversion factor of 1.6 for barley, which has a prolamin/glutelin ratio of 1.6, led to a reduction in overestimated gluten levels in ELISA tests. However, the values were still higher (R5: 41.9 to 34.0 g/100 g, G12: 30.7 to 24.9 g/100 g) than those obtained by RP-HPLC (4.9 to 15.9 g/100 g) due to differences in how R5 and G12 mAbs react to barley gluten. The overdetermination of gluten in barley with the R5 kit has already been demonstrated in several studies (Wieser and Koehler, 2009; Huang et al., 2016; Lexhaller et al., 2016; Huang et al., 2017; Amnuaycheewa et al., 2022). It could be shown that different hordein types, such as C-hordeins, show different reactivity towards the R5 ELISA (Huang et al., 2017). In addition, spiking barley flour to gluten-free oat flour, the prolamin concentration was overestimated 1.8 to 2.5 times with the gliadin standard (Huang et al., 2017).

Separating hordeins into prolamins and glutelins based solely on solubility (Osborne fractionation) is not applicable for hordeins, as shown in Figure 3.14, where there is no clear separation of B/γ-, C- and D-hordeins between the red. prolamins and glutelins. The same was observed for rye secalins (see section 3.2.1). While adjusting the conversion factor can improve gluten quantification, other factors like Ab specificity and using a barley-based RM are also important. A barley-based RM in combination with a suitable conversion factor also taking Ab specificity into account may improve the quantification of gluten.

3.3.2 Selection of representative barley cultivars

The chapter of cultivar selection shows the results gained from the selection procedure, with the inclusion of qualitative and quantitative data generated and shown in chapter 3.3.1. A typical RP-HPLC and GP-HPLC elution profile was the requirement for the selection. Based on the visual examination of the RP- and GP-HPLC chromatographic elution profiles, all 35 samples were considered for further selection as representative barley cultivars. The selection procedure based on the quantitative data aimed to select a small number of barley cultivars showing the most differences in quantitative data such as protein composition, gluten content and ELISA response (Tab. 5.27 to 5.29). For this reason, the statistical tool HCA was used as first criterion. This tool allows to identify similarities and differences in the results of the different cultivars.

The HCA resulted in the formation of 5 clusters (Tab. 5.3). Cluster C1 consisted of 20 different barley cultivars from seven countries, namely Austria (2, (number of cultivars)), Canada (2), Denmark (4), France (5), Germany (3), Hungary (2) and Latvia (2). All of the cultivars from France were present in cluster C1, which was attributed to their similar composition. The second cluster (C2) contained five cultivars (Canada (2), Germany (1) and Hungary (2)). In contrast, cluster C3 contained four cultivars, three of which were from Austria and one was from Germany. Cluster C4 consisted of four cultivars from Latvia (3) and Canada (1), while cluster C5 contained two cultivars, one from Hungary and one from Canada. The differences in the M_w distributions resulting from GP-HPLC measurements and the country of origin were used as further selection criteria for the representative cultivars. At least one sample was chosen from each cluster and country, with the exception of C1, which contained the most cultivars and had three cultivars selected (GKJ_HUN17, EVE_DEN20 and COC_FRA20). Additionally, JAK_GER20 (C2), EVE_AUS20 (C3), KOR_LAT19 (C4), CEL_CAN19 (C5) and PIX_FRA20, which showed significant differences in the M_w distribution of the prolamin and glutelin fractions compared to the others, were selected. In total, eight cultivars were chosen as representative cultivars for RM production (Tab. 3.8). The protein distribution and characterization of the flour mixture created based on the selection procedure is described in the following chapters.

Table 3.8: Content of protein and gluten fractions of the selected barley cultivars measured by RP-HPLC. With the barley protein fractions ALGL: albumins and globulins; D: D-hordeins; C: C-hordeins and B/γ: B/γ-hordeins. The values are given as means (n = 3), (g/100 g) and different capital letters indicate significant differences between the samples in each column (one-way ANOVA, Tukey's post hoc test, $p < 0.05$). Data already published in Xhaferaj et al. (2023b).

Samples	Protein[a]	Gluten[b]	Prolamins	Glutelins	AL/GL	D	C	B/γ	PROL/ GLUT[c]
				g/100 g					
PIX_FRA20	7.42 A	5.02 G	1.85 F	3.17 C	2.40 A	1.03 C	0.87 H	3.12 E	0.6
GKJ_HUN17	7.70 B	5.69 F	3.61 E	2.08 F	2.01 D	0.95 C	1.18 G	3.55 D	1.7
COC_FRA20	8.62 C	6.25 E	4.28 CD	1.97 F	2.37 AB	0.97 C	1.57 F	3.71 D	2.2
EVE_DEN20	9.04 D	6.80 D	3.65 E	3.15 C	2.24 C	1.02 C	1.76 E	4.02 C	1.2
JAK_GER20	10.00 F	7.54 C	4.05 D	3.49 B	2.46 A	1.25 B	2.06 D	4.23 C	1.2
CEL_CAN19	9.56 E	7.32 C	4.57 C	2.75 D	2.24 C	1.02 C	2.28 C	4.02 C	1.7
EVE_AUS20	11.10 G	8.72 B	6.28 B	2.43 E	2.38 AB	0.97 C	2.92 B	4.83 B	2.6
KOR_LAT19	18.14 G	15.87 A	11.29 A	4.57 A	2.27 BC	1.54 A	6.42 A	7.91 A	2.5
Mean	10.20	7.90	4.95	2.94	2.30	1.09	2.38	4.42	1.7

a Sum of reduced prolamins, glutelins, albumins and globulins measured by RP-HPLC

b Sum of reduced prolamins and glutelins measured by RP-HPLC

c Ratio of reduced prolamins and glutelins measured by RP-HPLC

3.3.2.1 Protein content and gluten composition

The eight selected cultivars and their flour mixture were compared in protein and gluten composition. The protein content measured by RP-HPLC ranged from 7.4 g/100 g (PIX_FRA20) to 18.4 g/100 g (KOR_LAT19) (Tab. 3.8). The highest gluten content was determined for KOR_LAT19 with 15.9 g/100 g, followed by EVE_AUS20 with 8.7 g/100 g. The AL/GL content, on the other hand, was similar across all samples, ranging from 2.0 to 2.5 g/100 g. The results indicated a strong correlation between the protein and gluten content ($r = 0.96$), which is consistent with the findings for all 35 cultivars. The prolamin content was higher than the glutelin content for all samples, except for PIX_FRA20, which had a prolamin/glutelin ratio of 0.6, while the others had ratios above one (Tab. 3.8).

The relative gluten composition of the eight selected samples followed the distribution pattern B/γ-hordeins > C-hordeins > D-hordeins, which was in accordance to the distribution of the 35 cultivars. It is noteworthy that PIX_FRA20 deviated from the expected trend as its D-hordein value (21%) was slightly higher than its C-hordein value (17%) and it had the highest overall percentage of B/γ-hordein (62%) (Fig. 3.7). On the other hand, KOR_LAT19 had the highest C-hordein percentage (40%), resulting in the lowest D- and B/γ-hordein percentages of 10% and 50%, respectively. The flour mixture (mixture of the eight selected flours in equal proportions) followed the hordein distribution of 18% D-hordeins, 29% C-hordeins and 53%

B/γ-hordeins. When comparing the flour mixture with the calculated means (mixture calc. in Fig. 3.7) both showed a similar hordein composition, considering the error bars. Within the group of selected samples, COC_FRA20, EVE_DEN20 and JAK_GER20 displayed comparable hordein distributions and CEL_CAN19 showed a hordein distribution that was similar to the calculated mixture (Fig. 3.7). This selection of samples demonstrates a significant degree of variability, which was the main aim in the selection process.

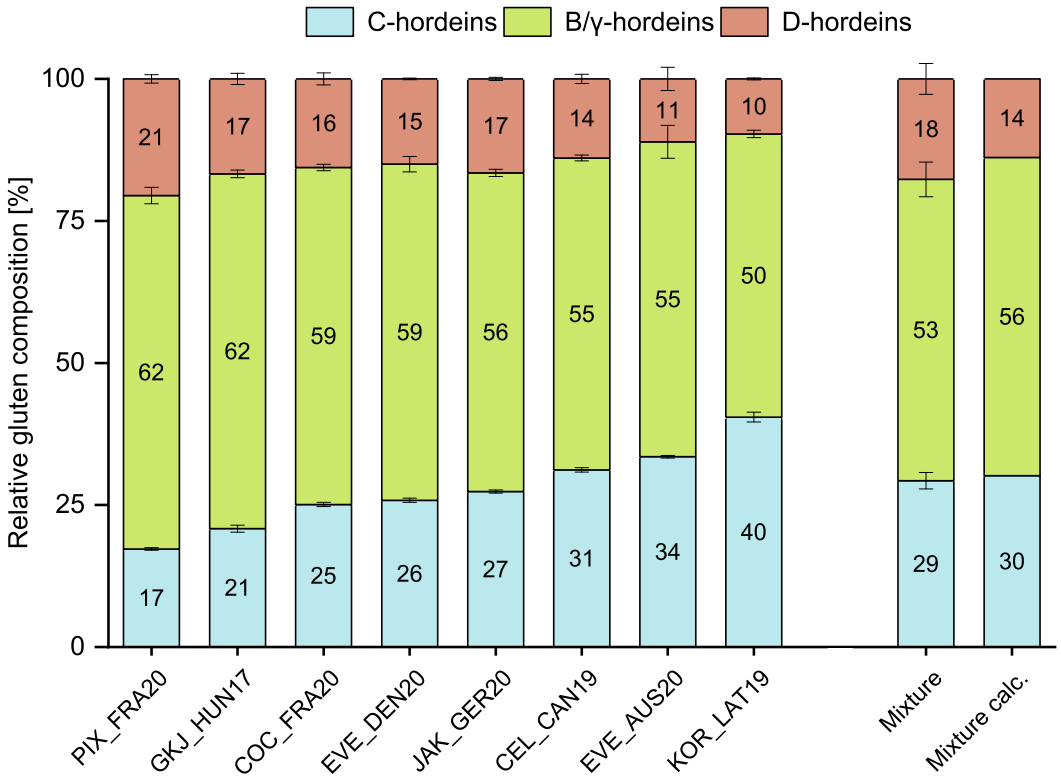

Figure 3.16: Relative gluten composition of selected barley cultivars and their mixture. The gluten composition was determined with RP-HPLC. The mixture consists of the flours of the selected eight cultivars in equal proportions. Mixture calc. is the calculated composition resulting from the mean values. Error bars indicate the standard deviations (n = 3). Modified from Xhaferaj et al. (2023b).

3.3.2.2 Molecular weight distribution by SDS-PAGE

For further characterization, the selected barley flours and their mixture were separated by their M_w using SDS-PAGE (Fig. 3.17). Specific band ranges were used to identify the hordeins such as 85–100 kDa (D-hordeins), 50–75 kDa (C-hordeins) and 30–50 kDa (B/γ-hordeins) (Schalk et al., 2018; Pont et al., 2020). Overall, a typical hordein distribution was observed for the eight cultivars as well as for the flour mixture which is consistent with previous research (Molina-Cano et al., 2001; Gessendorfer et al., 2009; Tanner et al., 2013; Schalk et al., 2018; Pont et al., 2020). The presence of D-hordeins was indicated by the

appearance of light bands at around 85 kDa in all samples, except for KOR_LAT19, which had a more prominent band located just below 85 kDa. In general, the bands for C-hordein and B/γ-hordein were more prominent in all samples. A band between 50 and 69 kDa, as well as a band below 60 kDa, were observed in all samples, indicating the presence of C-hordeins. In all samples two more prominent bands below 40 kDa and below 50 kDa were visible indicating B/γ-hordeins. The sample COC_FRA20 showed several more clearly separated bands below 50 kDa in the B/γ-hordein range, which was not seen in the other samples. More prominent bands around 40 kDa could be observed for three cultivars, EVE_DEN20, EVE_AUS20 and KOR_LAT19. The comparison of the individual sample distributions with the mixture showed that JAK_GER20 had a similar pattern, which is consistent with the relative gluten distribution (Fig. 3.17). The differences in the M_w distributions seen in the SDS-PAGE are due to protein polymorphisms between different cultivars (Echart-Almeida and Cavalli-Molina, 2000).

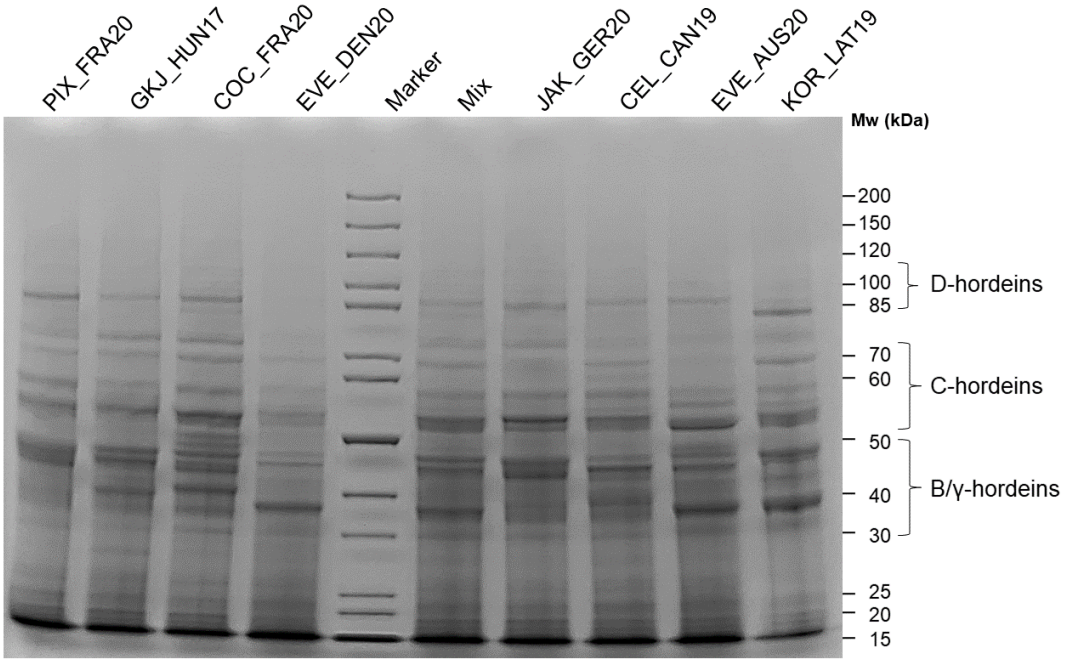

Figure 3.17: SDS-PAGE of 8 selected barley flours and their mixture. Mix: barley flour mixture, Mw: molecular weight. Modified from Xhaferaj et al. (2023b).

3.3.3 Isolation of barley hordeins

This chapter focuses on the production of isolates using a mixture of the seven chosen cultivars from a recent harvest (2022). The isolates were created by extracting hordeins from a flour mixture using different extraction procedures. It provides a comparison of the newly collected cultivars with the previous ones, as well as the protein content and yields of the four unique isolates. The results presented in this chapter are prepared for publication.

3.3.3.1 Influence of different harvest years

The cultivars selected in chapter 3.3.2 selected cultivars were grown in different harvest years (Tab. 3.2) and were investigated regarding the protein composition and M_w distribution. Since the gluten composition is highly influenced by environmental effects such as the soil conditions, weather and fertilization, differences in gluten composition are expected with different harvest years.

The hordein content measured by RP-HPLC of the eight samples of the first and second collection is shown in Figure 3.9. When comparing the gluten content, seven of the eight cultivars showed a significant difference between the first and the second collection, respectively (T-test, $p < 0.05$), except the cultivars EVE_AUS. It can be observed that the sample GKJ_HUN showed the greatest difference in gluten content with 5.7 g/100 g (harvest 2017) and 7.0 g/100 g (harvest 2022) (Fig. 3.9, Tab. 5.30 - 5.33). For most of the samples differences in the absolute and relative amounts of protein fractions (AL/GL, D-, C- and B/γ-hordeins) were determined statistically (Tab. 5.30). The gluten content can be seen in Figure 3.18, where the higher gluten amounts in KOR_LAT samples can also be observed. This shows the good comparability of the samples and the existing genetic similarity. For GKJ_HUN, EVE_AUS, KOR_LAT, COC_FRA and CEL_CAN there were no significant differences in D-hordein content when comparing the harvest years. This fraction, however, showed the highest standard deviations which leads to more similar results in the T-test. When comparing the mixtures of the different harvest years there is a significant difference in gluten content and composition. The hordein distribution in the mixtures from the first and second collection is the following 13.7% and 12.8% for the D-hordeins, 29.9% and 25.3% for the C-hordeins and 56.4% and 61.9% for B/γ-hordeins, respectively.

Table 3.9: Sample information of the first and the second collection of barley cultivars.

Cultivar	Sample code	Geographical origin	Year of collection
Celebration	CEL_CAN19	Canada	2019
	CEL_CAN22		2022
Coccinel	COC_FRA20	France	2020
	COC_FRA22		2022
Evelina	EVE_AUS20	Austria	2020
	EVE_AUS22		2022
Evergreen	EVE_DEN20	Denmark	2020
	EVE_DEN22		2022
GK Judy	GKJ_HUN17	Hungary	2017
	GKJ_HUN22		2022
Jakobus	JAK_GER20	Germany	2020
	JAK_GER22		2022
Kornelija	KOR_LAT19	Latvia	2019
	KOR_LAT22		2022
Pixel	PIX_FRA20	France	2020
	PIX_FRA22		2022

The hordein distribution when calculating the mean of both flour mixtures (mixture calc. 1 (earlier harvest)) and 2 (harvest 2022) showed comparable hordein distributions to the flour mixtures. The gluten content of the mixture calc. 1 and mixture calc. 2 lay in between both with 6.9 g/100 g (mixture 2) and 8.2 g/100 g (mixture 1). They show similar hordein distributions. The mean values indicate comparable hordein contents, which shows that flour mixtures reduce the effects of the year of cultivation on the gluten distribution. Our findings are in accordance to the literature showing that by mixing the flours the variability is reduced (Schall et al., 2020). The determined in chapter 3.3.1.2 hordein distribution pattern of B/γ-hordeins $>$ C-hordeins $>$ D-hordeins could be observed for all samples from the first and second collection as well as for the flour mixtures.

The M_w for the prolamins, red. prolamins and glutelins of each sample were analyzed and compared. The analysis was conducted within four M_w ranges for each fraction: (1) $>$ 66 kDa, (2) 66-29 kDa, (3) 29-12.4 kDa and (4) $<$ 12.4 kDa. The resulting percentages with the statistical ANOVA results are shown in Table 5.31. Within the prolamins, PIX_FRA showed the lowest percentage with 15.5% in range (1) for both harvest years compared to the others which were in the range of 19.6 - 28.8%. The percentages in range (2) were consistent with M_w percentages between 6.4 and 9.6%. The highest percentage resulted for M_w range (4), with the highest being 54.3% for PIX_FRA20. The M_w distribution in all ranges of PIX_FRA was quite comparable for both harvest years. For all of the samples from the second

collection the percentages for (3) were higher compared to the first collection. This trend for (3) could be observed for the glutelins as well. In all M_w ranges the sample EVE_DEN20 stood out the most with the lowest percentage for (1) and the highest percentages in (4) with 49.5% (prolamins), 78.1% (red. prolamins) and 67.0% (glutelins). No trends were apparent, when comparing the different harvest years (Tab. 5.31).

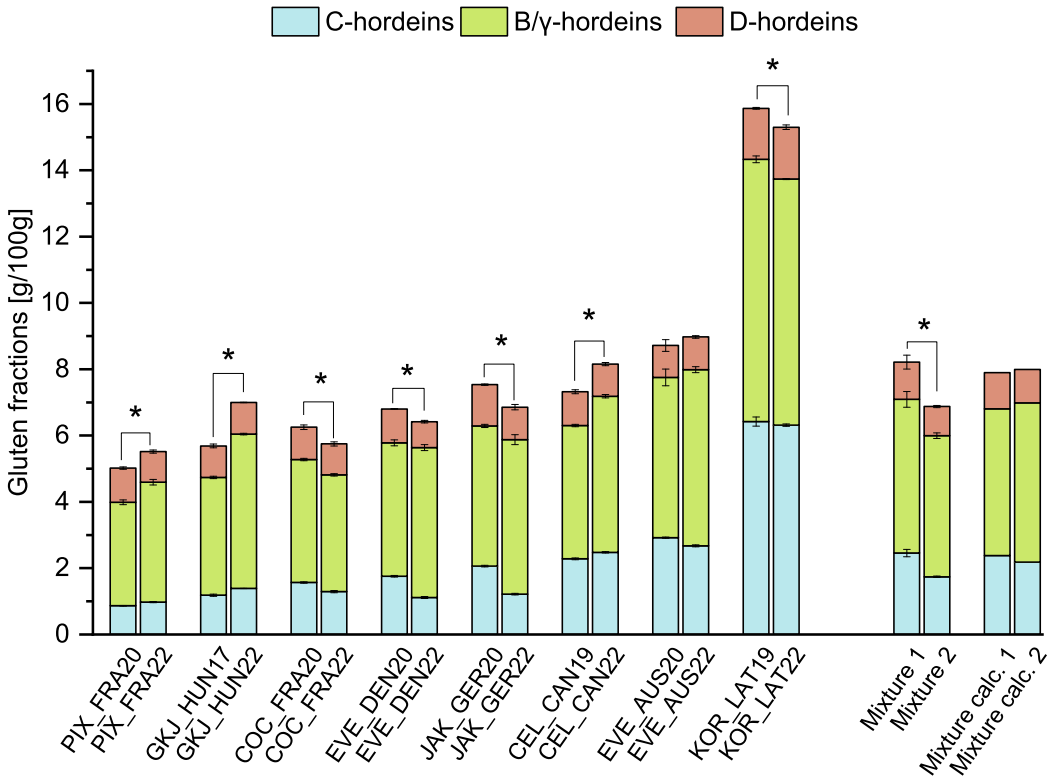

Figure 3.18: Hordein content of the eight selected barley cultivars and their mixture of different harvest years. The mixture consists of the flours of the selected eight varieties in equal proportions. Mixture calc. is the calculated composition resulting from the mean values. Error bars indicate the standard deviations ($n = 3$). The * indicate the significant difference between the gluten content (Two sample T-test, $p < 0.05$).

In general, the findings indicate notable variations in gluten content, distribution and M_w among samples obtained from different harvest years. Since the gluten composition can be impacted by several factors, including the harvest year the results were expected. Environmental factors like temperature, rainfall and sunlight during the growing period, as well as farming methods like pest management, fertilization and irrigation, can also influence gluten composition (Johansson et al., 2003).

3.3.3.2 Yield and protein content

The production of four distinct hordein isolates involved milling and extracting a mixture of eight selected barley grains (equal proportions). For gluten isolation, the selected barley cultivars from the second collection were chosen (see section 3.3.2). The isolate production was mostly based on Schalk et al. (2017b) and Batey et al. (1991), with a few modifications (section 5.3.4). The isolation process resulted in four different hordein isolates, which included prolamins ($PROL_{iso}$), glutelins ($GLUT_{iso}$), gluten (G_{iso}) and acetonitrile water-extractable proteins ($AWEP_{iso}$) (Tab. 3.10).

Prior to extraction and isolation, 100 g of flour mixture was defatted, which resulted in a weight reduction of 5%. The reduction in weight might be attributed to losses incurred during the defatting process, such as losses through the filter. The protein isolation of the flour mixture yielded 5.5% for G_{iso}, 2.9% for $AWEP_{iso}$, 3.9% for $GLUT_{iso}$ and 6.8% $PROL_{iso}$ (based on g isolate/ 100 g flour) (Tab. 3.10).

Table 3.10: Yield and protein content of the four barley isolates measured by Dumas and RP-HPLC. Values are given as means ($n = 3$). Yields are given as % and indicates g isolate/100 g flour. The crude protein and protein content measured by RP-HPLC are given as g protein/100 g isolate. The different capital letters indicate significant differences between the samples in each column (one-way ANOVA, Tukey's post hoc test, $p < 0.05$). SD: standard deviation, PC: protein content, hor: hordeins.

Sample	Yield	Crude protein		RP-HPLC (g/100 g)							
	%	g/100 g	SD	PC		C-hor		B-hor		B/γ-hor	
Gluten	5.5	74.2	0.5	95.9	B	8.4	A	24.5	C	63.0	A
AWEP	2.9	84.0	0.3	110.8	A	7.4	A	42.0	A	61.4	A
Prolamins	6.8	81.1	0.0	95.4	B	7.5	A	34.2	B	53.6	B
Glutelins	3.9	64.0	0.2	56.2	C	5.8	A	4.8	D	45.6	C

In order to estimate the protein content of the isolates, the protein content was measured and compared by RP-HPLC and the Dumas method. RP-HPLC analysis of the flour mixture indicated a prolamin and glutelin content of 4.2 g/100 g and 0.7 g/100 g, respectively, which leads to a total gluten content of 4.9 g/100 g (Tab.5.19). Additionally, the Dumas measurement of the flour mixture resulted in a crude protein content of 10.8%. Among the four isolates produced, $AWEP_{iso}$ (84.0%) and $PROL_{iso}$ (81.1%) had the highest crude protein content, followed by G_{iso} (74.2%) and $GLUT_{iso}$ (64.0%). The protein content measured by RP-HPLC was found to be higher than that measured by the Dumas method, except for $GLUT_{iso}$ (Tab. 3.10). This trend was also observed in the rye isolates described

in section 3.2.3.2. The protein content measured by RP-HPLC of $AWEP_{iso}$ was found to be too high at 110.8%. The reason for this overestimation could be attributed to the use of PWG-gliadin in the RP-HPLC calibration and differences in measurement principles of Dumas and RP-HPLC. The extraction of AWEP led to overestimation in RP-HPLC for rye protein isolation as well (section 3.2.3.2). However, the crude protein content determined by Dumas was considered to be more suitable for subsequent concentration calculations, particularly for gluten concentration measurements uisng ELISA.

Hydrolyzed prolamins from a barley flour were produced by Gessendorfer et al. (2009) in a similar manner. The crude protein content of the flour used for isolation was 5.9% and the hordein yield was 2.7 g/100 g of flour. The hordein isolate showed a crude protein content of 83.3% (Gessendorfer et al., 2009). In our study, the crude protein content (10.8%) and $PROL_{iso}$ content (4.18 g/100 g) of the flour mixture showed relatively similar yields compared to the literature, taking the protein content into account. The prolamin protein content (83.3%) is similar to our result (81.1%).

Regarding the glutelin fraction, little is known about yields out of barley flours in general. The protein content of the isolates produced is of great importance and should be taken into account, especially when using them for calibration in analytical methods. Compared to the PWG-gliadin (89.4%), $PROL_{iso}$ (81.8%) and $AWEP_{iso}$ (84.0%) are in a similar range of the crude protein content. It has proven to be more difficult to fully extract the gluten and glutelin proteins (G_{iso}, 74.2% and $GLUT_{iso}$, 56.2%). The extraction of $GLUT_{iso}$ and G_{iso} was done at higher temperatures and with a reducing agent. In the course of the extraction, other flour components could have been co-extracted, which would result in lower purity.

3.3.4 Hordein isolate characterization

The isolates $PROL_{iso}$, $GLUT_{iso}$, G_{iso} and $AWEP_{iso}$ were characterized further by SDS-PAGE, RP-HPLC, GP-HPLC and LC-MS/MS. The characterization was focused on the amount and distribution of the secalin fractions within the different isolates. An epitope search was performed and the reactivity of the isolates was tested against the R5 mAb ELISA kit. The results presented in this chapter are prepared for publication.

3.3.4.1 Molecular weight distribution by SDS-PAGE

The SDS-PAGE was used to further examine the isolates. The gel displayed bands for both the isolates and the flour mixture. The isolates showed a higher level of purity as indicated by the more distinct bands for the D-, C- and B/γ-hordeins. Conversely, the flour mixture showed more bands overall. The most prominent bands were found in the B/γ region (30 to 50 kDa), with two particularly strong bands at 47 kDa and 37 kDa and several weaker bands in between. In contrast, the C-hordein region only had faint bands, with all samples showing a slight band at 70 kDa, whcih was weakest in $GLUT_{iso}$. In general, the C-hordein region below 60 kDa does not tend to have bands, but rather areas that are slightly highlighted. The area between 50 and 55 kDa was more abundant in $PROL_{iso}$, G_{iso} and $AWEP_{iso}$. Among the isolates, the strongest band in the D-hordein range (85 to 110 kDa) was seen in G_{iso} and $GLUT_{iso}$, while $PROL_{iso}$ and $AWEP_{iso}$ only had a slight band between 85 and 100 kDa. It is also expected that $GLUT_{iso}$ and G_{iso} contain higher proportions of D-hordein compared to the other fractions.

Comparing the bad pattern of the isolates with the GPT results published by Schalk et al. (2017a), a comparable band pattern was observed for the B/γ-hordeins. The relatively light bands for C-hordeins in the isolates are not in accordance with the amounts found for the C-hordeins using RP-HPLC (Tab 3.10). However, when comparing the gel with literature, it was shown that the C-hordeins in general showed lighter bands compared to the B/γ-hordeins (Qi et al., 2006; Gessendorfer et al., 2009; Rallabhandi et al., 2015). The relatively lighter bands were observed for the homologous protein fraction of ω-secalins of the rye isolates as well. This could be due to the staining with Coomassie blue, which stains differently depending on the amino acid composition in the proteins (Tal et al., 1985).

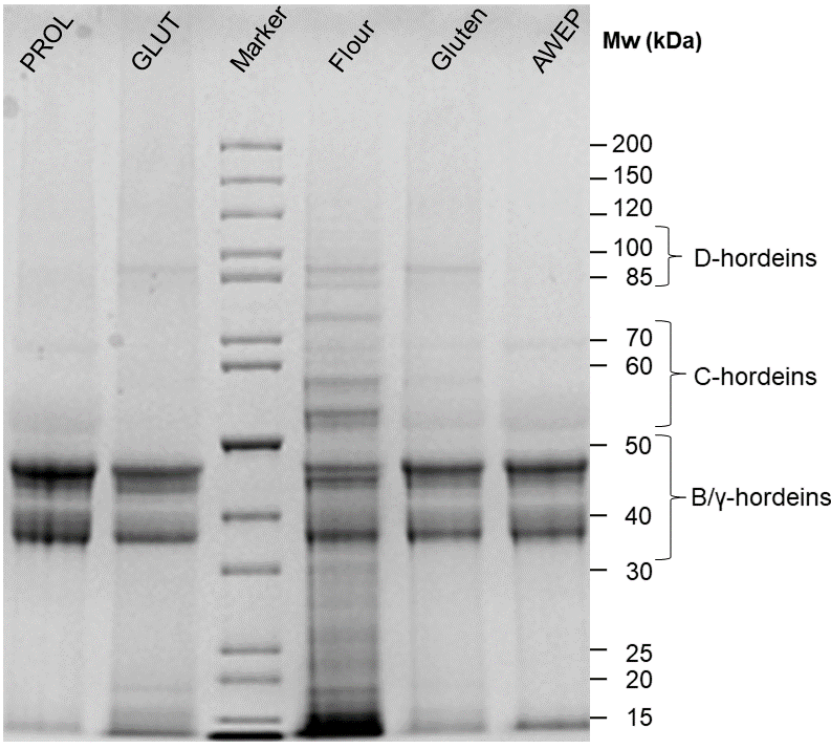

Figure 3.19: SDS-PAGE gel of the four isolates produced from the barley flour mixture. Mw: molecular weight.

3.3.4.2 Molecular weight distribution by GP-HPLC

The M_w of the four different isolates and the flour mixture measured by GP-HPLC were compared. The percentages found in different M_w ranges (>66 kDa (1) , 29-66 kDa (2), 12.4-29 kDa (3) and <12.4 kDa (4)) and the ANOVA (capital letters indicating significant differences between the samples in each column) are shown in (Tab. 3.11).

Within range (1) similar percentages were found for $PROL_{iso}$ and $PROL_{flour}$ as well as $AWEP_{iso}$ fractions ranging from 34.2 to 36.4%. The red. $PROL_{iso}$ and red. $PROL_{flour}$ showed significantly comparable percentages in range (1). The percentages in G_{iso}, $GLUT_{iso}$ and $GLUT_{flour}$ ranged from 10.2 to 17.9% for the M_w range (1). The smallest percentages were determined in M_w range (2) ranging from 3.1 to 9.1%. When comparing the different gluten fractions in the M_w range (3), it is apparent that most similarities occurred between the complementary gluten fractions such as $PROL_{flour}$ and $PROL_{iso}$ and $GLUT_{flour}$ and $GLUT_{iso}$. Within the isolates, the AWEP and the PROL fractions showed statistical similarities in M_w. The similarities are due to the lack of a reducing agent in both extraction solvents. The results indicate a comparable M_w distribution measured by GP-HPLC. This also shows that the loss of proteins during isolation is low and that the quality of the

isolate is suitable and representative for the flour. Loss of the sample or specific protein fraction also results from production on a larger scale (Van Eckert et al., 2006; Schall et al., 2020).

Table 3.11: The relative molecular weight distribution with GP-HPLC categorized for the four barley isolates (red. prolamins resulted after reduction of the prolamins fraction with DTT prior to measurement) and the barley flour mixtures according to four different molecular weight ranges. Values are given as means (n = 3) and percentages (%) referring to the relative percentages of the isolates in relation to the measured AUC at 210 nm by GP-HPLC. The different capital letters indicate significant differences between the samples in each column (one-way ANOVA, Tukey's post hoc test, $p < 0.05$).

Samples		> 66 kDa		29-66 kDa		12.4-29 kDa		< 12.4 kDa	
		%							
Isolates	Gluten	10.2	CD	5.3	D	46.8	B	37.7	B
	AWEP	34.2	A	6.0	C	33.8	DE	25.9	CD
	Prolamins	36.4	A	7.8	B	31.9	EF	23.9	D
	red. Prolamins	4.7	DE	5.1	D	52.9	A	37.3	B
	Glutelins	17.9	B	6.2	C	39.1	C	36.8	B
Flour mixture	Prolamins	35.1	A	6.2	C	30.1	F	28.6	C
	red. prolamins	2.2	E	3.1	E	49.5	AB	45.3	A
	Glutelins	15.0	BC	9.3	A	36.6	CD	39.1	B

3.3.4.3 Characterization with RP-HPLC

For further hordein characterization the hordein distribution of the four produced isolates and their corresponding flours were measured by RP-HPLC. The isolates were produced using different extraction solvents and procedures. These were used on the flour mixture as well. The comparison of the hordein distribution reveals information about the quality of the isolate procedure, as well as the losses during the lab-scale production. The comparison of the relative hordein distribution of the flours and the isolates are presented and compared with the results of the modified Osborne fractionation in Figure 3.20.

Significant differences were observed when comparing the D-hordein fraction of the isolates with the flours (Tab. 5.32). The isolates showed higher D-hordein percentages in the characterization, which resulted in 9% for G_{iso} and 14% for G_{flour}, in 7% for $AWEP_{iso}$ and 11% for $AWEP_{flour}$, as well as 8% for $PROL_{iso}$ and 11% for $PROL_{flour}$ and 10% for $GLUT_{iso}$ and 20% for $GLUT_{flour}$. As expected, the highest D-hordein contents were found in the GLUT samples, in both the isolates and flours.The C-hordein fraction also showed differences in composition in the ANOVA statistics, but these were not as pronounced as for the D-hordeins.

For the C-hordeins, comparable results were found for all extraction methods compared to the corresponding flours. The AWEP and PROL samples had higher C-hordein percentages (35 and 38%) than the G_{iso} and G_{flour} with 23% and 26%, respectively. Furthermore, the lowest content of C-hordeins was found in the $GLUT_{iso}$ and $GLUT_{flour}$. The isolates and flours had comparable percentages regarding the B/γ-hordein fraction. Similarly high B/γ-hordein contents were found for $AWEP_{iso}$ and $PROL_{iso}$ with 55% and 56%, respectively, which is in accordance with the flour. The B/γ content was higher in the isolates than in the flours in all four extraction methods. The highest B/γ percentages were found in the GLUT, followed by G and the lowest in the PROL and AWEP samples.

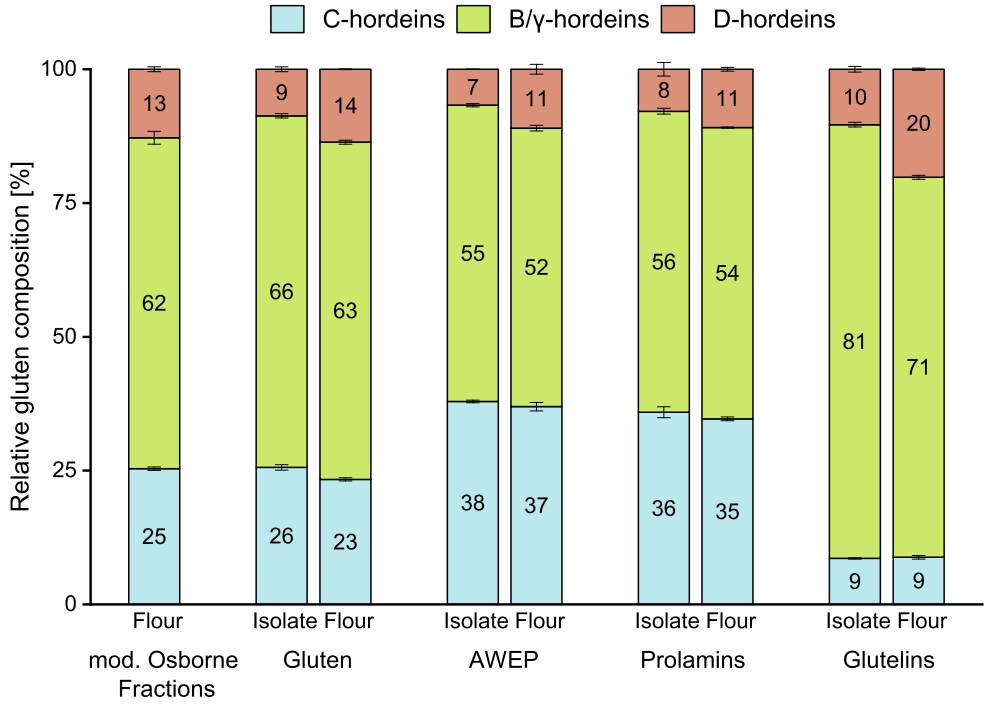

Figure 3.20: Comparison of the gluten composition between the lab-scale isolates and the corresponding characterized flours. Different extraction methods were used such as 60% ethanol for the prolamins and buffer solution with reducing agent for the glutelins (out of the prolamin residue) and for the total gluten extraction as well as the acetonitrile water extractable proteins (AWEP). Error bars indicate the standard deviations (n = 3).

The extraction according to the modified Osborne fractionation (section 5.3.5.1) of the flours resulted in 13% D-hordeins, 25% C-hordeins and 62% B/γ-hordeins. The gluten extraction method (G_{iso} and G_{flour}) showed the most similar hordein distribution to the results gained by the modified Osborne fractionation. Similar results were found in preliminary studies on the extraction methods (not shown). The extraction using the buffer only (after AL/GL extraction) leads to similar results and saves time in the lab since this method omits one

extraction step (60% EtOH). The extraction with the extraction buffer after AL/GL extraction is recommended in future hordein studies with RP-HPLC.

Overall, the results show similar and comparable hordein distributions in flours and isolates. This shows the quality of the isolation method also on the laboratory scale. When using different isolates, the composition must always be taken into account. The isolate which shows the most similar results in the hordein distribution to the corresponding flour, represents a general hordein composition. However, the selection of the RM for calibration strongly depends on the extraction method used for the food samples.

3.3.4.4 Characterization by untargeted LC-MS/MS

The goal of the untargeted LC-MS/MS characterization was to find the proteins and peptides with the highest response in the LC-MS/MS system. To achieve this, the isolates were reduced, alkylated and digested with trypsin before measurement (section 5.3.8.1). The data were processed and compared with MaxQuant and the UniprotKB database. The identification of peptides was based on a reference database of the tribe *Triticeae*, which contains 557477 protein entries (Uniprot FASTA-file 23.02.2023). More information on the MaxQuant settings can be found in chapter 5.3.8.3.

The search led to the identification of 396 protein groups, of which 20 were labelled as gluten proteins. Gluten proteins were identified by a fasta header search for keywords such as "gluten," "gliadin," "glutenin", "prolamin", "glutelin", "hordein" and "secalin". Among these groups, 42 were fragments, 17 were uncharacterised proteins and 1,246 peptides were identified across all samples.

The assignment of the protein groups to the hordein fractions (C-, D-, B and γ-hordeins) was performed using the fasta header and the homology of the amino acid sequence. Only protein groups derived from *Hordeum vulgare* and *Triticum aestivum* species were considered as appropriate for the selection. Since the entries in the databases do not always have the correct designation and the proteins are similar in their sequence, the proteins not derived from *Hordeum vulgare* were compared using the BLAST tool available from the UniProtKB webpage to find comparable protein groups. The identification of the protein groups is carried out using the Andromeda algorithm (probability-based), which calculates scores to each protein groups (Tyanova et al., 2016a). The higher the score is, the higher is the probability of the protein group being in the sample. Therefore, only protein groups with a score >20 were considered for relative quantification (Tab. 3.12). The relative quantification of the protein groups of the isolates was performed using the Intensity Based

Absolute Quantification (iBAQ) algorithm (Tyanova et al., 2016b). It estimates the relative abundance of the proteins within each sample. For comparison, a total sum normalization of iBAQ protein group intensities between samples was performed.

Table 3.12: Hordein protein groups identified by untargeted LC-MS/MS. The data was processed using MAXQuant and UniProt database using the reference database of the tribe *Triticeae*, which contains 557,477 protein entries (Uniprot FASTA-file 23.02.2023). The protein groups were then selected using own selection criteria.

UniProt KB accession	Species	UniProt Name	Score	iBAQ	Peptides
		C-hordeins			
I6TEV8	*Hordeum vulgare subsp. vulgare*	C hordein	7.91	3.1×10^7	1
		B-hordeins			
I6TMW4	*Hordeum vulgare*	B3 hordein	259.04	4.7×10^9	6
P06470	*Hordeum vulgare*	B1-hordein	214.51	6.7×10^9	4
I6TEV5	*Hordeum vulgare*	B3 hordein	21.91	6.4×10^6	5
		γ-hordeins			
P80198	*Hordeum vulgare*	Gamma-hordein-3	164.09	1.3×10^9	8
A0A8I6WAD5	*Hordeum vulgare subsp. vulgare*	AAI domain-containing protein	124.15	1.1×10^9	10
BLAST search: P17990 Gamma-hordein-1 (identity: 98.4%)					
E5KZT6	*Triticum aestivum*	75k gamma secalin	28.83	1.6×10^8	3
BLAST search: P17990 Gamma-hordein-1 (identity: 58.2%)					
I6TMV6	*Hordeum vulgare*	Gamma 1 hordein	23.22	7.0×10^8	9
		D-hordeins			
Q40054	*Hordeum vulgare*	D hordein	268.10	4.2×10^9	10

Only one protein group (I6TEV8) with one identified peptide could be assigned to the C-hordeins (Tab. 3.12). Although the score (7.91) of this protein group was less than 20 and did not meet the selection criteria, the protein was still chosen for the C-hordeins, because no other protein could be found to be assigned to the C-hordeins. Only one protein group was identified for the D-hordeins (Q40054), which is the protein group with the highest score and 10 identified peptides (score = 268.1). For the B-hordeins, 3 protein groups (I6TMW4, P06470 and I6TEV5) were identified, two of which showed the highest iBAQ intensity (Tab. 3.12). For the γ-hordeins four protein groups were identified. P80198 and A0A8I6WAD5 showed relatively high scores and high numbers of peptides identified with 8 and 10, respectively. For A0A8I6WAD5 the main protein was an AAI domain-containing protein but since it showed a high score and high number of identified peptides it was

subjected to the BLAST tool resulting in a 98.4% γ-hordein identity. Since E5KZT6 resulted as γ-75k-secalin, the BLAST tool was used to identify similar proteins. The BLAST resulted in a 58.2% similarity to γ-hordeins. Therefore it was assigned to the γ-hordeins.

The iBAQ of the selected protein groups (Tab. 3.12) was used to calculate the relative hordein distributions using a total sum normalization of iBAQ protein group intensities between samples (Fig. 3.21). The proportion of C-hordeins in all samples resulted in less than 0.01%, which is why they are not listed in the following order of distribution. The hordein distribution of G_{iso}, $AWEP_{iso}$ and $PROL_{iso}$ showed similar percentages ranging from 63.4-64.2 % B-hordeins, 16.0-22.2% γ-hordeins and 14.1-19.6% D-hordeins. The $GLUT_{iso}$ contained 46.4% B-hordeins, 14.1% γ-hordeins and 39.5% D-hordeins.

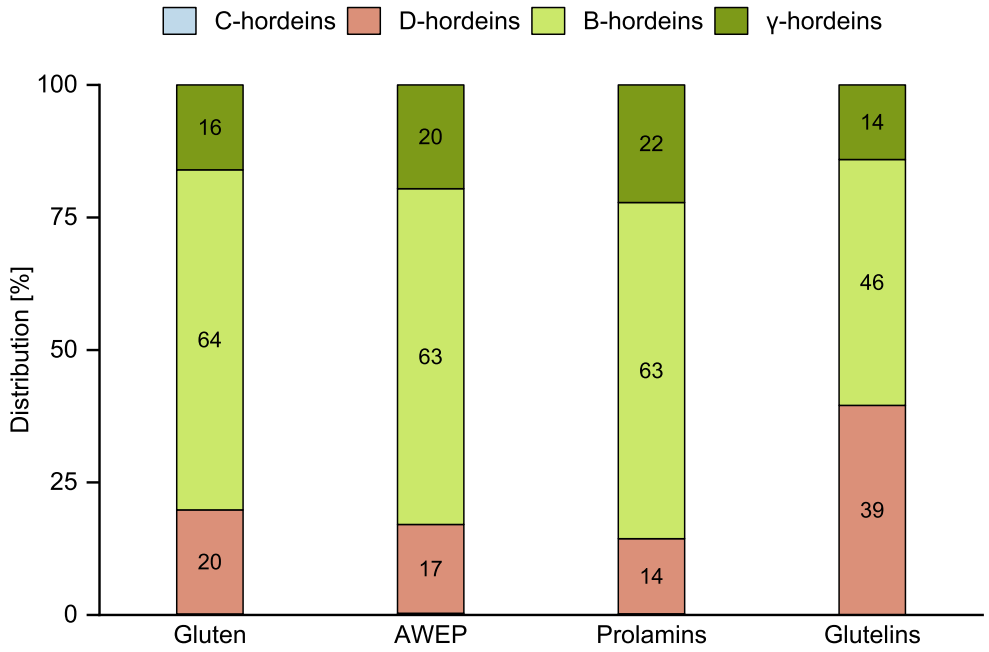

Figure 3.21: Relative hordein composition of four isolates identified by undirected LC-MS/MS. The relative quantification of the protein groups of the isolates was performed using the Intensity Based Absolute Quantification (iBAQ) algorithm. AWEP: acetonitrile water extractable proteins.

Protein groups I6TMW4, I6TEV5, P06470 and P80198 were also identified in the study of GPTs of wheat, rye and barley by Lexhaller et al. (2019) using LC-MS/MS (Lexhaller et al., 2019a). The protein group I6TMW4 was identified in B-, C- and γ-hordein protein types. It was the highest ranked protein group for the γ-hordeins in the study of Lexhaller et al. (2019a). Within the B-hordeins the protein group I6TMW4 was listed as the third highest detected protein group and showed a high number of identified proteins (102) (Lexhaller et al., 2019a). In our study the I6TMW4 was assigned to the B-hordeins according to the UniProt name (B3-hordein) and showed one of the highest iBAQ values. The protein

I6TEV5 (UniProt name: B3 Hordein) was found in the wheat GPT of the ω1,2-gliadins. The B1-hordein protein group, identified as P06470, was among the most prominent proteins identified in B-hordein GPT, but was also identified in D-, B-hordeins and ω1,2-gliadins. In other studies P06470 could be identified in purified proteins from barley and in beer samples. Another protein group P80198 was found in the γ-hordein-GPT and had a high number of peptides, similar to our findings. This protein group is found in beer and in wort as well (Colgrave et al., 2012, 2014).

However, LC-MS/MS investigation of hordein is only partially useful for characterization. Although some protein groups from our study were also identified in other studies, only one C-hordein was identified with a low score. From the RP-HPLC studies, however, a high amount of C-hordeins was found. The UniProtKB database may not provide adequate analysis of barley proteins for various reasons. The database does not include all barley proteins since some hordeins have not been characterized yet or added to the database. Furthermore, there may be errors in the naming of proteins that have highly similar sequences. As a result, it is necessary to approach the interpretation of the data with caution, considering that the quality of the results is based on the reliability of the database. Another reason is the probably insufficient digestion of C-hordeins with the digestive enzyme trypsin. Therefore, other enzymes for the digestion of gluten should also be investigated.

3.3.4.5 Identification of celiac disease-active peptides

The LC-MS/MS data processed using the MaxQuant software resulted in a list of 847 identified peptides throughout the four isolates. To identify the immunoactive peptides out of the 847 identified peptides and their major proteins, three search procedures were used. The first strategy involved searching for epitopes that can be recognized by the mAb R5 (Osman et al., 2001; Kahlenberg et al., 2006). The second strategy was based on the search for CD-relevant epitopes recognized by CD4+ T cells (Sollid et al., 2020) (Tab. 5.25). The third strategy involved searching for complete and partial matching sequence overlaps with previously known epitopes using the search strategy of the European Food Safety Authority (EFSA). Therefore, the peptide sequences were searched using the sequence Q-X1-P-X2, where X1 can represent L, Q, F, S or E and X2 can represent Y, F, A, V or Q. The matching peptides were identified as potentially harmful (Naegeli et al., 2017). The epitopes searched in the three strategies are listed in Table 5.25.

The search resulted in the identification of 23 peptides of which 13 peptides were found within the previously selected protein groups (section 3.2.4.4). Out of the 13 peptides associated with gluten proteins, no sequences were found to be recognized by CD4+ T cells. Three

sequences were found to be potentially recognized by the mAb R5 and ten were potentially harmful (Tab. 3.13). One peptide was found within the protein group of the C-hordein I6TEV8. Within the B-hordein protein groups three peptides were found for I6TMW4 and two were found for P06470 and I6TEV5. Within the γ-hordeins one peptide was found within P80198 and IT6MV6 whereas for A0A816WAD5 two peptides were found. One of the peptides within the γ-hordeins carries an epitope (QQPYP) that would likely be recognised by the R5 ELISA. Within the D-hordein protein group one peptide was found to carry a potentially harmful epitope.

Table 3.13: Potential immunoactive peptide sequences. The search procedure to identify immunactive epitopes within peptides identified by LC-MS/MS uses the following strategies: R5 mAb: The epitopes recognized by the R5 mAb ELISA (Osman et al., 2001; Kahlenberg et al., 2006; Amnuaycheewa et al., 2022), immunoactive peptides recognized by CD4+ T cells (Sollid et al., 2020) and potentially harmful epitopes selected based on the EFSA search strategy (Q-X1-P-X2; X1 = L, Q, F, S, E; X2 = Y, F, A, V, Q) (Naegeli et al., 2017). hor: hordeins; p. i.:potentially immunoreactive.

	Protein groups	**Sequence**	**Characteristics**
C-hor	I6TEV8	SQMLQQSSCHVLQQQCC**QIPE**QLR	p. i.
B-hor	I6TMW4	SQMLQQSSCYVLQQQCCQ**QLPQ**IPEQFR	p. i.
		PFPS**QQPFP**QQPPFWQQQPILSK	R5 ELISA epitope
		IARSQMLQQSSCHVLQQQCCQ**QLPQ**IPEQFRHEAIR	p. i.
	P06470	YPEQP**QQPFP**WQQPTIQLYLQQQLNPCK	R5 ELISA epitope
		IARSQMLQQSSCHVLQEQCCQ**QLPQ**IPEQFR	p. i.
	I6TEV5	QGVQIVQ**QQPQ**PQEVGQCVLVQGRDIVQPQQLAQMEAIR	p. i.
		QGVQIVQ**QQPQ**PQEVGQCVLVQGR	p. i.
γ-hor	P80198	DYLASLGA**QLPA**AAAGAK	p. i.
	A0A8I6WAD5	PFGQYQQPLT**QQPYP**QQQPLAQQQPSIEEQHQLNLCK	R5 ELISA epitope
		SQMLQQSSCHVLQQQCCQ**QLPQ**IPEQLRHEAVR	p. i.
	I6TMV6	SQMLQQSSCHVLQEQCCQ**QLPQ**IPEQFR	p. i.
D-hor	Q40054	IARSQMLQQSSCYVLQQQCCQ**QLPQ**IPEQFRHEAVR	p. i.

LC-MS/MS analysis of hordeins or peptides have been mostly used for the investigation of hordeins in malt products such as beer and soy sauce (Colgrave et al., 2012). The hordeins in such samples are highly hydrolyzed, which is why the R5 mAb cannot accurately quantify the gluten (Thompson and Méndez, 2008). Potential immunoactive peptide sequences of 60 commercially available beers have been reported by Colgrave et al. (2014). They identified 15 peptide fragments that share significant homology with immunoactive epitopes. In our experiment no sequences were found to be recognized by CD4+ T cells. There are several factors

influencing the number of identified peptides such as the enzyme used for digestion. The number of peptides identified depends on the selection of the enzyme used for digestion (Manfredi et al., 2015). A higher number and diverse peptides are expected to be found when using different enzymes for digestion such as chymotrypsin, thermolysin and pepsin (Manfredi et al., 2015; Colgrave et al., 2017a). In our study, at least one peptide containing at least one potentially harmful epitope was found in each protein group. These results, when using trypsin as enzyme, show that the peptides found are comparably long (18-40 amino acids) and that a study with different digestive enzymes is necessary for a more accurate analysis of hordeins.

3.3.5 New barley reference materials and ELISA responses

This chapter, similar to 3.2.5, presents a comparison of the reactivity of the R5 mAb to different barley isolates using the R5 sandwich ELISA according to Lexhaller et al. (2016). The results presented in this chapter are prepared for publication.

The sample preparation of the isolates was conducted in a similar manner as for the rye isolates (see section 3.2.5). To evaluate the reactivity of the isolates and the test kit standards the resulting absorbances were plotted on the same graph (Fig. 3.22). The prolamin, glutelin and gluten contents were determined using linear approximations (Tab. 3.14). The strength of the sensitivities between the isolates to the R5 mAb can be determined by the slope of the linear equation (Tab. 3.14).

The lowest reactivity was found for $GLUT_{iso}$ (slope$= 0.1627$, $R^2 = 0.9446$). The reactivity of the R5 mAb against $GLUT_{iso}$ was lower than the calibration standard. G_{iso}, $PROL_{iso}$ and $AWEP_{iso}$ showed slopes higher than 1 indicating greater reactivity of the R5 mAb against these isolates compared to the calibration standard. The isolates $AWEP_{iso}$ and $PROL_{iso}$ showed a relatively similar curve in Figure 3.22. According to Table 3.14, the $AWEP_{iso}$ (slope $= 5.5878$, $R^2 = 0.9995$) showed a slightly higher reactivity than $PROL_{iso}$ (slope $= 4.4056$, $R^2 = 0.9824$). The reactivity of G_{iso} (slope $= 5.5878$, $R^2 = 0.9995$) was between the highly reactive $PROL_{iso}$ and $AWEP_{iso}$ and the calibration standard. The reactivity of the isolates can be sorted in the following sequence with decreasing reactivity: $AWEP_{iso} \geq PROL_{iso} > G_{iso} > GLUT_{iso}$.

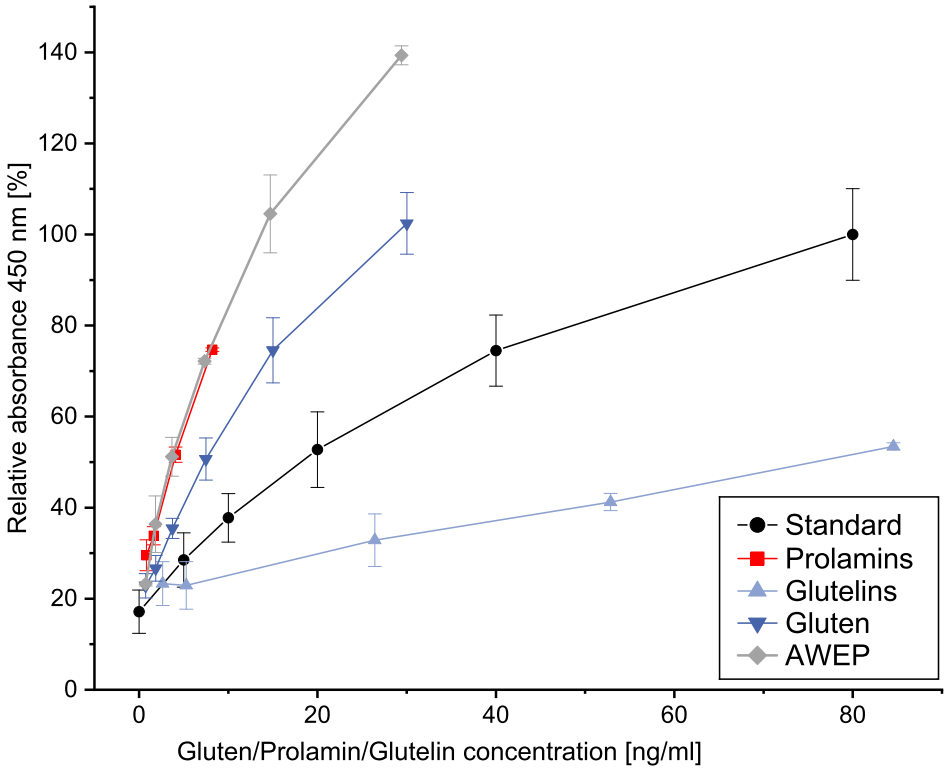

Figure 3.22: ELISA absorbances (λ = 450 nm) of the test kit standard, the prolamin, glutelin, gluten and acetonitrile water extractable proteins (AWEP) isolates of barley as a function of the concentrations quantified by RP-HPLC. Error bars indicate the minimum and the maximum value measured.

Table 3.14: Comparison of gluten reactivity between R5 ELISA and RP-HPLC. Linear fits parameters indicate the reactivity between the different barley protein isolates.

Calibration	Prolamins		Glutelins		Gluten		AWEP	
	ng/ml							
	RP-HPLC	ELISA	RP-HPLC	ELISA	RP-HPLC	ELISA	RP-HPLC	ELISA
1	0.41	< 5	2.6	< 5	0.8	< 5	0.7	5.0
2	0.82	< 5	5.3	< 5	1.9	5.49	1.8	10.8
3	1.64	5.51	26.4	5.73	3.8	10.23	3.7	22.0
4	4.10	15.15	52.8	10.41	7.5	21.58	7.4	41.8
5	8.21	36.53	84.6	17.46	15.0	44.73	14.7	> 80
6	-	-	-	-	30.0	> 80	29.4	> 80
Linear fit	y = 4.4056x - 0.7273 $R^2 = 0.9824$		y = 0.1627x + 2.7732 $R^2 = 0.9446$		y = 2.6462x + 1.8546 $R^2 = 0.9961$		y = 5.5878x + 0.8951 $R^2 = 0.9995$	

Previous research has studied the reactivity of rye, wheat and barley prolamins and glutelins on different ELISA test kits (Lexhaller et al., 2016). The findings showed that prolamin fractions of wheat, rye and barley exhibited higher reactivity than glutelins. Specifically, the

R5 ELISA test showed a higher reactivity for barley prolamins than for glutelins, likely due to the strong recognition of the QQPFP epitope by the R5 mAb.

The distribution of hordeins was evaluated in chapter 3.3.4.4. One protein group (Q40054) for D-hordeins was identified and it does not contain the QQPFP epitope in its amino acid sequence, according to UniProt. Given that D-hordeins make up to 39% of the $GLUT_{iso}$, it is not surprising that this isolate showed the lowest reactivity. A study conducted by Huang et al. (2017) investigated the reactivity of isolated C-, B- and D-hordein protein types against the R5 mAb. The results of the study showed that the reactivity of D-, C- and B-hordeins against the R5 mAb varied widely, with the order of reactivity being C-hordeins $>$ gliadin standard $>$ B-hordeins $>$ D-hordeins. The study also indicated that the proportion of C-hordeins is critical for accurate prolamin quantification because this fraction is mainly recognized by the R5 mAb (Huang et al., 2017). The protein group for C-hordeins (I6TEV8) contains 10 times the QQPFP sequence, according to UniProt. Besides the protein group E5KZT6 (13 times QQPFP), I6TEV8 showed the highest number of QQPFP epitopes compared to the other protein groups identified (below 4 times QQPFP). However, since the LC-MS/MS study did not identify the C-hordein fraction in high amounts, no conclusions could be drawn for the isolates.

3.3.6 Discussion of the barley reference materials

For the selection of the flours to produce barley-specific RMs the proteins of 35 different barley cultivars were characterized in-depth. Within the 35 barley flours from 7 different countries we found a relative hordein distribution of the following sequence: B/γ-hordeins > C-hordeins > D-hordeins. This sequence is in consistence with the values reported in previous literature (Šimić et al., 2007; Gessendorfer et al., 2009; Tanner et al., 2013; Huang et al., 2017; Schalk et al., 2017b; Pont et al., 2020). Prior to analysis the grain proteins were separated based on the Osborne fractionation. This separation is based on the solubility of the individual protein fractions into the two gluten fractions, the prolamins and glutelins. We found that in contrast to wheat gluten proteins, the distinction between prolamins and glutelins cannot be clearly confirmed in barley gluten proteins. More specifically, we found a minor part of the D-hordeins and large parts of the B-hordeins already present in the prolamin fraction by RP-HPLC. B- and D-hordeins occur as polymers connected by disulfide bonds and are expected to be found in majority in the glutelin fraction. Furthermore, the difficulty to clearly separate the B- and γ-hordeins in chromatography and SDS-PAGE has also been observed in the literature (Schalk et al., 2017b). The reason for this is the ability of the monomeric γ-hordeins to form disulfide bonds and create polymers with the B-hordeins which are mostly in majority polymeric fractions (Shewry and Tatham, 1990). Overall it is recommended to use the single protein types for defining the hordeins and not the prolamin and glutelin fractions.

The hordein distribution within the grain proteins is mainly influenced by genetic and environmental factors such as weather, harvest year and soil treatment (Echart-Almeida and Cavalli-Molina, 2000; Molina-Cano et al., 2001). In our study several cultivars out of seven countries were compared in protein distribution by RP-HPLC and GP-HPLC. We showed that more similarities were found in cultivars from the same country of origin. One of the samples showed a dissimilarity in the relative composition of hordeins compared to the other cultivars, with lower amounts of C-hordeins and higher amounts of D-hordeins. A major contributing factor to this variation in hordein distribution is the availability of nitrogen and sulphur in the soil. It was demonstrated that the levels of nitrogen have an impact on the ratio of B- and C-hordein composition (Molina-Cano et al., 2001). Differences in composition could be due to the differences in the capacity of the plant to take up and utilize sulphur, leading to differences in B- and C-hordein ratios. More sulphur in the soil may lead to higher B-hordein amounts which are rich in sulfur, while C-hordeins are poor in sulfur. The study by Molina-Cano et al. (2001) shows that the interplay of genetic and environmental influences is important in the distribution of proteins in barley. Unfortunately, it is not possible to make a precise statement about the individual factors in our collection, as the information about

the genetic and environmental effects of cultivation are not available. The only statement we can make from our data is the difference in composition in different harvest years of specific cultivars.

Out of the 35 cultivars eight cultivars were selected with a wide range of protein composition for RM production. The selection procedure was based on using statistical analysis and selection criteria, considering the protein composition and countries of origin (Tab. 5.30). The comparison of two harvest years of the eight selected cultivars showed notable differences in protein composition and M_w distribution, but the trends in protein content showed comparable amounts. However, the environmental factor seems to have a great impact on protein composition (Johansson et al., 2003). The eight cultivars were mixed and compared to the mixture of eight cultivars from a different harvest year. Mixing the flours is hypothesised to compensate the environmental effects on the protein composition (Schall et al., 2020). Statistically the cultivar mixtures (eight cultivars in each mixture) of each harvest year showed significant differences in protein content, but they showed considerable similarities to the calculated means of each mixture. The differences could be due to the different mixing and homogenization technique used for the mixtures. In the first collection the flours were mixed in equal proportions. It was different in the second batch, where a larger amount of each grain was mixed together before being milled, which may explain the differences in composition. The differences may occur due to of the improper homogenization when using flours only. The calculated mean, however, showed comparable protein contents and protein distribution. The mixture of the second batch was used for the barley RM production.

The ELISA is a widely used method for the quantification of gluten. However, this method is not without limitations, leading to different results with different ELISA test kits used for gluten determination. The use of different mAbs with different specificities, different RMs and extraction procedures leads to differences in results. Moreover, it has been observed that when testing barley-based foods, ELISA methods tend to over-determine the gluten content (Lexhaller et al., 2016, 2017). One reason are the structural differences of gluten proteins in different grains and therefore differences in reactivity to specific antibodies. The R5 is most specific towards the pentapeptide motifs QQPFP and QLPFP and the G12 mAb recognizes mainly the hexameric epitope QPQLPY (Sorell et al., 1998; Morón et al., 2008b).

Similar to previous studies, we found that the commonly used R5 and G12 sandwich ELISAs tend to overestimate the gluten content in the 35 barley flours studied. The structural differences between the gluten proteins of wheat and those of barley are the reason for the different affinity to the same Abs. They are also the reason why the calibration with

PWG-gliadin is not sufficient. Differences in the extraction of different homologous fractions to wheat have also been observed in the present study. Furthermore, in R5 ELISA the prolamin fraction is assumed to be 50% of the gluten content (Wieser and Koehler, 2009; Rzychon et al., 2017). The prolamin/glutelin ratio of barley samples ranged from 0.6 to 3.0, with an average of 1.6 $\pm$ 0.6, which explains the overestimation of gluten levels. The use of the conversion factor of 2 for the calculation has already been criticized in other studies (Wieser and Koehler, 2009; Kanerva et al., 2006). We showed that adjusting the conversion factor can improve gluten quantification but the results tend to be still higher. The use of a grain-specific factor is not always useful because it would require knowledge of the potential contamination, since ELISA test systems are used for the determination of traces of gluten in gluten-free labelled foods.

Grain-specific RMs have been proposed for the determination of hordeins in ELISA systems before (Kanerva et al., 2006; Huang et al., 2017). Huang et al. (2017) proposed a C-hordein RM for the determination of barley-contaminated oats. Since barley is the most common contaminant in oats, this standard would provide better performance compared to the PWG-gliadin (Koerner et al., 2011). This RM could also be used for hordein determination in purified barley starch, malt and malt extracts. The reactivity of GPTs in different ELISA test systems has been investigated by Lexhaller et al. (2017). The highest affinities in R5 ELISA have been shown for ω1,2-gliadins, C-hordeins, ω-secalins and γ-75k-secalins. Slightly different reactivities have been found for the G12 mAb that was most sensitive to ω1,2-gliadins, ω-secalins and C-hordeins. It was shown that the alcohol soluble fractions showed the highest sensitivity in both kits and this was found in other literature as well (Valdés et al., 2003; Van Eckert et al., 2010; Lexhaller et al., 2017). Since the R5 mAb was raised against the alcohol soluble fractions of the secalins this is not surprising. Our results are in accordance to the higher reactivity of the ethanol soluble fractions ($PROL_{iso}$) in the R5 ELISA.

The PWG-gliadin RM is an isolate which is of high purity, shows good solubility and high stability which are the reasons why it is used as a calibration standard for the R5 sandwich ELISA. We chose to produce species-specific isolates as new RMs. The isolation of hordeins from the flour mixture resulted in four different barley fractions: $PROL_{iso}$, $GLUT_{iso}$, G_{iso} and $AWEP_{iso}$. We showed that the isolation of the different fractions was possible on a laboratory scale. The yields were highest for $PROL_{iso}$ (6.8%) followed by G_{iso} (5.5%). These fractions also showed similar purities to the PWG-gliadin (Van Eckert et al., 2006). The yields of $AWEP_{iso}$ (2.9%) and $GLUT_{iso}$ (3.9%) were the lowest. The protein content was overestimated for $AWEP_{iso}$, which is due to the higher protein contents because of the higher total peak areas measured by RP-HPLC at 210 nm of $AWEP_{iso}$. Contaminations may be

an explanation for this findings since at 210 nm, other substances can also absorb, resulting in higher areas. Residues of the extraction agents, such as salts (residues from AL/GL extraction), can lead to contamination of the samples by containing substances that absorb at 210 nm (Anthis and Clore, 2013). The other isolates were produced in the same manner and this conclusion would mean that these were overestimated as well. However, since there is no study investigating $AWEP_{iso}$ and $GLUT_{iso}$ isolates no comparison can be made. Furthermore, we found that the isolation leads to similar relative hordein distributions when comparing the flours with the isolates. The D-hordein fraction, however, seems to be lower for all isolates. The loss of D-hordeins and the low yields of the $GLUT_{iso}$ indicate the more difficult extraction of the polymeric fractions. The most similarities in hordein distribution when comparing flour with the isolate were found for G_{iso}. It represents best the general hordein composition according to the modified Osborne fractionation. $AWEP_{iso}$ and $PROL_{iso}$ showed the most similarities in protein and M_w distribution. This may be due to the lack of reducing agents for both extraction methods, resulting in the extraction of monomeric proteins only.

The protein composition of the isolates was investigated by untargeted LC-MS/MS. We found eight protein groups that were assigned to be gluten proteins. Four of these have already been identified in studies on barley and barley products such as beer (Colgrave et al., 2012, 2014; Lexhaller et al., 2019a). A study by Lexhaller et al. (2019a) examined the protein groups found for individual GPTs. The assignment to the specific GPTs was for the most parts in accordance with our results (in terms of protein groups). Many protein groups were also found in different fractions such as P06470 which was present in B-hordeins, D-hordeins and ω1,2-gliadins. In our study only one protein group could be assigned to the C-hordeins. This C-hordein protein group was found in a very low concentration relative to the others by LS-MS/MS, leading to low percentages for all isolates. The relative composition of $AWEP_{iso}$ and $PROL_{iso}$ showed the following sequence: B-hordeins $>$ γ-hordeins $>$ D-hordeins. For $GLUT_{iso}$ and G_{iso}, higher values were found for the D-hordeins. These results are in contradiction with the RP-HPLC and ELISA results, indicating the limitations of the method used one of which is the database. More specifically, the plant protein databases such as UniProt are essential for proper identification of proteins and peptides and barley hordeins have yet to be characterised or added to the database. In addition, naming errors could occur for proteins with very similar sequences. Therefore, the interpretation of the data should be done with caution, as the reliability of the results depends on the accuracy of the database (Alves et al., 2019). An additional source of error could be the sample preparation which consists of many steps such as the extraction, the digestion and the cleanup using SPE. The loss during sample preparation has not been studied in the current investigation.

Out of 13, we found ten peptides that were characterized as potentially harmful and three that have epitopes that can be recognized by the R5 mAb based on the search strategy of the EFSA and studies on the R5 mAb (Osman et al., 2001; Kahlenberg et al., 2006; Naegeli et al., 2017). Most peptides were identified within the B-hordeins followed by γ-hordeins. The potentially harmful peptides are specific amino acid motifs with the potential to bind to HLA-DQ2.5 and stimulate gluten-specific T cells which are targets for the enzyme tissue transglutaminase 2 (Naegeli et al., 2017). No peptides were found to be recognized by CD4+ T cells (Sollid et al., 2020). The choice of enzyme has a great impact on peptide formation during sample preparation. When using different enzymes such as chymotrypsin, thermolysin and pepsin differences in the total number of identified peptides and differences in the peptide lengths and sequences could be observed (Colgrave et al., 2017a; Martínez-Esteso et al., 2017; Schalk et al., 2017a; Pasquali et al., 2019). This is due to the different enzyme specificity. Proline and glutamine residues, which are prevalent in gluten protein overall, are difficult for many enzymes to break down. Chymotrypsin cleaves at the C-terminal side of hydrophobic amino acids like tyrosine, phenylalanine and tryptophan while trypsin cleaves at the C-terminal side of arginine and lysine residues (Colgrave et al., 2017a). When using trypsin, peptides are longer and less common because gluten proteins have few trypsin cleavage sites in general. The use of other enzymes would result in a higher number of shorter peptides that can be compared and processed with the database. However, there may also be potentially more missed cleavages and the assignment may not be as clear and specific due to the shorter peptides.

This study was focused on the production of barley RMs for the improvement of ELISA test systems. The new barley isolates were tested on the R5 sandwich ELISA. The reactivity was highest for $AWEP_{iso}$ and $PROL_{iso}$ followed by G_{iso} and $GLUT_{iso}$. The higher reactivity of the prolamins versus the glutelins has been shown in earlier studies on R5 and G12 ELISA (Lexhaller et al., 2016). The G_{iso} represented the total gluten composition well and showed a higher reactivity than the calibration standard. Hordein reactivity against the R5 mAb has been studied using isolated C-, B- and D-hordein protein types (Huang et al., 2017).The study showed that the reactivity of D-, C- and B-hordeins against the R5 mAb varied widely. The reactivity order was as follows: C-hordeins $>$ gliadin standard $>$ B-hordeins $>$ D-hordeins. It was also shown that the C-hordein proportion is crucial for correct prolamin quantification because this fraction is mainly recognized by the R5 mAb (Huang et al., 2017). The reactivity of intact and partially hydrolyzed rye, wheat and barley GPTs was tested in sandwich and competitive ELISA by Gessendorfer et al. (2009). The prolamins were digested with pepsin and trypsin or pepsin and chymotrypsin to obtain the hydrolyzed prolamins. The intact hordeins showed no difference in reactivity when sandwich and competitive ELISA were

used. In contrast, the peptic-tryptic hordein digest showed a higher reaction in the competitive ELISA. Since the sandwich ELISA requires two binding epitopes, lower reactions were expected.

The findings highlight the difficulties of unifying definitions of gluten considering different grains. The produced RMs can be used for various research purposes, as RM for the production of new Abs against gluten proteins, for use in T cell assays or for further investigation of hordeins in general.

4 Conclusion and outlook

The safety of gluten-free products relies on accurate gluten analysis, most commonly using ELISA. These test kits are calibrated to gliadins or wheat gluten, because no well-defined reference materials based on rye and barley are available. This study aimed to fill this gap in producing rye and barley specific reference materials.

The analytical characterization and examination of three commercially available wheat protein reference materials from two production batches revealed substantial differences in gluten composition and protein content in comparison to the PWG-gliadin. Two gluten reference materials ($Gluten_{sigma}$ and $Gluten_{abcr}$) and one gliadin reference material ($Gliadin_{abcr}$) were investigated. While the $Gliadin_{abcr}$ showed a similar protein content in both batches, significant differences were observed in both gluten reference materials ($Gluten_{sigma}$ and $Gluten_{abcr}$). The $Gliadin_{abcr}$, however, did not show the typical elution profile in the RP-HPLC chromatogram. The division into protein types was therefore not possible, which is an important attribute when using the PWG-gliadin in order to characterize wheat prolamins. All wheat reference materials showed residues of albumins and globulins and exhibited lower protein content compared to PWG-gliadin. These findings indicate that the commercially available wheat reference materials show differences in composition, rendering them unreliable for calibration purposes. In order to be able to use them as calibration standards, prior characterization is required for each new batch. Moreover, there are no gluten reference materials consisting of rye and barley on the market. The findings highlight the lack of high quality reference materials available for the research on wheat gluten and the need for rye and barley based reference materials.

The composition of 32 rye and 35 barley cultivars from different counties was investigated in order to gain more knowledge on rye and barley gluten proteins and to select certain cultivars as a basis for reference material production. The gluten characterization using RP-HPLC revealed the expected distribution of gluten protein types in rye and barley. Within the 32 rye cultivars the γ-75k-secalins were the most abundant, followed by γ-40k-secalins, ω-secalins and HMW-secalins. For barley (35 cultivars) the B/γ-hordeins were the most abundant,

followed by C-hordeins and D-hordeins. Furthermore, the RP-HPLC analyses showed that the typical gluten separation into prolamins and glutelins based on the modified Osborne fractionation is not possible for rye and barley. The reason for this is the composition of the proteins. In rye, the γ-75k-secalins and minor parts of HMW-secalins are present in both fractions. For barley, minor parts of the D-hordeins and large parts of the B-hordeins are present in the prolamin fraction. Although they occur as polymers linked by disulfide bonds, they are soluble in 60% ethanol. This could be attributed to the hypothesis that the B-hordeins and D-hordeins are partially present as oligomers or monomers soluble in 60% ethanol. The findings enhance our understanding of secalins and hordeins by demonstrating that the division into the prolamins and glutelins cannot be accurately distinguished clearly through RP-HPLC. Therefore, it is important to consider the individual grain specific gluten protein types for protein characterization.

The gluten content for most of the rye (30) and barley (32) cultivars was overestimated by ELISA (R5 and G12) compared to the RP-HPLC results. For barley, for instance, the RP-HPLC recovery was up to 1747.8% (R5) and 1043.7% (G12). The overestimation can be explained by the higher reactivities of the antibodies towards rye and barley gluten proteins in general compared to wheat proteins. A further reason is the calculation of the gluten content when using the ELISA kits, assuming the prolamin and glutelin ratio to be 1:1 (multiplication of the prolamin content by factor 2). The prolamin/glutelin ratio (RP-HPLC) was on average 4.4:1 for rye and 1.6:1 barley. The results show that the conversion factor of 2 used in ELISA test systems is not appropriate for the gluten determination and should be revised to reduce the overestimation of rye and barley gluten. However, the change of the factor is not a complete solution to the problem of overdetermination, since the calibration standards should also be adjusted, especially if the source of contamination is known such as barley contamination in oats.

For the reference material production, the aim was to select a smaller number of rye and barley cultivars with highest variability in protein and gluten distribution. The highest variability was evaluated using statistical tools such as HCA and ANOVA on the data acquired from the in-depth characterization using RP-HPLC, GP-HPLC, ELISA and the Dumas method. Moreover, more criteria were defined such as the variability in the country of origin. Seven rye cultivars and eight barley cultivars from different countries were selected and used as the basis for the reference material production with the highest variability in gluten composition.

The selected clutivars were mixed in equal proportions and characterized in order to investigate the hypothesis that a mixture of selected cultivars is more suitable for the production of reference materials than single cultivars. To evaluate this hypothesis, the protein distribution of the mixture was compared with the mean values of the selected cultivars, comprising

seven rye cultivars and eight barley cultivars. Through laboratory-scale mixing of the selected flours in equal proportions, minor variations in the distribution of secalins were observed compared to the calculated mean values. Furthermore, when comparing the composition of the barley flour mixture with the calculated mean values, both showed similar hordein compositions. The influence of the harvest year was examined as well by comparing the selected cultivars from two different harvest years. Their mixtures were also compared. The results indicate that for rye, although the individual cultivars showed significant differences in gluten content and composition, there was no significant difference in the gluten content of the mixtures. However, for barley the mixtures did show significant differences in gluten content but not in gluten distribution. Although the investigations have only been carried out on a laboratory-scale the findings support the hypothesis that a mixture of selected cultivars is more suitable for the production of reference materials. The reasons are the stability in protein composition and a mixture representing the highest variability and an average gluten distribution.

The reference material production was carried out with the cultivar mixture each. Different extraction procedures were used resulting in four different reference materials each: the prolamins ($PROL_{iso}$), glutelins ($GLUT_{iso}$), gluten (G_{iso}) and acetonitrile water extractable proteins ($AWEP_{iso}$). The RP-HPLC characterization showed different protein distributions for each isolate. However, the isolate that showed the highest similarity to the results using the modified Osborne fractionation of the corresponding flour was the G_{iso} since both fractions (prolamin and glutelins) were extracted. For the barley isolates, $AWEP_{iso}$ and $PROL_{iso}$ showed the highest similarity in gluten distribution. For rye, the distribution of the secalins was more uniform for all isolates. Four different gluten isolates were successfully produced each with a gluten composition similar to the corresponding flour extraction.

The untargeted LC-MS/MS characterization of the isolates was carried out using a bottom up approach by analysing the peptides out of the enzymatic digest of the isolates and comparing the results with a protein database. The results revealed significant differences in gluten distribution for both rye and barley isolates compared to RP-HPLC measurements. All isolates showed low levels of C-hordeins (barley) and ω-secalins (rye). Notably, higher amounts of HMW-secalins/D-hordeins were observed in both species. In the case of rye isolates, $PROL_{iso}$ and $AWEP_{iso}$ showed similar secalin distribution patterns, while in barley, $PROL_{iso}$, $AWEP_{iso}$ and G_{iso} showed the most similarities in hordein distribution.

To investigate the peptide sequences of gluten proteins, three search strategies were employed. Firstly, the peptides were searched for epitopes recognized by the R5 monoclonal antibody (ELISA). The second search focused on celiac disease-relevant epitopes recognized

by CD4+ T cells. Lastly, the peptides were searched for complete and partial sequence overlaps with previously known epitopes, following the search strategy of the European Food Safety Authority (EFSA). Among the identified peptides, one was regarded to be recognized by the R5 monoclonal antibody and one was considered to be recognized by CD4+ T cells. Additionally, four peptides were identified as potentially harmful for celiac disease-patients. In the case of barley isolates, 13 peptides were discovered, with three of them being recognized by the R5 monoclonal antibody and ten identified as potentially harmful.

In terms of the untargeted LC-MS/MS characterization of either the gluten composition or the peptide sequences several factors influence the results. Firstly, the results are influenced by the quality of the database used for gluten protein assignments. The UniProtKB database may not include all the identified gluten proteins from barley and rye due to incomplete characterization or their absence in the database. Additionally, potential errors in protein nomenclature can arise when dealing with highly similar sequences as it is the case for gluten proteins. Therefore, the data must be used with care for interpretation. Moreover, the studies on the peptides depends on the enzyme used for digestion leading to different peptide lengths and cleavages and in turn to different results in the database comparison.

The reactivity of the isolates towards the R5 monoclonal antibody was tested and compared to the reactivity of the PWG-gliadin, which serves as the calibration standard in the R5 ELISA kit. Regarding rye isolates, the R5 monoclonal antibody showed the highest sensitivity towards $PROL_{iso}$, followed by $AWEP_{iso}$, G_{iso} and $GLUT_{iso}$. For barley isolates, the reactivity was highest for $AWEP_{iso}$ and $PROL_{iso}$ followed by G_{iso} and $GLUT_{iso}$. The results are in accordance to the literature but the reactivity of the $AWEP_{iso}$ to the R5 ELISA has not yet been investigated in other studies.

Overall, in this study the need for well characterized reference materials was demonstrated. Seven rye and eight barley cultivars were successfully identified for reference material production. The research involved an in-depth analytical characterization of a high number of rye flours that has not been previously conducted. The production and characterization of various protein isolates was demonstrated, which have the potential to be used in a variety of tests such as the production of antibodies for ELISA test systems, reference materials for analytical methods such as ELISA, RP-HPLC, LC-MS/MS and the development of T cell assays. Especially the use of the grain-specific reference materials on ELISA test systems is recommended when the source of contamination is known. Further, studies in relation to validation parameters such as accuracy, recovery and repeatability should be carried out using the produced isolates. Furthermore, this study contributes significantly to the understanding of rye secalins and barley hordeins. It is recommended that the assignment of secalins should be carried out

by allocating the subfractions, rather than using modified Osborne fractionation. Additionally, for MS analyses, further investigations using additional digestive enzymes are suggested for future research. It was also noted that insufficient research has been conducted on rye and barley proteins and the databases require revision, particularly in the naming of various protein groups, to establish a reliable basis for data evaluation.

5 Materials and methods

5.1 Chemicals and samples

Table 5.1: List of chemicals used and manufacturers.

Chemical	Purity	Provider
1-propanol	99.50%	VWR Chemicals (Radnor, USA)
2-chloroacetamide (CAA)	98%	Acros Organics (Waltham, USA)
2-propanol	≥99.9%	Carl Roth (Karlsruhe, Germany)
2-sodium hydrogen phosphate dihydrate	≥99.9%	VWR Chemicals (Radnor, USA)
3-(*N*-morpholino)propanesulfonic acid (MOPS)	98.5%	Fisher Scientific (Waltham, USA)
acetic acid	100.0%	Carl Roth (Karlsruhe, Germany)
	HPLC-grade	VWR Chemicals (Radnor, USA)
acetonitrile	≥99.9%	VWR Chemicals (Radnor, USA)
	LC-MS grade	Fisher Scientific (Waltham, USA)
Coomassie Brilliant Blue G-250	ACS	Sigma-Aldrich (St. Louis, USA)
dithiothreitol	-	AppliChem (Darmstadt, Germany)
ethanol	≥99.8%	VWR Chemicals (Radnor, USA)
ethylenediaminetetraacetic acid (EDTA)	99.4%	Fisher Scientific (Waltham, USA)
formic acid (FA)	≥99.0%	VWR Chemicals (Radnor, USA)
gliadin from wheat	-	Sigma Aldrich (Darmstadt, Germany)
gluten from wheat	-	Sigma Aldrich (Darmstadt, Germany)
gluten from wheat	-	ABCR (Karlsruhe, Germany)
hydrochloric acid 32%	p.A.	VWR Chemicals (Radnor, USA)
methanol	≥99.8%	VWR Chemicals (Radnor, USA)
n-pentane	≥99.0%	VWR Chemicals (Radnor, USA)
phenol red	≥99.0%	VWR Chemicals (Radnor, USA)
potassium chloride	≥99.5%	Carl Roth (Karlsruhe, Germany)
potassium dihydrogen phosphate (KH_2PO_4)	99.9%	VWR Chemicals (Radnor, USA)
sodium chloride	≥99.8%	VWR Chemicals (Radnor, USA)
sodium dodecyl sulfate	99.0%	VWR Chemicals (Radnor, USA)
sodium hydroxide	99.4%	VWR Chemicals (Radnor, USA)
sodium phosphate (Na_2PO_4)	99.0%	Fisher Scientific (Waltham, USA)
sucrose	99.0%	Fisher Scientific (Waltham, USA)

trichloroacetic acid (TFA)	≥99.5%	VWR Chemicals (Radnor, USA)
tris(hydroxymethyl)aminomethane (TRIS)	100.0%	VWR Chemicals (Radnor, USA)
TRIS-HCl	99.0%	Carl Roth (Karlsruhe, Germany)
urea	> 99.5 %	Carl Roth (Karlsruhe, Germany)
water	LC-MS grade	Fisher Scientific (Waltham, USA)
	HPLC grade	VWR Chemicals (Radnor, USA)

Table 5.2: General sample information of the 32 rye cultivars. Already published in Xhaferaj et al. (2023a).

Sample name	Cluster Nr.	Sample code	Geographical origin	Year of collection	Provider
Hancock	1	HAN_CAN17	Canada	2017	NDSU
Spooner	1	SPO_CAN17	Canada	2017	NDSU
Rymin	1	RYM_CAN17	Canada	2017	NDSU
Dacold	1	DAC_CAN17	Canada	2017	NDSU
Aroostock	1	ARO_CAN17	Canada	2017	NDSU
Hazlet	1	HAZ_CAN17	Canada	2017	NDSU
Wheeler	1	WHE_CAN17	Canada	2017	NDSU
Sangaste	1	SAN_EST19	Estonia	2019	Estonian Crop Research Institute
Vambo	1	VAM_EST19	Estonia	2019	Estonian Crop Research Institute
Daniello	1	DAN_GER19	Germany	2019	KWS Lochow GmbH
Performer	1	PER_GER19	Germany	2019	Saaten-Union GmbH
Dankowskie-Diament	1	DAD_HUN18	Hungary	2018	ELKH-ATK
Kaupo	1	KAU_LAT20	Latvia	2020	AREI
Dankowskie-Granat	1	DAG_POL20	Poland	2020	DANKO Hodowla Roślin Sp. z o.o
Dankowskie-Agat	2	DA_HUN17	Hungary	2017	ELKH-ATK
Dankowskie-Diament	2	DAD_HUN17	Hungary	2017	ELKH-ATK
Wibro	2	WIB_HUN17	Hungary	2017	Galga-Agrár Kft
Dankowskie-Rubin	2	DR_HUN19	Hungary	2019	ELKH-ATK
Elego	3	ELEG_AUS20	Austria	2020	Saatzucht Edelhof
Elect	3	ELE_AUS20	Austria	2020	Saatzucht Edelhof
Elvi	3	ELV_EST19	Estonia	2019	Estonian Crop Research Institute
Dankowskie-Diament	3	DAD_HUN19	Hungary	2019	ELKH-ATK
Dankowskie-Skand	3	DAN_HUN19	Hungary	2019	ELKH-ATK
Dankowskie-Rubin	3	DAR_POL20	Poland	2020	DANKO Hodowla Roślin Sp. z o.o
Dankowskie-Turkus	3	DAT_POL20	Poland	2020	DANKO Hodowla Roślin Sp. z o.o
Elias	4	ELI_AUS20	Austria	2020	Saatzucht Edelhof
Rettaa	4	RET_FIN20	Finland	2020	Boreal Plant Breeding Ltd.
Dankowskie-Turkus	4	DANT_HUN19	Hungary	2019	ELKH-ATK
Rye Food	4	RYEF_HUN19	Hungary	2019	Kruppa Mag
Wibro	4	WIB_HUN18	Hungary	2018	Galga-Agrár Kft
Wibro	4	WIB_HUN19	Hungary	2019	Galga-Agrár Kft
Rye Food	5	RYEF_HUN18	Hungary	2018	Kruppa Mag

NDSU: Carrington Research Extension Center, AREI: Institute of Agricultural Resources and Economics
ELKH-ATK: Eötvös Loránd Research Network - Agriculture Research

Table 5.3: General sample information of the 35 barley cultivars. Already published in Xhaferaj et al. (2023b).

Sample name	Cluster Nr.	Sample code	Geographical origin	Year of collection	Provider
Arcanda	1	ARC_AUS20	Austria	2020	Saatzucht Donau Ges.m.b.H & Co KG
Adalina	1	ADA_AUS20	Austria	2020	Saatzucht Donau Ges.m.b.H & Co KG
Conlon	1	CON_CAN19	Canada	2019	NDSU
Austerson	1	AUS_CAN19	Canada	2019	NDSU
Evergreen	1	EVE_DEN20	Denmark	2020	Nordic Seed
Feedway	1	FEE_DEN20	Denmark	2020	Nordic Seed
Newway	1	NEW_DEN20	Denmark	2020	Nordic Seed
Stairway	1	STA_DEN20	Denmark	2020	Nordic Seed
Pixel	1	PIX_FRA20	France	2020	Secobra Saatzucht GmbH
Coccinel	1	COC_FRA20	France	2020	Secobra Saatzucht GmbH
Memento	1	MEM_FRA20	France	2020	Secobra Saatzucht GmbH
Traveler	1	TRA_FRA20	France	2020	Secobra Saatzucht GmbH
Prunella	1	PRU_FRA20	France	2020	Secobra Saatzucht GmbH
Jallon	1	JAL_GER20	Germany	2020	Syngenta
Quadriga	1	QUA_GER20	Germany	2020	Secobra Saatzucht GmbH
Accordine	1	ACC_GER20	Germany	2020	Ackermann Saatzucht GmbH & Co. KG
GK Judy	1	GKJ_HUN17	Hungary	2017	Cereal Research Non-Profit Ltd.
MV Initium	1	MVI_HUN17	Hungary	2017	Centre for Agricultural Research
Jumara	1	JUM_LAT20	Latvia	2020	AREI
Saule PR	1	SAU_LAT20	Latvia	2020	AREI
Hockett	2	HOCK_CAN19	Canada	2019	NDSU
Tradition	2	TRA_CAN19	Canada	2019	NDSU
Jakobus	2	JAK_GER20	Germany	2020	Nordsaat Saatzucht GmbH
KWS Scala	2	KWSS_HUN17	Hungary	2017	Centre for Agricultural Research
Morex	2	MOR_HUN17	Hungary	2017	Centre for Agricultural Research
Estoria	3	EST_AUS20	Austria	2020	Saatzucht Edelhof
Evelina	3	EVE_AUS20	Austria	2020	Saatzucht Edelhof
Escalena	3	ESC_AUS20	Austria	2020	Saatzucht Edelhof
Antonella	3	ANT_GER20	Germany	2020	Nordsaat Saatzucht GmbH
Greenway	4	GRE_DEN20	Denmark	2020	Nordic Seed
Abava	4	ABA_LAT20	Latvia	2020	AREI
Irbe	4	IRB_LAT20	Latvia	2020	AREI
Kornelija	4	KOR_LAT19	Latvia	2019	AREI
Celebration	5	CEL_CAN19	Canada	2019	NDSU
Daishi Mochi	5	DAMO_HUN17	Hungary	2017	Centre for Agricultural Research

NDSU: Carrington Research Extension Center, AREI: Institute of Agricultural Resources and Economics

5.2 Instruments

RP-HPLC-System	Shimadzu Prominence HPLC
Manufacturer	Shimadzu (Kyoto, Japan)
Degasser	DGU-20A 5R
Pump	LC-20AD
Autosampler	SIL-20AC HAT
Column oven	CTO-20AC
Detector	SPD-M20A
Communication Module	CBM-20A
Software	LabSolutions V5.93
GP-HPLC-System	
Manufacturer	Shimadzu (Kyoto, Japan)
Degasser	DGU-405
Pump	LC-40D XS
Autosampler	SIL-40C XS
Column oven	CTO-40S
Detector	SPD-40
Communication Module	CBM-40
Software	LabSolutions V5.106
UHPLC-MS/MS-System	Vanquish
Manufacturer	Thermo Fisher Scientific (Waltham, USA)
Binary Pump	VF-P10-A
Autosampler	VF-A10-A
Column oven	VH-C10-A
Detector (DAD)	VF-D11-A
Mass analyzer	Q Exactive Plus Orbitrap
Software	Thermo Scientific Xcalibur V 4.2.47
Nitrogen Analyzer	DUMATHERM N Pro
Manufacturer	C. Gerhardt (Königswinter, Germany)
Software	Dumatherm Manager V8.11
Tecan	Infinite® 200 PRO
Manufacturer	Tecan Group (Männedorf, Switzerland)
Software	Tecan i-control V3.9.1.0
Image Reader and Analyzer	
Reader	LAS-3000, Fujifilm (Tokyo, Japan) V2.1
Analyzer	AIDA Image Analysis software V3.27.001 (Elysia-raytest, Angleur, Belgium)

5.3 Methods

5.3.1 Collection of the grains and flour preparation

The collection and milling of the grains was conducted by the Research Group of Cereal Science and Food Quality (Budapest, Hungary).

Flour preparation for the initial characterization

Different grains of rye (n = 32) and barley (n = 35) were collected from several geographical origins (Tab. 5.2 and 5.3). Wholemeal flours were produced by milling the kernels with a laboratory mill (Cyclotec Mill 99 1093, Foss Tecator AB, Höganäs, Sweden). The mill was cleaned mechanically and with compressed air after each sample and the first 10 g of newly milled sample were discarded. Wholemeal flours were stored in zip-lock bags at 22 °C until further use. For the rye and barley flour mixtures of the seven (rye cultivars) and eight (barley cultivars) selected cultivars (Tab. 3.1 and 3.8), 500 mg flour of each cultivar were mixed and homogenized for 24 h in an overhead shaker, respectively.

Flour preparation for the protein isolation

The selected cultivars (Tab. 3.1 and 3.8) were collected from the harvest year 2022. Equal amounts of kernels (4 kg) of the different cultivars were mixed prior to milling.

5.3.2 Crude protein content according to Dumas

The crude protein content of the flours and isolates was measured according to Dumas. The flour samples were measured in the laboratories of the project partners (Research Group of Cereal Science and Food Quality, Budapest, Hungary). Therefore, the measurements were performed using a Leco FP 528 nitrogen analyzer (Leco Corporation, St. Joseph, MO, USA) in duplicate according to ICC Standard No. 167. Similarly, the crude protein content of the isolates was determined on-site using a DUMATHERM N Pro nitrogen analyzer (C. Gerhardt, Königswinter, Germany) in triplicate according to ICC Standard No. 167. To determine the protein content, the nitrogen content was multiplied by 5.7 to obtain the crude protein content in both flours and isolates.

5.3.3 Determination of the fat and moisture content

Crude fat content measurement of the flours was performed in the laboratories of the project partners (Research Group of Cereal Science and Food Quality, Budapest, Hungary). The

samples were measured in duplicates according to ICC Standard No. 136 using a Soxtec System HT-1043 instrument (Foss Tecator AB, Höganäs, Sweden). The moisture content of the flours was determined by the oven-drying method in duplicates according to ICC Standard No. 109/1.

5.3.4 Protein isolation and reference material production

The mixture of the selected rye and barley flours was used to create four distinct isolates each utilizing various extraction methods. Protein extraction was based on Schalk et al. (2017b) and resulted in the production of the following four isolates: prolamins, glutelins, gluten (prolamins and glutelins extracted in one step) and acetonitrile water extractable proteins (AWEP). Prior to the extraction, the flours (100 g) were defatted with 250 mL n-pentane/ethanol (95/5, v/v) three times by stirring, followed by stirring once with 250 mL n-pentane at 22 °C for 30 min. To remove the extraction solution the suspension was filtered (Sartorius Stedim Biotech GmbH) under vacuum. The flour was spread evenly on a tray, dried overnight and carefully homogenized with a spatula. The production of the isolates was carried out using the buffers and solutions listed in Chapter 5.3.5.1 (Table 5.4).

5.3.4.1 Production of the prolamin and glutelin isolates

The isolation of the prolamins and glutelins was done on two 40 g batches each (in total 80 g rye flour and 80 g barley flour). Approximately 40 g of defatted flour was extracted first with 160 mL of Saline Solution by homogenizing with an Ultra Turrax blender (12000 rpm, IKA-Werke, Staufen, Germany) for 5 min and stirring for 10 min with a magnetic stirrer. The samples were then centrifuged at 3750 rcf and 22 °C for 25 min (Heraeus Multifuge X1, ThermoScientific) and the supernatant was separated and discarded. The extraction was repeated two times to remove the albumin and globulin fraction (chapter 5.3.5.1) The prolamins were extracted from the residue with 160 mL of Ethanol Solution by homogenizing with an Ultra Turrax blender for 5 min and stirring for 10 min with a magnetic stirrer. The samples were then centrifuged at 3750 rcf and 22 °C for 25 min and the extraction was repeated two times. The supernatants were combined. Lastly, the residue was used for glutelin extraction with 160 mL of extraction Buffer Solution by homogenizing under argon with an Ultra Turrax blender for 5 min and stirring for 30 min at 60 °C in a water bath. After centrifugation (3750 rcf and 22 °C for 25 min) the supernatant was collected and the extraction was repeated twice. The combined prolamin and glutelin supernatants were concentrated, dialyzed and lyophilized.

5.3.4.2 Production of the gluten isolate

The gluten isolation was done with approximately 50 g of defatted flour. The albumins and globulins were extracted according to chapter 5.3.5.1 and the supernatant was discarded. Then 200 mL Buffer Solution was used for extraction under argon by homogenizing with an Ultra Turrax blender (12 000 rpm) for 5 min and stirring for 30 min at 60 °C in a water bath. The samples were then centrifuged at 3750 rcf and 22 °C for 25 min. The extraction was conducted three times and the supernatants were combined, concentrated, dialyzed and lyophilized (Section 5.3.4.4).

5.3.4.3 Production of the AWEP isolate

The AWEP isolation was done with approximately 50 g of defatted flour. The albumins and globulins were extracted as described in chapter 1.4.2 and the supernatant was discarded. Then 200 mL of acetonitrile/water (1:1, v/v) containing 0.1% FA (v/v) was used for extraction by homogenizing with an Ultra Turrax blender (12 000 rpm) for 5 min. Then the sample was sonicated for 30 sec and stirred for 10 min with a magnetic stirrer. After centrifugation (3750 rcf and 22 °C for 25 min) the extraction was repeated two times without sonication and the supernatants were combined, concentrated, dialyzed and lyophilized (Section 5.3.4.4).

5.3.4.4 Concentration, dialyzation and lyophilization of the proteins

The combined supernatants of each fraction (prolamins, glutelins, gluten and AWEP) were concentrated (Rotavapor® R II, BÜCHI Labortechnik, Flawil, Switzerland) to approximately 300 mL. A membrane (Spectrum Labs™ Spectra/Por Dialysis Membrane, Fisher Scientific, Waltham, MA, USA) with a molecular weight cut-off of 12 - 14 kDa was used for dialysis. The protein solutions were dialyzed extensively against 0.01 mol/L acetic acid for 72 h and then against deionized water at 22 °C for 24 h. During dialysis, the 0.01 mol/L acetic acid was changed three times a day. The proteins were lyophilized for 48 h at -60 °C and 0.1 mbar (Beta 1-8 LSCplus, Martin Christ, Osterode am Harz, Germany). After drying, the isolates were stored at -20 °C until use.

5.3.5 Chromatographic protein characterization

5.3.5.1 Protein extraction procedure

The extraction of the proteins was performed according to the modified Osborne fractionation (Wieser et al., 1998). The stepwise extraction was conducted with approximately 100 mg of grain flour using three different extraction solvents (Table 5.4). The albumins and

globulins (AL/GL) were first extracted twice with 1 mL of salt solution, then vortexed for two min, followed by magnetic stirring for 10 min and centrifugation for 25 min at 25 °C and 3550 rcf. Prolamins were then extracted in triplicate using 0.5 mL of ethanol solution, followed by two min vortexing, 10 min magnetic stirring and centrifugation (25 min at 25 °C and 3550 rcf). Moreover, part of the prolamin fraction was reduced with 1% (w/v) dithiothreitol (DTT). Lastly, glutelins were obtained by extracting twice with 1 mL buffer solution under nitrogen. The extraction was done by vortexing for two min, magnetic stirring in a 60 °C water bath for 30 min and centrifugation (30 min at 25 °C,3550 rcf). The supernatant of AL/GL, prolamins, reduced prolamins and glutelins was diluted to 2 ml with the respective extraction solvents, filtered (0.45 μm Whatman SPARTAN, Cytiva Europe GmbH, Freiburg im Breisgau, Germany) and used for RP-HPLC and GP-HPLC analysis of the proteins, respectively.

Table 5.4: Buffers and solutions used for the protein extraction procedure.

Buffers and Solutions	**Composition**
Salt Solution (pH 7.6)	Solution 1 0.4 mmol/L NaCl + 0.07 mol/L Na_2HPO_4*$2H_2O$ Solution 2 0.4 mmol/L NaCl + 0.07 mol/L KH_2PO_4 Adjust pH to 7.6 of Solution 1 using Solution 2
Ethanol Solution	60% Ethanol + 40% H_2O (v/v)
Buffer Solution (pH 7.5)	50% 1-propanol + 50% 0.1 mol/L TRIS-HCl 2 mol/L Urea + 0.06 mol/L DTT

5.3.5.2 RP-HPLC protein characterization

The protein content and the distribution of the cereal proteins were investigated by RP-HPLC. The instrumental parameters and gradients used for the RP-HPLC separation of the grain proteins are listed in Table 5.5. Proteins were quantified using the corresponding absorbance (210 nm) areas of PWG-gliadin (c = 2.5 mg/ml) (Van Eckert et al., 2006). The sum of the reduced prolamins (reduced with DTT) and glutelins was used to calculate the gluten concentration. The protein types were quantified based on their percentage of the area under the curve (AUC). The chromatographic profiles were assessed and classified according to the published literature (Gellrich et al., 2003; Schalk et al., 2017b).

Table 5.5: RP-HPLC conditions and gradient parameters for the separation of the grain protein fractions (albumins, globulins, prolamins and glutelins).

RP-HPLC Conditons		
Detection	210 nm	
Column	YMC Triart Bio C18, 150 mm × 2.1 mm, 3 µm	
Eluent A	Water + 0.1% TFA	
Eluent B	Acetonitrile + 0.1% TFA	
Flow rate	0.5 ml/min	
Oven Temperature	60 °C	
Gradient for Prolamins and Glutelins		
Time [min]	Eluent A [%]	Eluent B [%]
0.0	95	5
0.4	95	5
0.5	70	30
16.0	40	60
16.1	0	100
22.1	0	100
22.2	95	5
35.0	95	5
Gradient for Albumins and Globulins		
Time [min]	Eluent A [%]	Eluent B [%]
0.0	100	0
0.4	100	0
0.5	80	20
8.0	40	60
8.1	0	100
13.0	0	100
13.1	100	0
27.0	100	0

5.3.5.3 GP-HPLC protein characterization

The GP-HPLC method (Table 5.6) was used for the characterization of the relative molecular weight (M_w) distribution of the grain proteins. Proteins with known M_w values (cytochrome C from horse heart (12.4 kDa), carbonic anhydrase from bovine erythrocytes (29 kDa) and albumin from bovine serum (66 kDa)) (gel filtration molecular weight markers 6–66 kDa, Sigma-Aldrich, St. Louis, MO, USA) were used to define specific M_w ranges for comparison. The M_w ranges used for sample comparison were the following: (1) >66 kDa; (2) 66–29 kDa; (3) 29–12.4 kDa; and (4) <12.4 kDa. The area AUC was integrated for each range and expressed as a percentage of the total area. Gluten, prolamin and glutelin fractions were

quantified using the corresponding absorbance areas of PWG-gliadin (Van Eckert et al., 2006).

Table 5.6: GP-HPLC conditions and parameters for the determination of the relative molecular weight distribution of the grain proteins.

GP-HPLC Conditons	
Detection	210 nm
Column	BioSep-SEC-s3000, 300 × 4.6 mm, 5µm
Eluent A	Water + 0.1% TFA
Eluent B	Acetonitrile + 0.1% TFA
Flow rate	0.3 ml/min
Oven Temperature	25 °C
Gradient	Isocratic (Eluent A/Eluent B, 50/50)

5.3.6 SDS-PAGE

The protein extraction for the SDS-PAGE was conducted using the solutions and buffers listed in Table 5.7. Approximately 20 mg of grain flour was extracted with 1 mL extraction buffer under reducing conditions (DTT, 50 mmol/L) overnight. The flour suspension was then shaken for 10 minutes at 60 °C and centrifuged for 5 minutes at a speed of 2370 rcf. A NuPAGE 4–12% BIS-TRIS protein gradient gel (1.0 mm, 10-well, Invitrogen, Carlsbad, CA, USA) was used for the electrophoresis. 10 µL of the extracted protein solutions and 5 µL of the marker (PageRuler Unstained Protein Ladder (Thermo Scientific, Bremen, Germany) covering a range of 10 to 200 kDa with 14 proteins) were loaded into the wells. DTT (5 mmol/L) was added to the MOPS running buffer as a reducing agent prior to use (Lagrain et al., 2012; Geisslitz et al., 2020). The gels were run at 200 V and 115 mA according to the manufacturer's guidelines (Thermo Scientific) with a running time of 30 min. The proteins were then stained with the staining solution for 30 minutes after being fixed for 30 minutes in the fixing solution. The destaining was conducted first with destaining solution I twice for 15 min and with destaining solution II until the bands were visible Kasarda et al. (1998). The gels were scanned with the LAS-3000, Fujifilm (Minato, Tokyo, Japan)and the M_w of the bands were estimated based on the marker proteins by the AIDA Image Analysis software V3.27.001 (Elysia-raytest, Angleur, Belgium).

Table 5.7: Buffers and solutions used for the SDS-PAGE.

Buffers and Solution	Composition
Extraction Buffer (pH 8.5)	293.3 mmol/L Sucrose 246.4 mmol/L Tris(hydroxymethyl)aminomethane 69.4 mmol/L Sodium dodecyl sulfate 0.51 mmol/L Ethylenediaminetetraacetic acid 0.22 mmol/L Brilliant Blue G-250 0.177 mmol/L Phenol red 0.105 mmol/L HCl
Running Buffer (pH 7.7)	50 mmol/L 3-(N-morpholino)propanesulfonic acid 50 mmol/L Tris(hydroxymethyl)aminomethane 3.5 mmol/L Sodium dodecyl sulfate 1 mmol/L Ethylenediaminetetraacetic acid
Fixing Solution	12% Trichloroacetic acid (v/v)
Staining Solution	3 mmol/L Coomassie Brilliant Blue G-250 in water/methanol/acetic acid (454/454/92, v/v/v)
Destaining Solution I	methanol/water/acetic acid (50/40/10, v/v/v)
Destaining Solution II	water/methanol/acetic acid (80/10/10, v/v/v)

5.3.7 Gluten quantification with ELISA

5.3.7.1 Flours

Rye and barley flour samples were measured in the laboratories of the project partners (Research Group of Cereal Science and Food Quality, Budapest, Hungary). The gluten content was quantified with two commercially available ELISA test kits: RIDASCREEN Gliadin Assay (R7001, R-Biopharm, Darmstadt, Germany; limit of detection (LOD): 0.5 mg/kg of gliadin, limit of quantification (LOQ): 2.5 mg/kg) and AgraQuant Gluten G12 Assay (COKAL0200, Romer Labs, Tulln, Austria; LOD: 2.0 mg/kg of gluten, LOQ: 4.0 mg/kg). The ELISA procedures were performed according to the manufacturer's instructions. To obtain a sample concentration within the calibration range the flours were additionally diluted 10.000-fold. The absorbances were determined using a microplate reader (iMarkTM Microplate Absorbance Reader, Bio-Rad, Hercules, CA, USA). The gluten concentrations were calculated from the absorbance values by the Bio-Rad Microplate Manager 6 software (Bio-Rad) using the curve fit and calculations suggested by the test kit manufacturer, respectively. The gluten content is calculated using the conversion factor of 2 (gliadin content $\times$ 2) for the RIDASCREEN Gliadin Assay.

5.3.7.2 Isolates

The responses of the gluten isolates toward the R5 mAb was tested using the RIDASCREEN Gliadin Assay (R7001, R-Biopharm, Darmstadt, Germany). For this purpose, the isolates were properly diluted (concentrations in Table 3.7 and 3.14) using the appropriate solvents and sample dilution buffers. The prolamin isolates were diluted 60% ethanol, the glutelins and the gluten isolates were diluted using the buffer solution (Tab. 5.4) and the AWEP isolates were diluted in acetonitrile/water (1:1, v/v) containing 0.1% FA (v/v). The final dilution step was carried out in accordance with the manufacturer's instructions using the sample dilution buffer included in the kit to make sure that all solutions contained the standard amount of the kit buffer (1:12.5). At least four serial dilutions of each extract were applied at least twice ($n = 2$) and the ELISA procedure was carried out strictly as described by the manufacturer. The ELISA absorbances were plotted against the protein concentrations of the isolate solutions, which were quantified by RP-HPLC while taking dilution factors into account. To compare the sensitivities of the isolates against the R5 mAb, the RP-HPLC concentrations were plotted against the ELISA concentrations using the respective kit standards for calculation without any additional conversion factor. Calibration curves were constructed using the cubic spline function of the Rida®Soft Win Software (R-Biopharm, Darmstadt, Germany).

5.3.8 Untargeted LC-MS/MS analysis of the isolates

5.3.8.1 Sample preparation

The untargeted LC-MS/MS study of proteins requires a number of sample preparation steps. The proteins must first be reduced and alkylated before being digested enzymatically. Peptides are generated from the proteins in this context. Before the sample is used for analysis, solid phase extraction is employed for purification. The detailed sample preparation and the needed solutions are shown in Table 5.8 and described in the following section.

Reduction and alkylation of the protein isolates

The isolates were dissolved in 500 µL TRIS-HCl and 500 µL 1-propanol and mixed with 100 µL TCEP solution. The solution was incubated for 30 min at 60 °C in a thermoshaker. After subsequent cooling, 100 µl CAA solution was added and the samples were shaken for 45 min at 37 °C. Finally, the samples were dried for approximately 3 h in the vacuum centrifuge.

Enzymatic digestion with trypsin

The samples were diluted in 1 mL of the Urea Solution (0.1 mol/L Tris-HCl and 0.04 mol/L Urea, pH 7.8). Trypsin (1:50 enzyme:protein w/w) was added for digestion and the samples

were further incubated for 18 h at 37 °C in the dark. The digestion was stopped by adding 10 µL TFA and the samples were evaporated to dryness (40 °C, 6 h, 8 mbar)(Martin Christ, Osterode am Harz, Germany).

Peptide purification by solid phase extraction

Purification was done using Discovery DSC 18 solid phase extraction columns (100 mg, Sigma Aldrich, MO, USA) according to manufacturer's instruction and peptides were eluted with 40% ACN containing 0.1% FA. The solvent was evaporated and the peptides were stored at 20 °C until further analysis.

Table 5.8: Buffers and solutions used for the LC-MS/MS sample preparation.

Buffers and Solutions	**Composition**
TRIS-HCl Solution (pH 8.5)	0.5 mol/L (Tris(hydroxymethyl)aminomethane Hydrochloride
TCEP Solution (pH 8.5)	0.05 mol/L Tris-(2-carboxyethyl)-phosphine
CAA Solution (pH 8.5)	0.5 mol/L 2-Chloroacetamide
Urea Solution (pH 7.8)	0.1 mol/L Urea
Enzyme for Digestion	Trypsin
	Solid Phase Extraciton
Equilibration Solution	80% Acetonitrile + 20% Water + 0.1% FA
Washing Solution	2% Acetonitrile + 98% Water + 0.1% FA
Elution Soltion	40% Acetonitrile + 60% Water + 0.1% FA

5.3.8.2 LC-MS/MS measurements

For the measurement, the residue was dissolved in 1 mL of 2% ACN, 0.1% FA in water and filtered (WhatmanTM filter, pore size 0.45 µm). Samples were then run on the UHPLC system coupled to a Q-Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer. The injection volume was 10 µL. The method and the instrumental details of the system are listed in section 4.5.

Table 5.9: UHPLC-MS/MS instrumental settings and method used for untargeted proteomics.

UHPLC Conditons			
Detection	210 nm		
Column	Aeris PEPTIDE XB-C18, 150x2.1 mm, 1.7 µm		
Eluent A	Water + 0.1% FA		
Eluent B	Acetonitrile + 0.1% FA		
Flow rate	0.2 ml/min		
Oven Temperature	30 °C		
	Gradient		
Time [min]	Eluent A [%]	Eluent B [%]	
0.0	95	5	
0.4	90	10	
32.0	68	32	
34.0	20	80	
37.0	20	80	
38.0	95	5	
45.0	95	5	
Q Exactive Plus Orbitrap Settings			
Full MS		*dd-MS*^{2}	
Polarity	Positive	Resolution	175000
Default charge state	2	AGC target	1E+05
Resolution	70000	Maximum IT	50 s
AGC target	3E+06	Loop count	15
Scan range	360-1300 m/z	TopN	15
		Isolation window	2.4 m/z
		Fixed first mass	140.0 m/z
		(N)CE/ stepped (N)CE	nce: 28
		Spectrum data type	Centroid

5.3.8.3 Data evaluation

The peptides and proteins in the isolate samples were identified and relatively quantified using MaxQuant (version 2.2.0.0). The search engine Andromeda was used to compare

the MS data to the *Triticeae* protein reference database derived from UniprotKB (*Poaceae*, download 23.02.23, protein entries 557,477). The workflow in MaxQuant was performed according to the workflow described in Tyanova et al. (2016a). Methionine oxidation and protein acetylation at the N-terminus were selected as variable modifications. Trypsin was designated as a proteolytic enzyme with a maximum of two permitted missed cleavage sites and carbamidomethylation on cysteines was set as a fixed modification. The data were filtered for a minimum length of seven amino acids and a 1% peptide and protein false discovery rate, with match-between runs enabled (matching time window 0.7 min, alignment time window 20 min) and the matching time window set to 0.7 min. For relative sample comparison, the Intensity-based Absolute Quantification (iBAQ) algorithm within the label free quantification selection was enabled. A total sum normalization of iBAQ protein intensities between samples was performed to correct for different total protein injection amounts.

5.3.9 Statistical analysis

Means and absolute standard deviations were computed for all quantitative values, with a sample size of three ($n = 3$). To determine the strength of the correlation between variables, Pearson correlation coefficients (r) were used, where $r \leq \pm 0.54$ indicated no correlation, $\pm 0.54 < r \leq \pm 0.67$ indicated a weak correlation, $\pm 0.67 < r \leq \pm 0.78$ indicated a medium correlation and $r > 0.78$ indicated a strong correlation Thanhaeuser et al. (2014). All reported correlations were statistically significant at $p < 0.05$. Additionally, differences in the means of the M_w distribution (GP-HPLC) and RP-HPLC data were analyzed using a one-way ANOVA with Tukey's post hoc test ($p < 0.05$). T-tests were performed with a significance level of $p = 0.05$. A hierarchical cluster analysis was conducted to identify similarities and differences among different barley cultivars and group them accordingly. All statistical analyses were performed using Origin 2021b software (OriginLab Cooperation, Northampton, MA, USA).

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Annex

Wheat Reference Materials

Table 5.10: Relative prolamin types composition of different reference materials obtained by the manufacturers Sigma Aldrich (Sigma) and abcr GmbH (abcr) and the PWG-gliadin. With the gluten types ω5-gliadins (ω5), ω1,2-gliadins (ω1,2), α-gliadin (α) and γ-gliadins (γ). The values are given as means ($n = 3$), (%) and different capital letters indicate significant differences between the samples in each column (one-way ANOVA, Tukey's post hoc test, $p < 0.05$).

Reference materials		ω5		ω1,2		α		γ	
		%							
$Gliadin_{sigma}$	1	10.1	B	4.9	C	73.5	A	11.5	F
	2	11.2	A	5.3	C	54.6	B	28.9	E
$Gluten_{abcr}$	1	8.7	D	5.3	C	46.0	D	40.0	C
	2	8.6	D	4.9	C	43.2	E	43.3	A
$Gluten_{sigma}$	1	8.3	D	5.0	C	46.2	D	40.5	BC
	2	9.5	C	13.4	A	41.8	E	35.3	D
PWG-gliadin		4.1	E	6.3	B	47.9	C	41.7	B

Table 5.11: Results of the independent two-sample T-test (null hypothesis) analysis performed with a significance level of 0.05 using Origin Pro 2019b. The factors show the significance differences between the absolute distribution of the protein fractions measured with RP-HPLC between the different gluten reference materials.

Sample	p-value	Significance	t(4)=t-Statistics
Albumins/Globulins			
Gliadin abcr	0.99134	No	0.01
Gluten Sigma	0.00006	Yes	17.93
Gliadin Sigma	0.15307	No	1.76
Prolamins			
Gliadin abcr	0.00928	Yes	4.70
Gluten Sigma	0.02360	Yes	3.56
Gliadin Sigma	0.55732	No	-0.64
Glutelins			
Gliadin abcr	0.13591	No	1.86
Gluten Sigma	0.05981	No	2.60
Gliadin Sigma	0.30142	No	1.19
Gluten			
Gliadin abcr	0.01223	Yes	4.34
Gluten Sigma	0.00328	Yes	6.28
Gliadin Sigma	0.89600	No	-0.14
Protein			
Gliadin abcr	0.01426	Yes	4.15
Gluten Sigma	0.00186	Yes	7.31
Gliadin Sigma	0.82416	No	0.24

Table 5.12: Results of the independent two-sample T-test (null hypothesis) analysis performed with a significance level of 0.05 using Origin Pro 2019b. The factors show the significance differences between the relative distribution of the protein fractions measured with RP-HPLC between the different gluten reference materials.

Sample	p-value	Significance	t(4)=t-Statistics
Albumins/Globulins			
Gluten Abcr	0.02453	Yes	-3.52
Gluten Sigma	0.00025	Yes	12.37
Gliadin Sigma	0.31629	No	1.14
Prolamins			
Gluten Abcr	0.33385	No	1.10
Gluten Sigma	0.72570	No	-0.38
Gliadin Sigma	0.35714	No	-1.04
Glutelins			
Gluten Abcr	0.81826	No	-0.25
Gluten Sigma	0.76271	No	-0.32
Gliadin Sigma	0.39135	No	0.96
Gluten			
Gluten Abcr	0.02453	Yes	3.52
Gluten Sigma	0.00025	Yes	-12.37
Gliadin Sigma	0.31629	No	-1.14

Table 5.13: The relative molecular weight distribution of three commercially available gluten and gliadin reference materials obtained by the manufacturers Sigma Aldrich (Sigma) and abcr GmbH (abcr) measured by with GP-HPLC. The fractions have been categorized according to the following molecular weight ranges: (1) > 66 kDa; (2) 66-29 kDa; (3) 29-12.4 kDa; (4) < 12.4 kDa. Values are given as means (n = 3) and percentages (%) referring to the relative percentages of the isolates in relation to the measured AUC at 210 nm by GP-HPLC. The capital letters indicate significant differences between the samples in each column (one-way ANOVA, Tukey's post hoc test, $p < 0.05$).

	> 66 kDa		29-66 kDa		12,4-29 kDa		<12,4 kDa	
			%					
Prolamins								
Gliadin Sigma	21.3	A	10.2	A	29.5	C	39.1	C
Gluten abcr	20.2	A	8.6	B	30.9	B	40.3	B
Gluten Sigma	11.9	B	5.4	C	35.1	A	47.5	A
Prolamins reduced								
Gliadin Sigma	3.1	A	4.8	B	24.5	C	67.6	A
Gluten abcr	3.6	A	7.6	A	44.7	B	44.0	B
Gluten Sigma	1.5	B	4.3	B	50.9	A	43.4	B
Glutelins								
Gliadin Sigma	14.8	B	7.0	B	27.3	C	50.9	A
Gluten abcr	17.1	A	13.0	A	38.9	B	31.0	B
Gluten Sigma	11.0	C	12.8	A	45.6	A	30.6	B

Table 5.14: Absolute contents of the protein fraction of three different wheat reference materials and their two batches. Values are given as means (n = 3), (g/100 g) and different capital letters indicate significant differences between the samples in each column (one-way ANOVA, Tukey's post hoc test, $p < 0.05$). PROL/GLUT: prolamin-to-glutelin rati0, AL/GL: albumins and globulins.

Sample		PROL/GLUT	Protein		Gluten		Prolamins		Glutelins		AL/GL	
							g/100 g					
$Gluten_{sigma}$	1	3.3	94.44	A	84.28	A	64.53	A	19.75	C	10.16	A
	2	3.9	93.88	A	84.68	A	67.56	A	17.13	C	9.19	A
$Gluten_{abcr}$	1	1.1	78.96	B	69.78	BC	36.17	B	33.61	B	9.17	A
	2	0.9	58.19	C	49.03	D	23.78	C	25.25	BC	9.16	A
$Gliadin_{sigma}$	1	0.6	84.34	AB	77.50	AB	28.83	BC	48.67	A	6.84	B
	2	0.6	60.53	C	57.59	CD	21.44	C	36.15	AB	2.94	C

Rye Reference Material

Table 5.15: Summary of the analytical characterization of 32 rye flours given as range and mean ($n = 3$) $\pm$ standard deviation (SD). [a]Ratio of reduced prolamins and glutelins measured by RP-HPLC; RP-HPLC: reversed-phase high-performance liquid chromatography; GP-HPLC: gel permeation HPLC; ALGL: albumins/globulins; HMW: high-molecular-weight; red: reduced with dithiothreitol. Data already published in Xhaferaj et al. (2023a).

g/100 g flour	Parameters	Range min		max	Mean $\pm$ SD		
	Moisture	8.17	-	13.02	10.36	$\pm$	1.04
	Fat	0.04	-	2.07	1.23	$\pm$	0.44
Protein content	Crude protein (N (Dumas) $\times$ 5.83)	5.24	-	13.19	9.09	$\pm$	1.67
	Protein (RP-HPLC)	4.21	-	11.19	7.21	$\pm$	1.60
Gluten content	Gluten (RP-HPLC)	2.57	-	7.83	4.33	$\pm$	1.32
	Gluten (GP-HPLC)	2.67	-	7.34	4.47	$\pm$	1.17
	Gluten (R5 ELISA)	2.34	-	59.12	23.06	$\pm$	13.71
	Gluten (G12 ELISA)	3.24	-	68.03	32.75	$\pm$	14.85
Osborne fractions	Prolamins (RP-HPLC)	1.96	-	6.71	3.53	$\pm$	1.18
	red. Prolamins (RP-HPLC)	2.06	-	6.75	3.54	$\pm$	1.17
	Prolamins (GP-HPLC)	2.23	-	6.89	3.89	$\pm$	1.24
	red. Prolamins (GP-HPLC)	2.21	-	6.38	3.69	$\pm$	1.05
	Glutelins (RP-HPLC)	0.51	-	1.15	0.79	$\pm$	0.18
	Glutelins (GP-HPLC)	0.46	-	1.17	0.78	$\pm$	0.17
	Prolamin/glutelin ratio[a]	2.97	-	6.26	4.44	$\pm$	0.80
Protein fractions	ALGL (RP-HPLC)	1.41	-	3.64	2.87	$\pm$	0.46
	ω-secalins (RP-HPLC)	0.44	-	2.34	1.04	$\pm$	0.50
	γ-75k-secalins (RP-HPLC)	1 .03	-	2.82	1.63	$\pm$	0.43
	γ-40k-secalins (RP-HPLC)	0.74	-	2.19	1.20	$\pm$	0.39
	HMW-secalins (RP-HPLC)	0.20	-	0.71	0.45	$\pm$	0.12

Table 5.16: Standard determination and ELISA and GP-HPLC results. The values are given as means (n = 3), The GP-HPLC values are given as means (n = 3), (g/100 g) and different capital letters indicate significant differences between the samples in each column (one-way ANOVA, Tukey's post hoc test, $p < 0.05$). Protein content measured by Dumas (5.83). Data already published in Xhaferaj et al. (2023a).

Sample code	Moisture	Crude Protein	Fat	ELISA		GP-HPLC							
				Gluten R5	Gluten G12	Gluten[a]		Prolamins		red. Prolamins		Glutelins	
	(%)			g/100 g		g/100 g							
HAN_CAN17	9.5 ± 0.5	8.0 ± 0.2	1.1 ± 0.1	9.2 ± 2.0	30.7 ± 5.6	3.05	MNO	2.46	OP	2.52	LMNOP	0.53	CD
SPO_CAN17	9.6 ± 0.1	9.1 ± 0.1	0.8 ± 0.0	15.1 ± 2.2	27.8 ± 0.9	4.45	FGH	3.24	KL	3.44	HIJK	1.01	A
RYM_CAN17	9.5 ± 0.2	8.5 ± 0.0	0.7 ± 0.1	10.4 ± 1.3	38.8 ± 0.7	3.67	JKLM	2.92	LM	2.94	JKLM	0.73	BC
DAC_CAN17	9.6 ± 0.1	8.4 ± 0.1	1.0 ± 0.1	9.6 ± 3.9	30.4 ± 3.3	3.65	JKLM	2.83	MN	2.74	LMNO	0.91	AB
ARO_CAN17	9.5 ± 0.3	7.8 ± 0.1	0.9 ± 0.2	10.5 ± 3.1	28.2 ± 1.1	3.49	KLMN	2.45	OP	2.50	MNOP	0.99	A
HAZ_CAN17	9.5 ± 0.3	10.4 ± 0.1	1.7 ± 0.2	13.0 ± 2.1	31.1 ± 2.0	4.94	EFG	4.21	GH	4.06	EFG	0.88	AB
WHE_CAN17	9.3 ± 0.6	8.3 ± 0.1	1.9 ± 0.0	19.3 ± 0.9	20.6 ± 1.2	3.40	KLMN	2.60	MNO	2.61	LMNO	0.79	AB
SAN_EST19	9.9 ± 0.3	6.9 ± 0.0	1.1 ± 0.0	15.5 ± 1.5	19.0 ± 0.4	2.67	O	2.23	P	2.21	OP	0.46	D
VAM_EST19	10.4 ± 0.4	7.9 ± 0.1	0.9 ± 0.1	22.3 ± 0.7	27.4 ± 1.3	3.39	KLMN	3.37	JK	2.89	KLMN	0.50	CD
DAN_GER19	10.4 ± 0.3	7.2 ± 0.0	0.3 ± 0.1	10.5 ± 2.3	26.8 ± 1.7	3.97	HIJK	3.20	KL	3.10	IJKL	0.88	AB
REF_GER19	12.8 ± 0.0	5.2 ± 0.2	0.0 ± 0.1	10.9 ± 3.1	27.8 ± 1.5	3.76	IJKL	2.69	MNO	2.75	LMNO	1.01	A
DAD_HUN18	9.9 ± 0.4	7.2 ± 0.0	2.0 ± 0.3	23.6 ± 1.0	29.6 ± 1.0	2.86	NO	2.44	OP	2.33	NOP	0.53	CD
KAU_LAT20	10.0 ± 0.1	8.7 ± 0.0	1.4 ± 0.1	20.6 ± 0.4	26.7 ± 1.0	4.60	FGH	4.02	H	3.81	EFGH	0.79	AB
DAG_POL20	9.8 ± 0.3	8.1 ± 0.1	1.3 ± 0.3	21.4 ± 0.6	28.6 ± 1.8	4.42	FGH	3.62	IJ	3.43	HIJK	0.99	A
DA_HUN17	10.9 ± 0.1	7.9 ± 0.1	1.2 ± 0.1	8.9 ± 0.5	3.2 ± 0.1	3.54	JKLM	2.69	MNO	2.82	LMN	0.72	BC
DAD_HUN17	11.2 ± 0.1	8.1 ± 0.1	1.0 ± 0.0	9.8 ± 0.3	10.6 ± 1.1	3.19	LMNO	2.58	MNOP	2.47	MNOP	0.73	BC
WIB_HUN17	13.0 ± 0.1	7.5 ± 0.1	1.3 ± 0.3	2.3 ± 0.4	9.3 ± 0.3	3.07	MNO	2.49	NOP	2.60	LMNOP	0.46	D
DR_HUN19	10.3 ± 0.1	10.0 ± 0.0	1.3 ± 0.0	5.6 ± 1.2	15.9 ± 1.0	5.00	DEF	4.67	EF	4.28	DEF	0.72	BC
ELEG_AUS20	9.8 ± 0.4	11.1 ± 0.0	1.1 ± 0.2	31.9 ± 1.5	34.7 ± 0.4	6.05	B	5.75	C	5.52	B	0.53	CD
ELE_AUS20	10.2 ± 0.2	10.1 ± 0.1	1.2 ± 0.2	30.2 ± 0.7	38.8 ± 1.3	5.73	BC	4.92	E	4.82	CD	0.91	AB
ELV_EST19	10.9 ± 0.2	8.9 ± 0.1	1.1 ± 0.1	24.0 ± 0.9	40.2 ± 1.1	4.55	FGH	5.79	C	3.72	FGH	0.83	AB
DAD_HUN19	10.0 ± 0.1	9.9 ± 0.1	2.1 ± 0.0	37.0 ± 1.9	37.8 ± 0.5	4.95	DEFG	4.24	GH	4.04	EFG	0.91	AB
DAN_HUN19	10.3 ± 0.1	9.8 ± 0.1	1.2 ± 0.0	37.4 ± 0.7	38.7 ± 1.2	4.78	EFG	4.11	GH	3.99	EFGH	0.79	AB
DAR_POL20	11.6 ± 0.1	8.3 ± 0.1	1.2 ± 0.2	30.2 ± 1.8	42.1 ± 0.8	4.36	GHI	3.47	JK	3.52	GHIJ	0.84	AB
DAT_POL20	11.8 ± 0.1	10.8 ± 0.2	1.4 ± 0.1	30.8 ± 1.5	41.2 ± 1.3	4.15	HIJ	3.93	HI	3.69	GHI	0.46	D
ELI_AUS20	11.2 ± 0.3	13.2 ± 0.1	1.8 ± 0.0	40.6 ± 1.2	51.7 ± 0.9	7.11	A	6.89	A	6.38	A	0.73	BC
RET_FIN20	9.0 ± 0.1	12.4 ± 0.0	1.4 ± 0.4	39.9 ± 1.8	48.0 ± 2.0	6.72	A	6.46	B	5.87	AB	0.84	AB
DANT_HUN19	11.1 ± 0.4	9.0 ± 0.0	1.1 ± 0.0	42.8 ± 2.7	41.4 ± 0.3	4.86	EFG	4.19	GH	4.08	EFG	0.79	AB
RYEF_HUN19	8.2 ± 0.5	11.0 ± 0.1	1.7 ± 0.0	28.8 ± 2.3	56.6 ± 0.7	5.69	BC	4.89	E	4.70	CD	0.99	A
WIB_HUN18	11.7 ± 0.0	11.2 ± 0.1	1.2 ± 0.0	47.5 ± 0.3	49.2 ± 1.8	5.75	BC	5.35	D	4.88	C	0.88	AB
WIB_HUN19	9.4 ± 0.5	10.2 ± 0.2	1.3 ± 0.0	33.2 ± 1.7	56.6 ± 1.2	5.38	CDE	4.43	FG	4.37	CDE	1.01	A
RYEF_HUN18	10.7 ± 0.4	10.8 ± 0.1	1.9 ± 0.0	59.1 ± 3.2	68.0 ± 2.1	5.58	BCD	4.89	E	4.79	CD	0.79	AB

Table 5.17: Determinations of the 32 rye cultivars with RP-HPLC. Gluten: Sum of reduced prolamins and glutelins. Protein: Sum of reduced prolamins glutelins. albumins and globulins (ALGL). Values are given as means (n = 3). (g/100 g) and different capital letters indicate significant differences between the samples in each column (one-way ANOVA. Tukey's post hoc test. $p < 0.05$). Data already published in Xhaferaj et al. (2023a).

Sample code	PROL/ GLUT	Protein		Gluten		Prolamins		reduced Prolamins		Glutelins		AL/GL		ω-secalins		γ-75k-secalins		γ-40k-secalins		HMW-secalins	
HAN_CAN17	4.1	6.00	PQ	2.88	RS	2.21	PQ	2.32	VW	0.56	MN	3.12	CDE	0.47	ST	1.60	LMN	0.38	P	0.43	EFGH
SPO_CAN17	4.2	6.84	KL	3.69	M	2.94	L	2.98	PQ	0.71	IJKL	3.15	BCDE	0.68	OP	2.11	D	0.48	O	0.42	EFGH
RYM_CAN17	4.3	6.61	LM	3.41	NO	2.64	M	2.77	QR	0.65	JKLM	3.20	BCD	0.58	QR	1.89	EFGH	0.43	OP	0.51	DEF
DAC_CAN17	4.5	6.29	NO	3.18	OPQ	2.57	MN	2.60	RST	0.58	MN	3.11	CDE	0.60	PQ	1.78	IJ	0.38	P	0.26	KL
ARO_CAN17	4.1	5.80	QR	2.77	ST	2.14	QR	2.23	WX	0.55	MN	3.03	DEF	0.44	T	1.53	MNO	0.38	P	0.42	EFGH
HAZ_CAN17	4.3	7.99	H	4.75	I	3.78	I	3.86	IJ	0.90	EFG	3.23	BCD	0.93	IJK	2.73	A	0.61	N	0.48	DEFG
WHE_CAN17	4.3	6.14	NOP	3.06	PQR	2.35	OPQ	2.49	STUV	0.57	MN	3.08	DE	0.50	QRST	1.71	JK	0.38	P	0.47	DEFG
SAN_EST19	4.0	4.75	T	2.57	T	1.96	R	2.06	X	0.51	N	2.18	K	0.53	QRS	1.03	R	0.74	M	0.27	JKL
VAM_EST19	5.1	6.17	NOP	3.74	LM	3.07	L	3.13	OP	0.61	LMN	2.43	J	0.89	JKL	1.49	NO	0.98	HI	0.39	GHIJ
DAN_GER19	3.0	5.46	S	3.60	MN	2.63	M	2.69	RS	0.91	DEFG	1.85	L	0.68	OP	1.60	KLM	1.04	GH	0.28	IJKL
REF_GER19	3.9	4.21	U	2.79	ST	2.20	PQ	2.22	WX	0.57	MN	1.41	M	0.49	RST	1.17	PQ	0.94	IJ	0.20	L
DAD_HUN18	3.9	5.59	RS	2.97	QRS	2.40	NOP	2.37	UVW	0.61	LMN	2.62	IJ	0.70	NO	1.13	QR	0.75	M	0.99	A
KAU_LAT20	3.5	7.02	JK	4.57	I	3.64	IJ	3.55	LMN	1.02	BCD	2.45	J	1.00	HI	1.86	FGHI	1.28	E	0.43	EFGH
DAG_POL20	4.2	6.61	LM	3.82	LM	3.10	L	3.08	P	0.74	IJK	2.79	GHI	0.86	KLM	1.48	O	1.08	FG	0.40	FGH
DA_HUN17	3.4	6.08	OPQ	3.33	O	2.66	M	2.58	RSTU	0.75	IJ	2.75	GHI	0.83	LM	1.25	P	0.87	KL	0.38	GHIJK
DAD_HUN17	3.3	5.98	PQ	3.25	OP	2.52	MNO	2.50	STUV	0.76	HIJ	2.73	HI	0.70	NO	1.25	P	0.90	JK	0.40	FGHI
WIB_HUN17	4.1	5.80	QR	2.99	QRS	2.53	MNO	2.40	TUVW	0.59	MN	2.81	FGHI	0.79	MN	1.04	R	0.82	L	0.34	HIJK
DR_HUN19	3.7	8.55	EF	5.33	FG	4.20	GH	4.17	GH	1.15	A	3.23	BCD	1.27	E	1.95	EF	1.67	C	0.44	EFGH
ELEG_AUS20	5.8	9.34	C	6.28	C	5.35	C	5.36	C	0.92	CDEF	3.05	DE	1.45	D	2.32	C	2.04	B	0.34	HIJK
ELE_AUS20	5.6	8.22	GH	5.27	G	4.53	F	4.47	F	0.80	GHI	2.96	EFG	1.26	E	1.82	GHI	1.65	C	0.53	DE
ELV_EST19	4.9	6.76	KL	4.27	J	3.51	JK	3.55	KLM	0.72	IJKL	2.48	J	0.96	HIJ	1.70	JKL	1.24	E	0.37	GHIJK
DAD_HUN19	4.2	8.32	FG	5.01	H	4.02	H	4.04	HI	0.97	BCDE	3.31	BC	1.16	F	1.90	EFG	1.41	D	0.53	DE
DAN_HUN19	4.5	7.50	I	4.55	I	3.75	I	3.72	JKL	0.83	FGHI	2.95	EFGH	1.12	FG	1.76	IJ	1.27	E	0.40	FGHI
DAR_POL20	3.6	7.15	J	4.23	JK	3.32	K	3.32	NO	0.90	DEFG	2.93	EFGH	1.01	HI	1.61	KLM	1.13	F	0.47	DEFG
DAT_POL20	5.4	6.40	MN	3.98	KL	3.33	K	3.35	MNO	0.62	KLMN	2.42	J	1.03	GH	1.52	MNO	1.04	GH	0.39	GHIJ
ELI_AUS20	6.3	11.19	A	7.83	A	6.71	A	6.75	A	1.08	AB	3.36	B	2.12	B	2.82	A	2.19	A	0.71	B
RET_FIN20	5.8	10.39	B	7.15	B	6.15	B	6.10	B	1.05	AB	3.24	BCD	1.92	C	2.55	B	2.01	B	0.66	BC
DANT_HUN19	4.6	7.64	I	4.60	I	3.81	I	3.78	JK	0.82	FGHI	3.04	DE	1.20	EF	1.79	HIJ	1.21	E	0.32	HIJK
RYEF_HUN19	4.5	9.39	C	5.75	E	4.69	EF	4.71	E	1.04	ABC	3.64	A	1.42	D	2.13	D	1.62	C	0.58	CD
WIB_HUN18	5.6	9.04	D	6.01	D	5.10	D	5.09	D	0.92	DEF	3.03	DEF	2.34	A	1.98	E	1.00	H	0.68	BC
WIB_HUN19	4.0	8.73	E	5.38	FG	4.27	G	4.31	FG	1.07	AB	3.36	B	1.29	E	1.94	EF	1.61	C	0.54	DE
RYEF_HUN18	5.4	8.62	E	5.57	EF	4.77	E	4.70	E	0.87	EFGH	3.04	DE	2.18	B	1.79	HIJ	0.90	JK	0.71	B

Table 5.18: The relative molecular weight distribution with GP-HPLC categorized according to the following molecular weight ranges: (1) > 66 kDa; (2) 66-29 kDa; (3) 29-12.4 kDa; (4) < 12.4 kDa. Values are given as means (n = 3) and percentages (%) referring to the relative percentages of the isolates in relation to the measured AUC at 210 nm by GP-HPLC. The capital letters indicate significant differences between the samples in each column (one-way ANOVA, Tukey's post hoc test, $p < 0.05$). Data already published in Xhaferaj et al. (2023a).

Sample code	Prolamins				Reduced Prolamins				Glutelins			
	1	2	3	4	1	2	3	4	1	2	3	4
HAN_CAN17	30.2 FGHI	11.1 DE	22.2 N	36.6 EFGH	5.4 CDEF	7.0 QR	53.3 DEFGH	34.3 CDE	9.8 OP	9.6 M	31.7 I	48.9 AB
SPO_CAN17	27.9 LMN	11.0 EF	22.8 LMN	38.3 D	5.1 DEFGH	7.4 PQR	54.9 ABCD	32.7 DEF	11.1 IJKLMNO	10.9 IJK	32.8 FGHI	45.2 CD
RYM_CAN17	28.1 KLMN	10.9 EF	22.8 LMN	38.2 D	5.5 CDEF	7.7 NOPQR	54.2 BCDE	32.6 DEF	10.8 JKLMNOP	11.5 FGHI	33.7 EFGH	44.0 DE
DAC_CAN17	28.5 JKL	11.0 EF	23.3 KLM	37.2 DEFG	3.8 IJKL	6.8 R	55.4 A BC	34.0 DEF	9.8 MNOP	9.5 M	31.4 IJ	49.2 AB
ARO_CAN17	31.3 EF	11.7 B	22.6 MN	34.4 I	5.6 CDEF	7.7 NOPQR	54.0 BCDEF	32.7 DEF	10.1 LMNOP	10.0 KLM	32.6 GHI	47.2 BC
HAZ_CAN17	27.7 LMNO	10.6 GHI	23.7 JK	37.9 D	5.3 CDEF	7.6 OPQR	55.1 A BCD	32.0 FG	12.7 GH	11.6 FGHI	34.6 CDEF	41.1 FGH
WHE_CAN17	30.9 FGH	10.8 EFG	22.1 N	36.2 FGH	5.1 DEFGH	7.4 PQR	54.0 BCDEF	33.5 DEF	9.5 P	9.7 M	31.4 IJ	49.3 A B
SAN_EST19	30.3 FGHI	11.5 BC	21.0 O	37.2 DEFG	5.7 CDE	8.2 MNOP	51.4 HIJKL	34.7 BCD	11.6 HIJKL	10.9 IJK	34.0 DEFGH	43.6 DEF
VAM_EST19	29.3 IJKO	11.0 EF	23.9 JK	35.9 GH	5.5 CDEF	8.1 MNOPQ	50.1 KLM	36.2 BC	11.0 IJKLMNOP	10.8 IJKL	34.5 DEFG	43.8 DE
DAN_GER19	37.1 A	12.0 A	27.5 EF	23.4 O	4.2 GHIJK	12.8 EF	55.7 A B	27.3 IJ	15.0 CDE	18.2 A	36.7 A B	30.2 N
REF_GER19	33.8 BC	8.9 M	27.4 EF	29.9 LM	3.3 KL	9.7 JKL	54.0 BCDEF	33.0 DEF	16.0 ABCD	16.7 B	31.4 IJ	35.9 KL
DAD_HUN18	30.5 FGHI	11.0 EF	22.6 MN	35.9 GH	7.2 B	13.5 E	50.2 JKLM	29.1 HI	11.3 HIJKLMN	10.2 KLM	31.7 I	46.8 BC
KAU_LAT20	31.0 FG	9.1 M	29.2 CD	30.8 L	4.1 HIJKL	10.1 IJK	51.9 GHIJK	34.0 DEF	16.6 A B	16.6 B	32.7 GHI	34.1 LM
DAG_POL20	34.1 BC	10.6 GHI	28.0 E	27.4 N	4.7 EFGHIJ	11.5 G	52.0 FGHIJK	31.8 FG	15.6 BCDE	15.1 CD	31.4 IJ	37.9 IJK
DA_HUN17	27.3 LMNO	10.2 JK	22.6 MN	39.9 C	9.4 A	21.8 A B	47.0 NO	21.8 K	11.6 GHIJK	11.2 HIJ	32.3 HI	44.8 CD
DAD_HUN17	29.7 HIJ	10.2 JK	22.8 LMN	37.4 DEF	6.5 BC	11.4 G	53.2 DEFGH	28.9 HI	12.4 GHI	11.3 GHIJ	32.8 FGHI	43.5 DEF
WIB_HUN17	25.4 Q	10.5 HI	22.8 LMN	41.3 B	9.5 A	19.3 C	45.0 O	26.2 J	10.3 KLMNOP	9.8 LM	29.6 JK	50.3 A
DR_HUN19	26.6 OPQ	10.1 JK	23.5 JKL	39.7 C	8.8 A	22.1 A	49.8 LM	19.3 L	12.4 GHI	12.0 EFGH	33.6 EFGH	42.0 EFG
ELEG_AUS20	33.1 CD	10.0 K	28.8 D	28.0 N	6.0 CD	10.9 GHI	50.8 IJKL	32.3 EF	14.8 DE	15.4 CD	34.0 DEFGH	35.8 KL
ELE_AUS20	31.2 EF	11.1 E	26.8 F	30.9 KL	5.6 CDEF	9.6 JKL	46.0 O	38.8 A	14.4 EF	11.3 HIJ	28.4 K	45.9 CD
ELV_EST19	29.9 GHI	11.1 E	23.4 KL	35.6 HI	5.3 DEFG	8.5 LMNOP	54.3 BCDE	31.9 FG	11.1 IJKLMNO	12.0 EFGH	37.9 A	39.0 HIJ
DAD_HUN19	28.5 JKL	10.3 IJK	24.0 IJK	37.2 DEFG	7.4 B	15.7 D	53.9 BCDEFG	23.1 K	11.8 GHIJ	12.3 EF	35.4 BCDE	40.5 GHI
DAN_HUN19	27.6 LMNO	10.4 IJ	24.3 IJ	37.7 DE	5.2 DEFGH	8.7 LMNO	52.2 EFGHIJ	33.8 DEF	11.3 HIJKLM	11.7 EFGHI	36.4 A BC	40.6 GH
DAR_POL20	28.2 KLM	9.6 L	32.9 A	29.4 M	2.9 L	11.4 GH	56.8 A	28.8 HI	17.4 A	18.8 A	32.1 HI	31.7 MN
DAT_POL20	34.8 B	10.7 FGH	29.8 BC	24.6 O	5.0 DEFGHI	11.7 FG	53.4 CDEFGH	30.0 GH	16.4 A BC	14.5 D	29.8 JK	39.3 HI
ELI_AUS20	32.4 DE	10.6 GHI	29.6 CD	27.4 N	4.5 FGHIJ	10.2 HIJK	48.5 MN	36.8 A B	13.1 FG	14.7 D	35.6 BCD	36.7 JKL
RET_EST20	19.9 R	10.4 IJ	15.6 P	54.2 A	3.6 JKL	10.8 GHIJ	52.3 EFGHIJ	33.3 DEF	15.7 BCDE	16.1 BC	33.1 FGHI	35.1 L
DANT_HUN19	28.0 KLMN	10.4 IJ	25.2 GH	36.4 EFGH	5.3 CDEF	8.8 LMN	52.5 EFGHI	33.3 DEF	11.4 HIJKL	12.2 EFG	36.8 A B	39.6 GHI
RYEF_HUN19	27.1 MNO	10.5 HI	24.8 HI	37.7 DE	9.0 A	20.9 B	51.6 HIJKL	18.5 L	12.1 GHIJ	12.5 EF	35.2 BCDE	40.2 GHI
WIB_HUN18	27.4 LMNO	10.6 GHI	29.8 BC	32.2 JK	5.7 CDE	8.4 MNOP	52.2 EFGHIJ	33.6 DEF	11.5 HIJKL	10.3 JKLM	34.3 DEFG	43.8 DE
WIB_HUN19	26.9 NOP	9.7 L	25.7 G	37.8 DE	5.7 CDE	9.3 KLM	55.7 A B	29.3 HI	11.9 GHIJ	12.7 E	35.0 BCDE	40.4 GHI
RYEF_HUN18	25.7 PQ	11.4 CD	30.4 B	32.5 J	7.4 B	16.2 D	50.6 IJKL	25.9 J	9.8 NOP	9.8 LM	35.4 BCDE	45.0 CD

Table 5.19: Determinations of the seven rye cultivars of different harvest years and their mixtures with RP-HPLC. Gluten: Sum of reduced prolamins and glutelins. Protein: Sum of reduced prolamins glutelins. albumins and globulins (ALGL). Values are given as means (g/100 g) ($n = 3$) and different capital letters indicate significant differences between the samples in each column (one-way ANOVA. Tukey's post hoc test. $p < 0.05$).

Sample code	Protein		Gluten		Prolamins		reduced Prolamins		Glutelins		ALGL		ω-secalins		γ-75k-secalins		γ-40k-s secalins		HMW-secalins	
DAT_POL20	6.40	GH	3.98	FG	3.33	G	3.35	H	0.62	DE	2.42	E	1.03	F	1.52	HI	1.04	F	0.39	EF
DAT_POL22	7.03	F	4.04	F	3.29	G	3.50	GH	0.54	E	2.99	CD	1.28	D	1.60	GH	1.04	F	0.44	DEF
DAN_GER19	5.46	J	3.60	G	2.63	HI	2.69	IJ	0.91	BC	1.85	F	0.68	G	1.60	GH	1.04	F	0.28	F
DAN_GER22	6.86	FG	4.04	F	2.74	H	3.33	H	0.72	D	2.82	D	1.45	C	1.43	I	1.02	FG	0.73	A
ELI_AUS20	11.19	A	7.83	A	6.71	A	6.75	A	1.08	A	3.36	AB	2.12	A	2.82	A	2.19	A	0.71	ABC
ELI_AUS22	6.96	F	3.70	FG	2.89	H	2.98	I	0.72	D	3.26	ABC	1.09	EF	1.45	HI	1.00	FG	0.39	EF
RET_FIN20	10.39	B	7.15	B	6.15	B	6.10	B	1.05	A	3.24	ABC	1.92	B	2.55	B	2.01	B	0.66	ABCD
RET_FIN22	8.87	C	5.39	D	4.61	DE	4.49	EF	0.90	BC	3.48	A	1.54	C	2.05	CD	1.54	C	0.48	BCDEF
RYEF_HUN18	8.62	CD	5.57	D	4.77	D	4.70	DE	0.87	C	3.04	BCD	2.18	A	1.79	EF	0.90	GH	0.71	AB
RYEF_HUN22	7.76	E	5.31	D	4.38	EF	4.66	DE	0.65	DE	2.45	E	1.84	B	1.92	DE	1.40	D	0.59	ABCDE
WHE_CAN17	6.14	HI	3.06	H	2.35	I	2.49	J	0.57	E	3.08	BCD	0.50	H	1.71	FG	0.38	I	0.47	CDEF
WHE_CAN22	9.06	C	5.69	D	4.57	DE	4.99	D	0.71	D	3.37	AB	1.77	B	2.17	C	1.59	C	0.73	A
WIB_HUN17	5.80	IJ	2.99	H	2.53	HI	2.40	J	0.59	DE	2.81	D	0.79	G	1.04	J	0.82	H	0.34	F
WIB_HUN22	10.01	B	6.57	C	5.15	C	5.51	C	1.06	A	3.44	A	2.20	A	2.22	C	1.89	B	0.66	ABCD
Mixture 1	8.09	DE	4.83	E	3.59	G	3.81	G	1.02	AB	3.27	ABC	1.19	DEF	1.71	FG	1.23	E	0.70	ABC
Mixture 2	8.07	E	4.83	E	4.03	F	4.18	F	0.65	DE	3.23	ABC	1.21	DE	1.78	EF	1.36	D	0.48	BCDEF

Table 5.20: Relative secalin distributions in the four isolates compared to the flours measured by RP-HPLC. Values are given as means ($n = 3$), (%) and different capital letters indicate significant differences between the relative secalin distribution of different isolates (one-way ANOVA, Tukey's post hoc test, $p < 0.05$).

Samples		ω/HMW-secalins		γ-75k-secalins		γ-40k-secalins	
Isolates	Gluten	31.7	C	39.2	D	29.1	B
	AWEP	28.7	E	53.4	A	17.9	D
	Prolamins	32.3	BC	35.6	EF	32.1	A
	Glutelins	30.2	D	46.3	C	23.5	C
Flours	R7 Gluten	32.9	B	37.4	DE	29.7	B
	R7 AWEP	29.9	D	47.7	BC	22.5	C
	R7 Prolamins	37.0	A	35.0	F	28.0	B
	R7 Glutelins	22.5	F	49.0	B	28.5	B

Table 5.21: The relative molecular weight distribution with GP-HPLC categorized for 7 rye cultivars with different collection years according to the following molecular weight ranges: (1) > 66 kDa; (2) 66-29 kDa; (3) 29-12.4 kDa; (4) < 12.4 kDa. Values are given as means (n = 3) and percentages (%) referring to the relative percentages of the isolates in relation to the measured AUC at 210 nm by GP-HPLC. The capital letters indicate significant differences between the samples in each column (one-way ANOVA, Tukey's post hoc test, $p < 0.05$).

Sample code	Prolamins				Reduced Prolamins				Glutelins			
	1	2	3	4	1	2	3	4	1	2	3	4
DAT_POL20	34.8 AB	10.7 BCD	29.8 BC	24.6 EF	5.0 CDE	11.7 CD	53.4 AB	30.0 DE	16.4 A	14.5 B	29.8 G	39.3 CDE
DAT_POL22	30.6 ABCD	10.9 BC	26.7 E	31.8 CDE	4.5 CDEF	8.2 E	44.2 CDE	43.2 BC	10.0 DEF	10.7 CDE	37.8 BCD	41.5 CD
DAN_GER19	37.1 A	12.0 A	27.5 DE	23.4 F	4.2 CDEF	12.8 C	55.7 A	27.3 E	15.0 AB	18.2 A	36.7 CDE	30.2 F
DAN_GER22	27.8 BCDE	10.6 CD	27.1 E	34.5 BCD	2.1 G	8.4 E	44.7 CDE	44.8 ABC	7.9 G	11.0 CDE	42.9 A	38.2 DE
ELI_AUS20	32.4 ABC	10.6 CD	29.6 BC	27.4 DEF	4.5 CDEF	10.2 D	48.5 BCD	36.8 CD	13.1 BC	14.7 B	35.6 CDE	36.7 DE
ELI_AUS22	23.3 EF	10.2 CD	28.5 CD	38.0 BC	3.9 CDEF	6.8 E	36.1 F	53.2 A	8.5 FG	9.3 E	33.8 EF	48.5 AB
RET_FIN20	19.9 F	10.4 CD	15.6 H	54.2 A	3.6 DEFG	10.8 D	52.3 AB	33.3 DE	15.7 A	16.1 B	33.1 EFG	35.1 EF
RET_FIN22	27.6 BCDE	10.6 CD	28.5 CD	33.3 CD	3.2 FG	7.6 E	44.3 CDE	45.0 ABC	11.8 CD	11.3 CD	37.9 BCD	38.9 CDE
RYEF_HUN18	25.7 CDEF	11.4 AB	30.4 B	32.5 CD	7.4 B	16.2 B	50.6 ABC	25.9 E	9.8 EFG	9.8 DE	35.4 CDE	45.0 ABC
RYEF_HUN22	27.2 CDE	10.6 CD	28.9 C	33.4 CD	3.2 FG	7.6 E	42.9 DE	46.2 AB	10.1 DEF	12.0 C	39.0 BC	38.9 CDE
WHE_CAN17	30.9 ABC	10.8 BCD	22.1 G	36.2 BC	5.1 CD	7.4 E	54.0 AB	33.5 DE	9.5 EFG	9.7 DE	31.4 FG	49.3 A
WHE_CAN22	30.5 ABCDE	10.3 CD	24.8 F	34.3 BCD	5.5 C	8.0 E	39.7 EF	46.8 AB	12.2 C	10.5 CDE	34.9 DEF	42.3 BCD
WIB_HUN17	25.4 CDEF	10.5 CD	22.8 G	41.3 B	9.5 A	19.3 A	45.0 CDE	26.2 E	10.3 DEF	9.8 DE	29.6 G	50.3 A
WIB_HUN22	23.5 DEF	10.1 D	32.4 A	34.0 BCD	3.3 EFG	8.3 E	44.2 CDE	44.2 ABC	11.4 CDE	15.0 B	40.5 AB	33.1 EF

Table 5.22: Results of the independent two-sample T-test (null hypothesis) analysis performed with a significance level of 0.05 using Origin Pro 2019b. The factors show the significance differences between the total amounts (g/100 g (RP-HPLC)) between the first and the second cultivar collection analyzed with RP-HPLC (2022).

Sample	p-value	Significant	t(4)=t-Statistics	p-value	Significant	t(4)=t-Statistics
	Protein				γ-75k-secalins	
Wheeler	2.46×10^{-5}	Yes	-22.15	7.24×10^{-3}	Yes	-5.05
Daniello	4.11×10^{-5}	Yes	-19.46	1.84×10^{-3}	Yes	7.34
Dankowskie-T.	9.47×10^{-5}	Yes	-15.76	2.34×10^{-3}	Yes	-6.88
Wibro	1.24×10^{-5}	Yes	-26.30	3.88×10^{-5}	Yes	-19.75
Elias	8.89×10^{-6}	Yes	28.60	1.07×10^{-6}	Yes	48.61
Reetta	1.84×10^{-3}	Yes	7.33	4.97×10^{-5}	Yes	18.55
Rye Food	7.12×10^{-4}	Yes	9.41	7.86×10^{-3}	Yes	-4.93
Mixture	9.04×10^{-1}	No	0.13	1.56×10^{-1}	No	-1.75
	Gluten				γ-40k-secalins	
Wheeler	1.10×10^{-4}	Yes	-15.18	1.11×10^{-5}	Yes	-27.08
Daniello	5.56×10^{-3}	Yes	-5.44	4.36×10^{-1}	No	0.86
Dankowskie-T.	5.01×10^{-2}	No	-2.77	7.99×10^{-1}	No	0.27
Wibro	8.30×10^{-6}	Yes	-29.10	3.72×10^{-5}	Yes	-19.96
Elias	3.04×10^{-7}	Yes	66.62	2.10×10^{-5}	Yes	23.05
Reetta	1.25×10^{-5}	Yes	26.28	4.63×10^{-6}	Yes	33.70
Rye Food	2.57×10^{-2}	Yes	3.47	2.70×10^{-6}	Yes	-38.58
Mixture	9.67×10^{-1}	No	-0.04	8.68×10^{-3}	Yes	-4.80
	Prolamins				ω-secalins	
Wheeler	8.51×10^{-5}	Yes	-16.19	6.35×10^{-4}	Yes	-9.69
Daniello	7.97×10^{-4}	Yes	-9.13	2.32×10^{-3}	Yes	-6.90
Dankowskie-T.	7.97×10^{-4}	Yes	-9.13	4.29×10^{-3}	Yes	5.84
Wibro	1.32×10^{-6}	Yes	-46.15	1.94×10^{-6}	Yes	-41.90
Elias	6.42×10^{-7}	Yes	55.25	7.27×10^{-8}	Yes	95.30
Reetta	2.18×10^{-5}	Yes	22.82	6.19×10^{-5}	Yes	17.55
Rye Food	3.84×10^{-1}	No	0.98	5.03×10^{-4}	Yes	10.29
Mixture	6.04×10^{-2}	No	-2.59	5.25×10^{-1}	No	-0.70
	Glutelins				HMW-Secalins	
Wheeler	3.08×10^{-3}	Yes	-6.39	4.37×10^{-3}	Yes	-5.81
Daniello	2.71×10^{-4}	Yes	12.06	1.70×10^{-3}	Yes	-7.49
Dankowskie-T.	1.75×10^{-2}	Yes	3.90	2.14×10^{-1}	No	-1.48
Wibro	1.27×10^{-3}	Yes	-8.08	7.12×10^{-3}	Yes	-5.07
Elias	1.24×10^{-3}	Yes	8.15	1.01×10^{-4}	Yes	15.51
Reetta	1.42×10^{-3}	Yes	7.85	1.16×10^{-3}	Yes	8.27
Rye Food	1.89×10^{-3}	Yes	7.29	3.76×10^{-1}	No	0.99
Mixture	1.43×10^{-3}	Yes	7.84	7.03×10^{-2}	No	2.45
	Albumins/Globulins					
Wheeler	8.19×10^{-2}	No	-2.31			
Daniello	2.64×10^{-6}	Yes	-38.77			
Dankowskie-T.	2.01×10^{-4}	Yes	-13.02			
Wibro	2.28×10^{-4}	Yes	-12.61			
Elias	4.21×10^{-1}	No	0.90			
Reetta	2.52×10^{-1}	No	-1.34			
Rye Food	3.27×10^{-4}	Yes	11.50			
Mixture	4.42×10^{-1}	No	0.85			

Table 5.23: Results of the independent two-sample T-test (null hypothesis) analysis performed with a significance level of 0.05 using Origin Pro 2019b. The factors show the significance differences between the relative distribution of the secalin fractions measured with RP-HPLC between the samples of the first and the second cultivar collection (2022).

Sample	p-value	Significant	t(4)=t-Statistics
	γ-75k-secalins		
Wheeler	9.95×10^{-6}	Yes	27.80
Daniello	1.71×10^{-4}	Yes	13.56
Dankowskie-Turkus	2.21×10^{-2}	Yes	-3.63
Wibro	6.41×10^{-2}	No	2.54
Elias	7.25×10^{-3}	Yes	-5.05
Reetta	2.07×10^{-6}	Yes	-41.22
Rye Food	7.69×10^{-5}	Yes	-16.61
Mixture	8.89×10^{-2}	No	-2.24
	γ-40k-secalins		
Wheeler	5.14×10^{-7}	Yes	-58.43
Daniello	7.12×10^{-3}	Yes	5.07
Dankowskie-Turkus	1.53×10^{-1}	No	1.76
Wibro	7.63×10^{-2}	No	-2.38
Elias	4.66×10^{-1}	No	0.81
Reetta	1.62×10^{-2}	Yes	-3.99
Rye Food	5.98×10^{-7}	Yes	-56.25
Mixture	8.41×10^{-3}	Yes	-4.84
	γ-secalins		
Wheeler	2.94×10^{-3}	Yes	-6.47
Daniello	1.86×10^{-2}	Yes	-3.83
Dankowskie-Turkus	1.92×10^{-3}	Yes	7.26
Wibro	8.53×10^{-2}	No	-2.27
Elias	6.37×10^{-5}	Yes	17.42
Reetta	2.40×10^{-3}	Yes	6.83
Rye Food	1.86×10^{-3}	Yes	7.32
Mixture	2.33×10^{-1}	No	-1.41
	HMW-Secalins		
Wheeler	7.91×10^{-2}	No	2.34
Daniello	9.80×10^{-4}	Yes	-8.66
Dankowskie-Turkus	2.50×10^{-1}	No	-1.34
Wibro	3.76×10^{-1}	No	1.00
Elias	3.38×10^{-2}	Yes	-3.17
Reetta	2.86×10^{-1}	No	1.23
Rye Food	4.92×10^{-1}	No	0.76
Mixture	3.27×10^{-2}	Yes	3.21

Table 5.24: Results of the independent two-sample T-test (null hypothesis) analysis performed with a significance level of 0,05 using Origin Pro 2019b. The factors show the significance differences between the relative distribution of the secalin fractions measured with RP-HPLC between the Isolates and the flours.

Sample	p-value	Significance	t(4)=t-Statistics
	γ-75k-secalins		
Prolamins	0.62	No	0.53
Glutelins	1.78×10^{-4}	Yes	-13.42
Gluten	1.73×10^{-4}	Yes	13.53
AWEP	1.11×10^{-4}	Yes	15.13
	γ-40k-secalins		
Prolamins	1.08×10^{-2}	Yes	4.50
Glutelins	1.82×10^{-5}	Yes	-23.88
Gluten	1.22×10^{-2}	Yes	-4.34
AWEP	1.10×10^{-3}	Yes	-8.40
	γ/HMW-secalins		
Prolamins	7.45×10^{-4}	Yes	-9.30
Glutelins	1.03×10^{-6}	Yes	49.06
Gluten	8.64×10^{-5}	Yes	-16.13
AWEP	1.03×10^{-2}	Yes	-4.57

Table 5.25: Search procedure to identify immunactive epitopes within peptides identified by LC-MS/MS. R5 mAb: The epitopes recognized by R5 mAb ELISA (Osman et al., 2001; Kahlenberg et al., 2006; Amnuaycheewa et al., 2022), Immunactive Peptides recognized by CD4+ T cells (Sollid et al., 2020). Potentially dangerous epitopes selechted based on the EFSA search strategy (Q-X1-P-X2; X1 = L, Q, F, S, E; X2 = Y, F, A, V, Q) (Naegeli et al., 2017).

R5 mAb	Immunoactive peptides		Potentially dangerous peptides
QQPFP	EGSFQPSQE	PFPQPQQPF	QLPY
QQQFP	EGSFQPSQE	PFPQPQQPF	QQPQ
LQPFP	EGSFQPSQQ	PFPQQPEQI	QFPQ
QLPFP	EGSFQPSQQ	PFSEQEQPV	QSPQ
QLPYP	EQPQQPFPQ	PFSEQQQPV	QLPQ
QLPTF	EQPQQPYPE	PFSQQEQPV	QEPQ
QQSFP	EQPQQPYPQ	PFSQQQQPV	QQPA
QQTFP	PFSEQEQPV	PQPELPYPQ	QFPV
QQPYP	PFSEQQQPV	PQPEQEFPQ	QEPA
PQPFP	PFSQQEQPV	PQPEQPFCQ	QQPV
QQPFPQ	PFSQQQQPV	PQPEQPFPQ	QQPF
QQPFPL	PQQSFPEQE	PQPEQPFPQ	QFPA
PQQPFP	PQQSFPEQE	PQPEQPFPW	QSPA
SQQPFP	PQQSFPEQQ	PQPEQQFPQ	QLPA
QLPFPQ	PQQSFPEQQ	PQPQLPYPQ	QEPF
QRPFAQ	PQQSFPQQE	PQPQQEFPQ	QLPV
QQSFPQ	PQQSFPQQE	PQPQQPFCQ	QLPF
QXPW/FP	PQQSFPQQQ	PQPQQPFPQ	QSPF
	PQQSFPQQQ	PQPQQPFPQ	QFPF
	QGSFQPSQE	PQPQQPFPW	QSPY
	QGSFQPSQE	PQPQQQFPQ	QSPV
	QGSFQPSQQ	PQQSFPEQQ	QFPY
	QGSFQPSQQ	PQQSFPQQQ	QQPY
	QGSVQPQQL	PYPEQEEPF	QEPY
	QGYYPTSPQ	PYPEQEQPF	QEPV
	QGYYPTSPQ	PYPEQEQPI	
	QQPQQPFPQ	PYPEQPQPY	
	QQPQQPYPE	PYPEQQEPF	
	QQPQQPYPQ	PYPEQQQPF	
	QYSQPEQPI	PYPEQQQPI	
	QYSQPQQPI	PYPQPELPY	
	FRPEQPYPQ	PYPQPQLPY	
	FRPQQPYPQ	PYPQQPQPY	
	IQPEQPAQL	QQPEQPFPQ	
	IQPQQPAQL	QQPEQPYPQ	
	LQPEQPFPQ	QQPFPEQPQ	
	LQPQQPFPQ	QQPFPQQPQ	
	PFPEQPEQI	QQPQQPFPQ	
	PFPQPELPY	QQPQQPYPQ	
	PFPQPEQPF	SQPEQEFPQ	
	PFPQPEQPF	SQPEQQFPQ	
	PFPQPEQPF	SQPQQEFPQ	
	PFPQPQLPY	SQPQQQFPQ	
	PFPQPQQPF		

Barley Reference Material

Table 5.26: Summary of the analytical characterization of 35 barley flours given as range and mean (n = 3) ± standard deviation (SD). [a]Ratio of reduced prolamins and glutelins measured by RP-HPLC; RP-HPLC: reversed-phase high-performance liquid chromatography; GP-HPLC: gel permeation chromatography; ALGL: albumins/globulins; HMW: high-molecular-weight. Data already published in Xhaferaj et al. (2023b).

g/100 g flour	Parameters	Range min		max	Mean ± SD		
	Moisture	8.75	-	11.42	10.21	±	0.76
	Fat	1.25	-	2.22	1.67	±	0.25
Protein content	Crude protein (Dumas, 5.83)	8.77	-	19.93	11.57	±	2.05
	Protein (RP-HPLC)	7.11	-	18.14	9.50	±	1.99
Gluten content	Gluten (RP-HPLC)	4.89	-	15.87	7.21	±	1.94
	Gluten (GP-HPLC)	4.40	-	15.46	6.71	±	1.91
	Gluten (R5)	3.40	-	166.84	41.88	±	33.79
	Gluten (G12)	7.31	-	94.04	30.68	±	22.44
Osborne fractions	Prolamins (RP-HPLC)	1.85	-	11.29	4.35	±	1.69
	red. Prolamins (GP-HPLC)	1.59	-	9.03	3.97	±	1.40
	Glutelins (RP-HPLC)	1.53	-	4.57	2.86	±	0.64
	Glutelins (GPC)	1.34	-	6.43	2.74	±	0.88
	Prolamins/glutelins Ratio[a]	0.58	-	3.02	1.59	±	0.63
Protein fractions	ALGL (RP-HPLC)	2.01	-	2.65	2.29	±	0.14
	D-hordeins (RP-HPLC)	0.76	-	1.54	1.03	±	0.15
	C-hordeins (RP-HPLC)	0.87	-	6.42	1.91	±	0.98
	B/γ-hordeins (RP-HPLC)	3.00	-	7.91	4.27	±	0.91

Table 5.27: Standard determination as well as ELISA and GP-HPLC results of the 35 barley flours. The values are given as means (n = 3), The GP-HPLC values are given as means (n= 3), (g/100 g) and different capital letters indicate significant differences between the samples in each column (one-way ANOVA, Tukey's post hoc test, $p < 0.05$). Crude Protein measured by Dumas (5.83). Data already published in Xhaferaj et al. (2023b).

Sample code	Moisture	Crude Protein	Fat	ELISA		GP-HPLC							
				Gluten R5	Gluten G12	Gluten [a]		Prolamins		red. Prolamins		Glutelins	
	%			g/100 g		g/100 g							
ARC_AUS20	10.8 ± 0.2	11.8 ± 0.0	1.6 ± 0.1	40.8 ± 1 0.7	28.8 ± 4.3	5.74	IJKL	5.17	GH	4.87	CD	0.88	MN
ADA_AUS20	10.9 ± 0.1	8.8± 0.0	1.4 ± 0.2	18.9 ± 1.1	7.3 ± 1.4	4.43	OP	3.52	NOPQ	3.42	LMNOP	1.01	M
CON_CAN19	8.8 ± 0.2	11.6± 0.1	1.7 ± 0.0	11.8 ± 0.3	27.4 ± 2.9	7.39	CD	4.00	KLM	3.71	IJKLMN	3.68	B
AUS_CAN19	9.3 ± 0.3	9.4 ± 0.2	1.7 ± 0.1	11.9 ± 5.0	22.5 ± 4.7	5.26	LMN	3.80	KLMNO	3.62	JKLMNOP	1.64	L
EVE_DEN20	10.8 ± 0.1	11.5 ± 0.1	1.6 ± 0.2	34.6 ± 0.5	26.5 ± 2.3	6.21	GHIJK	3.38	OPQR	3.19	MNOPQ	3.01	CDEF
FEE_DEN20	11.0 ± 0.1	10.3 ± 0.0	1.4 ± 0.2	43.8 ± 2.7	9.8 ± 1.3	5.68	IJKLM	4.05	JKLM	3.85	FGHIJKLM	1.83	KL
NEW_DEN20	10.8 ± 0.3	11.3 ± 0.1	1.6 ± 0.1	36.2 ± 4.3	24.6 ± 4.2	6.47	EFGHI	3.99	KLM	3.74	HIJKLM	2.73	GHIJ
STA_DEN20	11.1 ± 0.5	11.3 ± 0.1	1.3 ± 0.0	48.0 ± 10.7	15.0 ± 1.0	6.41	FGHIJ	3.92	KLMN	3.77	GHIJKLM	2.64	IJ
PIX_FRA20	9.6 ± 0.1	9.8 ± 0.0	1.5 ± 0.2	14.0 ±2.8	9.9 ± 1.2	4.81	NO	1.73	U	1.58	S	3.22	CD
COC_FRA20	9.3 ± 0.5	10.4 ± 0.0	1.6 ± 0.0	24.3 ± 1.8	16.6 ± 1.8	5.64	JKLM	4.09	JKL	3.72	HIJKLMN	1.92	KL
MEM_FRA20	9.6 ± 0.4	10.3 ± 0.2	1.5 ± 0.1	14.8 ± 0.8	11.0 ± 0.2	5.40	LMN	2.68	S	2.51	QR	2.89	EFGHI
TRA_FRA20	8.8 ± 0.5	10.2 ± 0.0	2.0 ± 0.1	18.5 ± 1.4	11.3 ± 2.6	4.87	MNO	3.13	QR	2.93	NOPQ	1.94	K
PRU_FRA20	8.9 ± 0.3	12.7 ± 0.0	2.2 ± 0.0	33.7 ± 4.0	18.3 ± 1.4	6.95	CDEFG	5.18	GH	4.48	DEFGHI	2.47	J
JAL_GER20	9.8 ± 0.2	13.6 ± 0.1	1.8 ± 0.1	51.2 ± 7.7	11.9 ± 1.3	5.96	HIJKL	5.90	DE	5.49	BC	0.46	P
QUA_GER20	10.5 ± 0.0	9.0 ± 0.1	1.5 ± 0.2	34.5 ± 3.0	11.5 ± 1.9	3.57	Q	3.82	KLMNO	2.85	PQ	0.72	NOP
ACC_GER20	10.6 ± 0.2	11.3 ± 0.0	2.0 ± 0.1	34.0 ± 1.3	20.6 ± 1.5	3.72	PQ	3.93	KLMN	2.87	OPQ	0.84	MN
GKJ_HUN17	10.4 ± 0.1	9.5 ± 0.0	1.6 ± 0.1	3.4 ± 0.9	14.4 ± 0.9	5.40	KLMN	3.64	MNOP	3.55	KLMNOP	1.86	KL
MVI_HUN17	10.6 ± 0.3	11.2 ± 0.1	1.7 ± 0.1	7.0 ± 0.5	17.7 ± 1.0	4.75	NO	4.13	JK	4.03	EFGHIJKL	0.72	NOP
JUM_LAT20	9.9 ± 1.2	9.5 ± 0.2	1.3 ± 0.2	23.3 ± 2.2	12.4 ± 1.1	4.81	NO	2.19	T	2.03	RS	2.78	FGHI
SAU_LAT20	10.6 ± 0.1	9.5 ± 0.0	1.3 ± 0.1	28.4 ± 2.1	14.7 ± 0.5	4.75	NO	2.13	UT	1.96	RS	2.80	FGHI
HOCK_CAN19	9.7 ± 0.7	11.6 ± 0.0	1.5 ± 0.1	17.7 ± 9.3	39.1 ± 3.9	7.23	CDE	3.68	LMNO	3.41	LMNOP	3.82	B
TRA_CAN19	8.9 ± 0.4	11.8 ± 0.1	1.7 ± 0.0	18.9 ± 4.0	37.2 ± 3.8	7.33	CD	4.45	IJ	4.23	DEFGHIJK	3.10	CDE
JAK_GER20	10.7 ± 0.2	11.5 ± 0.4	1.5 ± 0.0	38.5 ± 2.4	52.0 ± 3.2	4.64	NO	4.89	GHI	3.65	KLMNO	0.99	MN
KWSS_HUN17	11.4 ± 0.2	11.1 ± 0.1	2.1 ± 0.0	21.6 ± 7.5	56.8 ± 7.5	6.70	DEFGH	3.20	PQR	3.14	MNOPQ	3.56	B
MOR_HUN17	10.1 ± 0.0	11.5 ± 0.1	1.7 ± 0.2	5.2 ± 1.0	68.1 ± 3.3	7.00	CDEFG	4.67	I	4.35	DEFGHIJ	2.65	HIJ
EST_AUS20	11.4 ± 0.2	11.4 ± 0.1	2.0 ± 0.0	56.1 ± 6.2	49.7 ± 5.4	5.31	LMN	6.16	CD	4.58	DEF	0.72	NOP
EVE_AUS20	10.6 ± 0.6	13.6 ± 0.1	2.0 ± 0.1	85.0 ± 4.4	91.0 ± 4.4	6.42	EFGHIJ	6.47	BC	5.89	B	0.53	OP
ESC_AUS20	11.2 ± 0.4	13.4 ± 0.1	1.5 ± 0.0	58.9 ± 3.7	68.2 ± 10.0	7.07	CDEF	6.66	B	6.16	B	0.91	MN
ANT_GER20	11.0 ± 0.1	11.7 ± 0.1	1.7 ± 0.0	63.2 ± 1.1	52.2 ± 2.8	5.30	LMN	6.06	CDE	4.51	DEFGH	0.79	MNO
GRE_DEN20	10.7 ± 0.4	10.5 ± 0.2	1.3 ± 0.1	66.0 ± 2.7	14.9 ± 1.8	5.65	JKLM	2.99	RS	2.83	PQ	2.82	EFGHI
ABA_LAT20	10.0 ± 0.4	14.1 ± 0.2	1.8 ± 0.0	69.3 ± 1.8	26.5 ± 1.0	7.60	C	5.68	EF	4.68	DE	2.93	EFGH
IRB_LAT20	10.5 ± 0.2	13.3 ± 0.0	1.7 ± 0.1	59.7 ± 4.9	26.5 ± 1.4	7.45	CD	5.30	FG	4.55	DEFG	2.90	EFGHI
KOR_LAT19	10.2 ± 0.2	19.9 ± 0.1	2.0 ± 0.1	77.6 ± 3.6	27.9 ± 0.6	15.46	A	11.12	A	9.03	A	6.43	A
CEL_CAN19	10.0 ± 0.3	12.0 ± 0.0	1.7 ± 0.1	127.9 ± 5.0	37.3 ± 4.2	7.50	CD	4.80	HI	4.55	DEFG	2.95	DEFG
DAMO_HUN17	9.2 ± 0.3	14.9 ± 0.1	2.2 ± 0.1	166.8 ± 14.6	94.0 ± 3.3	9.34	B	6.74	B	6.08	B	3.25	C

Table 5.28: Determinations of the 35 barley cultivars with RP-HPLC. Gluten: Sum of reduced prolamins and glutelins. Protein: Sum of reduced prolamins glutelins. albumins and globulins (AIGL). Values are given as means (n = 3), (g/100 g) and different capital letters indicate significant differences between the samples in each column (one-way ANOVA, Tukey's post hoc test, $p < 0.05$. Data already published in Xhaferaj et al. (2023b).

Sample code	PROL/ GLUT	Protein		Gluten		Prolamins reduced		Glutelins		AL/GL		D-hordeins		C-hordeins		B/γ-hordeins	
ARC_AUS20	2.1	10.09	EFG	7.75	FGH	5.26	EFG	2.49	KL	2.35	CDEFGH	1.02	DEFGHIJ	2.22	EFG	4.51	EFG
ADA_AUS20	1.1	7.11	S	4.89	R	2.58	Q	2.31	LMN	2.22	IJKLM	0.85	IJK	1.04	PQR	3.00	S
CON_CAN19	1.1	9.47	HIJKLM	7.36	GHIJK	3.86	LMN	3.51	CD	2.11	MNO	0.97	DEFGHIJK	1.69	KLM	4.70	DE
AUS_CAN19	2.3	7.25	RS	5.08	R	3.55	NO	1.53	Q	2.17	JKLMN	0.76	K	1.18	OP	3.14	RS
EVE_DEN20	1.2	9.04	LMN	6.80	LMN	3.65	MNO	3.15	EFG	2.24	HIJKL	1.02	DEFGHIJ	1.76	JKL	4.02	JKL
FEE_DEN20	2.1	8.70	NO	6.48	NO	4.38	IJKL	2.11	NO	2.21	JKLM	1.09	BCDEFGH	1.52	MN	3.88	LM
NEW_DEN20	1.5	9.39	IJKLM	7.26	HIJKL	4.33	IJKL	2.93	FGHI	2.13	LMN	1.13	BCDEFG	2.03	GHI	4.10	IJKL
STA_DEN20	1.5	9.26	JKLM	6.94	KLMN	4.16	JKLM	2.78	IJ	2.31	DEFGHI	1.17	BCD	1.97	HIJ	3.81	LMN
PIX_FRA20	0.6	7.42	QRS	5.02	R	1.85	R	3.17	EFG	2.40	CD	1.03	CDEFGHI	0.87	R	3.12	RS
COC_FRA20	2.2	8.62	NO	6.25	O	4.28	IJKL	1.97	OP	2.37	CDEF	0.97	DEFGHIJK	1.57	LMN	3.71	MNO
MEM_FRA20	0.9	7.97	PQ	5.67	PQ	2.71	PQ	2.96	EFGHI	2.30	DEFGHI	0.88	HIJK	0.87	R	3.91	KLM
TRA_FRA20	1.6	7.44	QRS	5.16	QR	3.20	OP	1.96	OP	2.28	EFGHIJ	0.81	JK	1.12	PQ	3.23	QRS
PRU_FRA20	2.2	10.42	DE	8.08	EF	5.53	DEF	2.55	JKL	2.34	CDEFGH	1.09	BCDEFGH	1.91	HIJK	5.08	C
JAL_GER20	2.0	11.04	C	8.47	DE	5.64	DE	2.83	HIJ	2.57	AB	1.28	B	2.69	D	4.50	EFGH
QUA_GER20	1.1	7.49	QRS	5.20	QR	2.74	PQ	2.46	L	2.28	DEFGHIJ	0.93	EFGHIJK	0.90	QR	3.37	PQR
ACC_GER20	0.8	9.32	IJKLM	6.97	KLMN	3.17	OP	3.80	B	2.35	CDEFGH	0.91	GHIJK	1.54	LMN	4.52	EFG
GKJ_HUN17	1.7	7.70	PQR	5.69	PQ	3.61	NO	2.08	NO	2.01	O	0.95	DEFGHIJK	1.18	OP	3.55	NOP
MVI_HUN17	1.2	9.75	FGHIJ	7.53	GHIJ	4.06	KLMN	3.47	CD	2.22	IJKLM	1.14	BCDEF	2.05	GHI	4.34	FGHI
JUM_LAT20	0.8	7.38	RS	5.26	QR	2.31	QR	2.95	FGHI	2.12	LMNO	0.90	HIJK	1.02	PQR	3.34	PQR
SAU_LAT20	0.8	7.48	QRS	5.36	QR	2.36	QR	3.00	EFGHI	2.11	MNO	0.98	DEFGHIJK	0.93	QR	3.46	OPQ
HOCK_CAN19	1.0	9.50	HIJKLM	7.24	HIJKLM	3.58	NO	3.66	BC	2.26	FGHIJK	0.97	DEFGHIJK	1.59	LMN	4.68	DE
TRA_CAN19	1.5	9.67	GHIJK	7.12	IJKLM	4.22	IJKL	2.90	GHI	2.55	AB	0.95	EFGHIJK	1.97	HIJ	4.20	HIJK
JAK_GER20	1.2	10.00	EFGH	7.54	GHIJ	4.05	KLMN	3.49	CD	2.46	BC	1.25	BC	2.06	FGH	4.23	GHIJ
KWSS_HUN17	0.8	9.15	KLMN	7.00	JKLMN	3.13	OP	3.88	B	2.15	KLMN	0.95	DEFGHIJK	1.44	N	4.61	DEF
MOR_HUN17	1.6	9.86	FGHI	7.59	FGHI	4.62	HIJ	2.97	EFGHI	2.27	EFGHIJK	1.27	B	2.28	EF	4.04	IJKL
EST_AUS20	2.9	9.00	MN	6.71	MNO	5.01	FGH	1.70	PQ	2.29	DEFGHIJ	0.98	DEFGHIJ	2.02	GHI	3.70	MNO
EVE_AUS20	2.6	11.10	C	8.72	CD	6.28	BC	2.43	LM	2.38	CDE	0.97	DEFGHIJK	2.92	C	4.83	CD
ESC_AUS20	3.0	11.01	C	8.70	CD	6.53	B	2.17	MNO	2.31	DEFGHI	1.14	BCDE	2.72	CD	4.84	CD
ANT_GER20	1.5	10.23	EF	7.83	FG	4.74	GHI	3.09	EFGH	2.40	CD	1.09	BCDEFGH	1.84	IJK	4.90	CD
GRE_DEN20	1.1	8.24	OP	6.18	OP	3.17	OP	3.01	EFGHI	2.06	NO	0.92	FGHIJK	1.38	NO	3.87	LM
ABA_LAT20	1.8	11.66	B	9.02	C	5.83	CD	3.19	EF	2.65	A	1.13	BCDEFG	2.29	E	5.60	B
IRB_LAT20	1.8	10.92	CD	8.39	DE	5.37	DEF	3.02	EFGHI	2.53	AB	0.95	EFGHIJK	2.35	E	5.09	C
KOR_LAT19	2.5	18.14	A	15.87	A	11.29	A	4.57	A	2.27	EFGHIJK	1.54	A	6.42	A	7.91	A
CEL_CAN19	1.7	9.56	GHIJKL	7.32	GHIJKL	4.57	HIJK	2.75	IJK	2.24	GHIJKL	1.02	DEFGHIJ	2.28	EF	4.02	JKL
DAMO_HUN17	2.0	12.10	B	9.74	B	6.50	B	3.23	DE	2.36	CDEFG	1.03	CDEFGHI	3.19	B	5.52	B

Table 5.29: The relative molecular weight distribution with GP-HPLC categorized according to the following molecular weight ranges: (1) > 66 kDa; (2) 66-29 kDa; (3) 29-12.4 kDa; (4) < 12.4 kDa. Values are given as means (n = 3) and percentages (%) referring to the relative percentages of the isolates in relation to the measured AUC at 210 nm by GP-HPLC. The capital letters indicate significant differences between the samples in each column (one-way ANOVA, Tukey's post hoc test, $p < 0.05$). Data already published in Xhaferaj et al. (2023b).

Sample code	Prolamins								Reduced Prolamins								Glutelins			
	1		2		3		4		1		2		3		4		1		2		3		4	
ARC_AUS20	34.6	CDE	5.5	O	28.9	EF	31.1	LMN	1.8	GHIJ	2.5	OPQ	44.1	DE	51.6	KL	12.4	EFGHIJK	8.7	EFGHI	31.4	EFGH	47.5	JKLM
ADA_AUS20	33.6	DEFG	7.1	IJK	24.5	IJK	34.8	IJ	3.3	ABCDE	3.5	JKL	42.2	FG	51.1	KLMN	13.9	DEF	8.5	FGHIJK	30.3	GHIJ	47.3	JKLM
CON_CAN19	27.9	KL	7.5	GH	28.9	DEF	35.6	HI	1.9	FGHIJ	3.7	GHIJK	45.8	CD	48.7	PQ	9.4	MNOP	7.3	JKLM	38.4	A	45.0	MNOP
AUS_CAN19	41.2	A	8.9	BC	21.5	LMN	28.3	OP	3.2	ABCDEF	4.3	CDE	36.9	HI	55.7	HI	13.5	DEFG	8.8	EFGHI	28.2	KL	49.5	IJ
EVE_DEN20	23.3	NO	8.2	D	19.0	OPQ	49.5	BC	0.7	J	1.5	S	19.6	Q	78.1	B	5.1	R	3.8	O	24.1	QR	67.0	A
FEE_DEN20	32.7	DEFGH	8.6	C	18.6	OPQ	40.1	EF	2.3	CDEFGHI	2.8	O	23.6	NO	71.3	D	11.1	HIJKLMN	7.3	JKLM	25.5	OP	56.0	CDE
NEW_DEN20	23.7	NO	7.7	FG	19.6	OP	49.1	BC	1.1	HIJ	2.0	R	21.7	P	75.2	C	5.9	QR	4.8	O	25.1	PQ	64.3	AB
STA_DEN20	25.1	NO	8.2	DE	15.9	R	50.8	B	0.9	IJ	1.0	T	18.0	Q	80.1	A	5.2	R	3.8	O	23.8	R	67.2	A
PIX_FRA20	15.5	Q	7.9	F	21.5	LMN	55.1	A	1.9	EFGHIJ	3.8	EFGHIJ	31.8	J	62.4	F	19.8	A	10.5	ABC	30.5	GHI	39.1	R
COC_FRA20	34.2	CDE	9.2	B	19.5	OP	37.1	GH	3.3	ABCDE	4.2	DEFG	26.3	LM	66.3	E	16.5	BC	10.3	BCD	23.6	R	49.6	IJ
MEM_FRA20	31.3	GHIJ	9.2	B	18.2	PQ	41.3	E	3.6	ABC	4.2	CDEF	26.7	KL	65.4	E	12.3	EFGHIJK	8.7	EFGHIJ	26.2	NOP	52.8	FGH
TRA_FRA20	32.2	EFGHI	8.2	DE	19.1	OPQ	40.5	EF	3.4	ABCD	3.8	EFGHIJ	26.8	KL	65.9	E	10.4	JKLMNO	8.1	HIJKL	25.6	OP	55.8	CDEF
PRU_FRA20	34.7	CD	8.8	C	17.6	QR	38.9	FG	2.2	DEFGHI	3.6	IJKL	22.9	OP	71.4	D	11.7	FGHIJKL	8.7	EFGHI	23.3	R	56.3	CD
JAL_GER20	29.7	JKL	5.8	N	33.4	A	31.1	LMN	2.7	BCDEFG	3.3	KLM	47.9	AB	46.0	R	14.8	CD	11.0	AB	33.5	C	40.7	QR
QUA_GER20	41.2	A	7.7	FG	21.5	LMN	29.6	NOP	2.3	CDEFGH	2.7	OP	43.7	EF	51.3	KLM	14.0	DE	9.9	BCDE	30.6	GHI	45.5	LMNO
ACC_GER20	24.9	NO	6.8	KL	30.8	BC	37.6	GH	1.1	HIJ	2.1	QR	47.7	AB	49.1	OP	7.4	PQR	6.2	MN	32.0	EF	54.5	CDEFG
GKJ_HUN17	37.2	B	9.6	A	19.3	OP	33.9	IJK	4.2	A	3.7	HIJK	37.1	H	54.9	I	10.9	IJKLMN	7.7	IJKL	27.5	LM	53.9	DEFG
MVI_HUN17	27.6	LM	8.7	C	29.1	DE	34.6	IJK	3.9	AB	4.9	AB	41.5	G	49.7	MNOP	17.3	B	11.8	A	27.4	LMN	43.5	NOPQ
JUM_LAT20	23.6	NO	7.3	HI	19.4	OP	49.7	BC	1.6	GHIJ	2.9	MNO	24.7	MN	70.9	D	9.5	LMNOP	7.6	IJKL	29.3	JK	53.6	DEFG
SAU_LAT20	24.3	NO	7.9	EF	20.1	NO	47.7	CD	2.0	EFGHIJ	3.3	LMN	23.7	NO	71.1	D	9.2	NOP	7.2	KLM	26.2	OP	57.4	C
HOCK_CAN19	25.4	MN	7.1	IJK	29.8	CDE	37.6	GH	1.6	GHIJ	3.7	HIJKL	46.2	BC	48.5	PQ	10.7	IJKLMN	8.4	GHIJKL	37.9	A	42.9	OPQ
TRA_CAN19	30.9	HIJ	7.0	IJK	29.3	CDE	32.8	JKL	2.1	DEFGHI	4.0	EFGHI	42.0	FG	51.9	KL	12.7	DEFGHIJ	8.7	EFGHIJ	30.3	GHIJ	48.3	IJKL
JAK_GER20	22.7	O	5.9	N	30.6	BCD	40.8	EF	2.0	EFGHIJ	2.3	PQR	48.4	A	47.3	QR	11.1	HIJKLMN	9.2	CDEFGH	37.4	AB	42.3	PQ
KWSS_HUN17	18.4	P	7.9	DEF	27.3	FG	46.4	D	2.2	CDEFGHI	2.7	OP	42.2	FG	52.8	JK	11.3	GHIJKLMN	8.6	FGHIJK	31.9	EF	48.3	JKL
MOR_HUN17	30.0	IJKL	8.8	C	25.2	HIJ	36.0	HI	2.4	CDEFGH	4.5	BCD	35.9	HI	57.1	H	14.3	CDE	9.6	CDEFG	31.5	EFG	44.7	MNOP
EST_AUS20	33.9	CDEF	7.2	I	26.1	GHI	32.8	JKL	2.3	CDEFGHI	2.8	NO	44.1	DE	50.8	LMNO	12.7	DEFGHI	9.8	BCDEF	31.3	EFGH	46.2	KLMN
EVE_AUS20	31.6	FGHIJ	5.9	N	32.1	AB	30.4	MNO	1.2	HIJ	2.1	QR	44.4	DE	52.3	KL	8.2	OPQ	7.1	LM	31.9	EF	52.9	EFGH
ESC_AUS20	36.2	BC	6.5	LM	29.6	CDE	27.8	P	1.2	HIJ	1.9	RS	42.2	FG	54.7	I	11.6	FGHIJKLM	9.1	DEFGH	29.8	IJ	49.5	IJ
ANT_GER20	39.8	A	6.1	MN	23.1	KL	31.0	LMN	1.6	GHIJ	2.2	QR	41.9	G	54.3	IJ	10.8	IJKLMN	8.1	HIJKL	30.8	FGHI	50.3	HIJ
GRE_DEN20	23.5	NO	7.9	DEF	20.3	MNO	48.2	CD	1.0	HIJ	2.0	R	23.3	NOP	73.6	C	6.0	QR	4.9	NO	26.5	MNO	62.6	B
ABA_LAT20	32.6	DEFGH	7.2	HI	24.3	JK	35.9	HI	2.7	BCDEFG	4.1	EFGH	32.6	J	60.6	G	10.8	IJKLMN	8.7	FGHI	36.5	B	44.0	NOP
IRB_LAT20	30.2	IJK	6.8	JK	21.9	LM	41.1	EF	4.0	AB	4.6	BC	28.2	K	63.2	F	13.3	DEFGH	10.3	BCD	33.2	CD	43.2	NOPQ
KOR_LAT19	20.1	P	6.4	M	33.2	A	40.3	EF	2.2	DEFGHI	3.8	FGHIJ	36.8	HI	57.2	H	10.1	KLMNO	11.8	A	26.6	MNO	51.5	GHI
CEL_CAN19	30.1	IJK	7.1	IJ	30.4	BCDE	32.4	KLM	3.5	ABCD	5.2	A	42.0	G	49.3	NOP	12.8	DEFGHI	8.6	EFGHIJ	30.3	HIJ	48.3	JKL
DAMO_HUN17	30.0	IJKL	7.8	FG	26.6	GH	35.6	HI	3.4	ABCD	4.0	EFGH	35.4	I	57.1	H	10.9	IJKLMN	7.7	IJKL	32.1	DE	49.3	IJK

Table 5.30: Determinations of the eight barley cultivars of different harvest years and their mixtures with GP-HPLC. Gluten: Sum of reduced prolamins and glutelins. Protein: Sum of reduced prolamins glutelins. albumins and globulins (ALGL). Values are given as means (n = 3). (g/100 g) and different capital letters indicate significant differences between the samples in each column (one-way ANOVA. Tukey's post hoc test. $p < 0.05$).

Sample code	Protein		Gluten		reduced Prolamins		Glutelins		ALGL		D-hordeins		C-hordeins		B/γ-hordeins	
CEL_CAN19	9.56	F	7.32	EF	4.57	F	2.75	GHI	2.24	FG	1.02	CD	2.28	E	4.02	F
CEL_CAN22	10.84	D	8.16	D	5.63	D	2.53	I	2.69	B	0.97	CDE	2.48	D	4.71	DE
COC_FRA20	8.62	IJ	6.25	I	4.28	FG	1.97	K	2.37	DEFG	0.97	CD	1.57	H	3.71	G
COC_FRA22	8.45	J	5.75	J	3.20	J	2.55	I	2.70	B	0.93	CDE	1.29	IJ	3.52	G
EVE_AUS20	11.10	CD	8.72	C	6.28	C	2.43	IJ	2.38	CDEFG	0.97	CDE	2.92	B	4.83	D
EVE_AUS22	11.36	C	8.97	C	5.95	CD	3.03	FGH	2.39	CDEF	0.98	CD	2.67	C	5.32	C
EVE_DEN20	9.04	GH	6.80	GH	3.65	I	3.15	EFG	2.24	G	1.02	CD	1.76	G	4.02	F
EVE_DEN22	8.92	HI	6.42	HI	2.64	K	3.78	C	2.50	CD	0.78	E	1.11	K	4.52	E
GKJ_HUN17	7.70	K	5.69	J	3.61	I	2.08	JK	2.01	H	0.95	CDE	1.18	JK	3.55	G
GKJ_HUN22	9.38	FG	7.00	FG	3.70	HI	3.30	DEF	2.39	CDEFG	0.95	CDE	1.39	I	4.65	DE
JAK_GER20	10.00	E	7.54	E	4.05	GH	3.49	CDE	2.46	CD	1.25	B	2.06	F	4.23	F
JAK_GER22	9.37	FG	6.86	G	2.48	K	4.38	B	2.52	C	0.98	CD	1.22	JK	4.66	DE
KOR_LAT19	18.14	A	15.87	A	11.29	A	4.57	B	2.27	EFG	1.54	A	6.42	A	7.91	A
KOR_LAT22	17.76	B	15.30	B	9.68	B	5.62	A	2.46	CD	1.56	A	6.31	A	7.42	B
PIX_FRA20	7.42	K	5.02	K	1.85	L	3.17	EF	2.40	CDE	1.03	CD	0.87	L	3.12	H
PIX_FRA22	8.56	IJ	5.52	J	1.83	L	3.69	CD	3.05	A	0.92	DE	0.98	L	3.61	G
Mixture 1	10.73	D	8.21	D	5.10	E	3.11	EFG	2.52	C	1.12	BC	2.46	D	4.63	DE
Mixture 2	9.27	FGH	6.88	G	4.19	G	2.69	HI	2.40	CDE	0.88	DE	1.74	G	4.25	F

Table 5.31: The relative molecular weight distribution with GP-HPLC categorized according to the following molecular weight ranges: (1) > 66 kDa; (2) 66-29 kDa; (3) 29-12.4 kDa; (4) < 12.4 kDa. Values are given as means (n = 3) and percentages (%) referring to the relative percentages of the isolates in relation to the measured AUC at 210 nm by GP-HPLC. The capital letters indicate significant differences between the samples in each column (one-way ANOVA, Tukey's post hoc test, $p < 0.05$).

Sample code	Prolamins				red. Prolamins				Glutelins			
	1	2	3	4	1	2	3	4	1	2	3	4
CEL_CAN19	30.1 DE	7.1 EF	30.4 D	32.4 GHI	3.5 BC	5.2 BC	42.0 F	49.3 G	12.8 E	8.6 EFG	30.3 F	48.3 D
CEL_CAN22	37.8 A	7.9 D	22.3 GH	32.0 HIJ	6.6 A	6.4 A	42.7 EF	44.3 I	21.1 A	10.2 BCD	30.0 F	38.6 H
COC_FRA20	34.2 BC	9.2 B	19.5 I	37.1 F	3.3 BCD	4.2 F	26.3 I	66.3 B	16.5 CD	10.3 BCD	23.6 H	49.6 CD
COC_FRA22	35.9 AB	6.8 F	23.0 G	34.3 G	4.0 B	3.9 FG	42.7 EF	49.4 G	11.9 EFG	8.5 EFGH	32.8 E	46.8 DE
EVE_AUS20	31.6 CDE	5.9 H	32.1 BC	30.4 J	1.2 EF	2.1 I	44.4 E	52.3 F	8.2 H	7.1 I	31.9 E	52.9 B
EVE_AUS22	32.3 CD	7.2 E	29.0 E	31.5 IJ	3.4 BCD	4.9 DE	49.0 CD	42.8 J	10.9 FG	7.2 HI	37.3 C	44.6 EF
EVE_DEN20	23.3 F	8.2 D	19.0 I	49.5 B	0.7 F	1.5 J	19.6 J	78.1 A	5.1 I	3.8 J	24.1 H	67.0 A
EVE_DEN22	24.6 F	7.3 E	31.2 CD	37.0 F	2.0 CDEF	3.4 H	54.3 A	40.3 K	10.5 G	7.8 FGHI	38.8 B	42.9 F
GKJ_HUN17	37.2 AB	9.6 A	19.3 I	33.9 GH	4.2 B	3.7 GH	37.1 G	54.9 E	10.9 EFG	7.7 GHI	27.5 G	53.9 B
GKJ_HUN22	37.9 A	8.8 C	25.5 F	27.8 K	4.4 B	5.4 B	51.5 B	38.6 L	15.6 D	9.3 CDE	35.4 D	39.8 GH
JAK_GER20	22.7 FG	5.9 H	30.6 D	40.8 DE	2.0 DEF	2.3 I	48.4 CD	47.3 H	11.1 EFG	9.2 DEF	37.4 C	42.3 FG
JAK_GER22	19.6 H	7.5 E	31.8 C	41.1 D	4.3 B	4.5 E	52.3 B	38.9 L	18.1 BC	10.6 ABC	41.3 A	29.9 I
KOR_LAT19	20.1 GH	6.4 G	33.2 A	40.3 DE	2.2 CDE	3.8 G	36.8 G	57.2 D	10.1 G	11.8 A	26.6 G	51.5 BC
KOR_LAT22	28.8 E	7.3 E	24.9 F	39.0 EF	3.2 BCD	4.6 E	47.2 D	44.9 I	12.7 EF	9.6 BCDE	34.9 D	42.7 FG
PIX_FRA20	15.5 I	7.9 D	21.5 H	55.1 A	1.9 DEF	3.8 FG	31.8 H	62.4 C	19.8 AB	10.5 ABC	30.5 F	39.1 H
PIX_FRA22	15.5 I	7.5 E	33.0 AB	43.9 C	4.3 B	5.1 CD	49.5 C	41.2 K	20.2 A	10.9 AB	38.1 BC	30.8 I

Table 5.32: The hordein distribution with RP-HPLC categorized for the four barley isolates and flours by RP-HPLC. Values are given as means (n = 3), (%) and different capital letters indicate significant differences between the samples in each column (one-way ANOVA, Tukey's post hoc test, $p < 0.05$).

Samples		D-hordeins		C-hordeins		B/γ-hordeins	
Isolates	Gluten	8.7	DE	25.6	D	65.7	C
	AWEP	6.7	E	37.9	A	55.4	EF
	Prolamins	7.9	E	35.9	BC	56.2	E
	Glutelins	10.4	CD	8.6	F	81.1	A
Flours	Gluten	13.6	B	23.4	E	63.0	D
	AWEP	11.0	C	37.0	AB	52.0	G
	Prolamins	10.9	C	34.7	C	54.4	F
	Glutelins	20.2	A	8.8	F	71.0	B

Table 5.33: Results of the independent two-sample T-test (null hypothesis) analysis performed with a significance level of 0.05 using Origin Pro 2019b. The factors show the significance differences between the total amounts (g/100 g (RP-HPLC)) between the first and the second cultivar collection analyzed with RP-HPLC.

Sample	p-value	Significant	t(4) = t-Statistics	p-value	Significant	t(4) = t-Statistics
	Protein				Albumins/Globulins	
GK Judy	1.18×10^{-6}	Yes	-47.45	5.36×10^{-4}	Yes	-10.12
Evelina	5.00×10^{-2}	Yes	-2.78	8.51×10^{-1}	No	-0.20
Jakobus	6.15×10^{-3}	Yes	5.28	2.99×10^{-1}	No	-1.19
Kornelija	4.55×10^{-2}	Yes	2.87	1.42×10^{-3}	Yes	-7.86
Evergreen	3.20×10^{-1}	No	1.13	1.40×10^{-5}	Yes	-25.53
Pixel	1.37×10^{-3}	Yes	-7.93	2.60×10^{-4}	Yes	-12.19
Coccinel	6.17×10^{-2}	No	2.57	3.12×10^{-3}	Yes	-6.37
Celebration	4.77×10^{-5}	Yes	-18.74	5.70×10^{-5}	Yes	-17.92
Mixture	1.16×10^{-4}	Yes	14.97	3.65×10^{-3}	Yes	6.10
	Gluten				D-hordeins	
GK Judy	2.82×10^{-5}	Yes	–21.40	9.21×10^{-1}	No	–0.11
Evelina	1.02×10^{-1}	No	–2.12	8.69×10^{-1}	No	–0.18
Jakobus	8.90×10^{-3}	Yes	4.76	5.17×10^{-3}	Yes	5.55
Kornelija	1.76×10^{-2}	Yes	3.90	6.07×10^{-1}	No	–0.56
Evergreen	2.40×10^{-2}	Yes	3.54	4.20×10^{-4}	Yes	10.78
Pixel	1.01×10^{-2}	Yes	–4.59	3.90×10^{-2}	Yes	3.02
Coccinel	3.35×10^{-4}	Yes	11.42	4.87×10^{-1}	No	0.76
Celebration	3.19×10^{-4}	Yes	–11.56	3.24×10^{-1}	No	1.12
Mixture	1.95×10^{-4}	Yes	13.12	1.08×10^{-3}	Yes	8.44
	Prolamins				C-hordeins	
GK Judy	3.60×10^{-1}	No	–1.03	5.71×10^{-4}	Yes	–9.96
Evelina	2.01×10^{-2}	Yes	3.74	2.09×10^{-3}	Yes	7.09
Jakobus	1.63×10^{-5}	Yes	24.55	1.64×10^{-6}	Yes	43.72
Kornelija	2.03×10^{-3}	Yes	7.15	2.62×10^{-1}	No	1.31
Evergreen	1.03×10^{-4}	Yes	15.42	8.99×10^{-6}	Yes	28.52
Pixel	6.45×10^{-1}	No	0.50	7.30×10^{-4}	Yes	–9.35
Coccinel	3.25×10^{-5}	Yes	20.64	2.55×10^{-4}	Yes	12.26
Celebration	3.23×10^{-4}	Yes	–11.53	7.47×10^{-4}	Yes	–9.29
Mixture	9.48×10^{-7}	Yes	50.12	3.49×10^{-6}	Yes	36.15
	Glutelins				B/γ-hordeins	
GK Judy	3.85×10^{-6}	Yes	–35.28	1.61×10^{-6}	Yes	–43.91
Evelina	6.82×10^{-4}	Yes	–9.51	3.18×10^{-2}	Yes	–3.24
Jakobus	1.99×10^{-3}	Yes	–7.18	8.97×10^{-3}	Yes	–4.75
Kornelija	1.35×10^{-2}	Yes	–4.22	1.34×10^{-3}	Yes	7.97
Evergreen	2.86×10^{-4}	Yes	–11.89	2.54×10^{-3}	Yes	–6.73
Pixel	5.05×10^{-3}	Yes	–5.58	1.56×10^{-3}	Yes	–7.66
Coccinel	1.58×10^{-3}	Yes	–7.64	2.77×10^{-3}	Yes	6.58
Celebration	7.56×10^{-4}	Yes	9.26	4.32×10^{-5}	Yes	–19.22
Mixture	9.67×10^{-3}	Yes	4.65	4.04×10^{-3}	Yes	5.94

Table 5.34: Results of the independent two-sample T-test (null hypothesis) analysis performed with a significance level of 0.05 using Origin Pro 2019b. The factors show the significant differences between the relative distribution of the hordein fractions mesured with RP-HPLC between the samples of the first and the second cultivar collection.

Sample	p-value	Significant	$t(4) =$ t-Statistics
	D-hordeins		
GK Judy	3.81×10^{-3}	Yes	6.03
Evelina	3.52×10^{-5}	Yes	20.23
Jakobus	7.35×10^{-7}	Yes	53.42
Kornelija	4.75×10^{-2}	Yes	-2.83
Evergreen	1.24×10^{-7}	Yes	83.30
Pixel	9.33×10^{-2}	No	-2.19
Coccinel	2.97×10^{-3}	Yes	6.45
Celebration	5.80×10^{-3}	Yes	5.37
Mixture	1.61×10^{-5}	Yes	24.66
	C-hordeins		
GK Judy	3.81×10^{-3}	Yes	6.03
Evelina	3.52×10^{-5}	Yes	20.23
Jakobus	7.35×10^{-7}	Yes	53.42
Kornelija	4.75×10^{-2}	Yes	-2.83
Evergreen	1.24×10^{-7}	Yes	83.30
Pixel	9.33×10^{-2}	No	-2.19
Coccinel	2.97×10^{-3}	Yes	6.45
Celebration	5.80×10^{-3}	Yes	5.37
Mixture	1.61×10^{-5}	Yes	24.66
	B/γ-hordeins		
GK Judy	2.32×10^{-3}	Yes	-6.89
Evelina	5.51×10^{-2}	No	-2.68
Jakobus	1.01×10^{-5}	Yes	-27.72
Kornelija	2.07×10^{-3}	Yes	7.11
Evergreen	4.18×10^{-6}	Yes	-34.57
Pixel	1.14×10^{-4}	Yes	-15.05
Coccinel	1.26×10^{-2}	Yes	-4.31
Celebration	1.49×10^{-3}	Yes	-7.76
Mixture	1.33×10^{-5}	Yes	-25.87

Table 5.35: Results of the independent two-sample T-test (null hypothesis) analysis performed with a significance level of 0.05 using Origin Pro 2019b. The factors show the significance differences between the relative distribution of the hordein fractions measured with RP-HPLC between the Isolates and the flours.

Sample	p-value	Significance	$t(4) =$ t-Statistics
	D-hordeins		
Prolamins	3.19×10^{-2}	Yes	-3.23
Glutelins	1.64×10^{-5}	Yes	-24.53
Gluten	1.16×10^{-4}	Yes	-14.96
AWEP	2.75×10^{-3}	Yes	-6.59
	C-Hordeins		
Prolamins	1.78×10^{-1}	No	1.63
Glutelins	4.36×10^{-1}	No	-0.86
Gluten	6.04×10^{-3}	Yes	5.31
AWEP	1.80×10^{-1}	No	1.62
	B/γ-hordeins		
Prolamins	1.09×10^{-2}	Yes	4.50
Glutelins	1.73×10^{-5}	Yes	24.18
Gluten	2.39×10^{-3}	Yes	6.84
AWEP	1.43×10^{-3}	Yes	7.83