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Development of targeted mass spectrometric methods for the quantitation of gluten-specific peptides in foods

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A Winner Is A Dreamer Who Never Gives Up.

- Nelson Mandela -

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Vorabveröffentlichungen

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Abbreviations

AGA	Anti-gliadin antibody
APC	Antigen-presenting cell
ATI	Amylase-trypsin-inhibitor
CD	Coeliac disease
DNA	Deoxyribonucleic acid
DTT	Dithiothreithol
EC	European Commission
ELISA	Enzyme-linked immunosorbent assay
EMA	Endomysial antibody
ESPGHAN	European Society for Paediatric Gastroenterology Hepatology and Nutrition
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FDA	Food and Drug Administration
FDR	Food and Drug Regulation
FLD	Fluorescence detection
FSANZ	Food Standards Australia and New Zealand
FSC	New Zealand and Australian Food Standard Codes
GP	Gel-permeation
GS	Glutenin subunits
HLA	Human leukocyte antigen
HMW	High-molecular-weight
HPLC	High-performance liquid chromatography
IBS	Irritable bowel syndrome
IEL	Intraepithelial lymphocytes
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LC	Liquid chromatography
LMW	Low-molecular-weight

LOD	Limit of detection
LOQ	Limit of quantitation
MALDI	Matrix-assisted laser desorption/ionization
MHC	Major histocompatibility complex
MMW	Medium-molecular-weight
MoniQA	International Association for Monitoring and Quality Assurance in the Total Food Supply Chain
M_r	Relative molecular weight
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NCGS	Non-coeliac gluten sensitivity
PCR	Polymerase chain reaction
PEP	Prolyl-endopeptidase
PWG	Prolamin Working Group
RF	Response factor
RP	Reversed-phase
SDS	Sodium dodecyl sulphate
SIDA	Stable isotope dilution assay
TCEP	Tris(2-carboxyethyl)-phosphine
TNF	Tumor necrosis factor
TOF	Time-of-flight
tTG	Tissue transglutaminase
tTGA	anti-tissue transglutaminase antibody
UPEX	Universal prolamin and glutelin extraction solvent
UV	Ultraviolet
WDEIA	Wheat dependent, exercise-induced anaphylaxis
WHO	World Health Organization

One letter code for amino acids

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

1 Introduction

1.1 Coeliac disease

1.1.1 Definitions and history

Coeliac disease (CD) is defined as “a chronic small intestinal immune-mediated enteropathy precipitated by exposure to dietary gluten in genetically predisposed individuals” (the so-called “Oslo-definition”) according to Ludvigsson et al. [2013].
In general, gluten is defined as the rubbery mass that remains, when wheat dough is washed with water or salt solution to remove starch granules and water-soluble constituents [Wieser, 2007]. In the field of CD, gluten is defined as “a protein fraction from wheat, rye, barley, oats or their crossbred varieties and derivatives thereof, to which some persons are intolerant and that is insoluble in water and 0.5 mol/L NaCl” [Codex Stan 118, 2015].

In 1888, Samuel Gee was the first who introduced and specified the clinical syndrome of CD. Later, Willem K. Dicke [1950] described that the ingestion of wheat, barley and rye is responsible for CD. In 1953, van de Kamer et al. performed the fractionation of wheat dough into water-soluble albumins, gluten and starch and *in vivo* testing of all obtained fractions showed that gluten was toxic whereas albumins and starch were not. In 1954, abnormalities of mucosal tissue from the small intestine of CD patients were elucidated for the first time by John W. Paulley, which were confirmed by Margot Shiner [1956] and William H. Crosby [1957]. These observations were important milestones for the ongoing research in the field of CD.

1.1.2 Epidemiology

In the past, CD was classified as rare childhood disease. The diagnosis was only based on typical symptoms, such as chronic diarrhea, steatorrhea, abdominal pain and vomiting. Subsequently, improved diagnostic techniques, such as intestinal biopsy and serological tests became available, which showed that CD can actually occur at any age. Currently, CD has a prevalence of 1 in 100-300 individuals. CD

shows a high prevalence in European countries and the USA, were the diet is traditionally based on gluten-containing cereal food products. CD was perceived as a rare disorder in South America, North Africa and Asia, but epidemiological studies showed that CD was frequently underdiagnosed due to lack of diagnostic facilities [Fasano and Catassi, 2001].

The epidemiology of CD can be demonstrated based on the “iceberg model” [Logan, 1992; Fasano and Catassi, 2001] (see Figure 1.1).

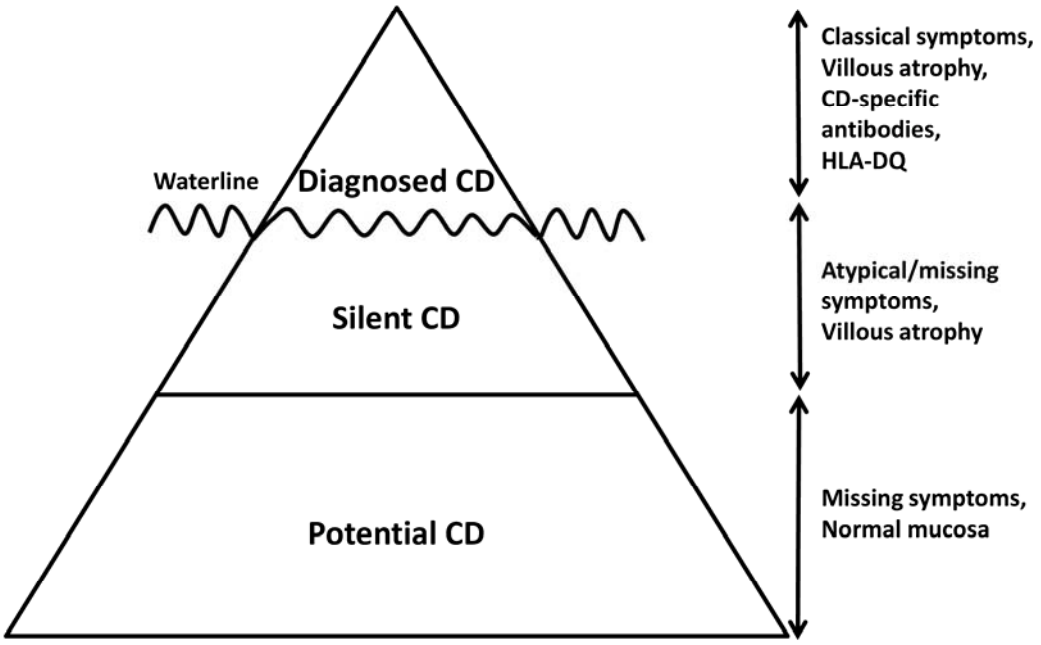

Figure 1.1: The iceberg model of coeliac disease [modifed according to Logan, 1992]

The iceberg is divided into three levels of CD. In all of these 3 forms, CD patients carry the human leukocyte antigen (HLA)-DQ2/DQ8 alleles and show a positive anti-tissue transglutaminase antibody (tTGA)-test (see 1.1.3 and 1.1.5). The tip of the iceberg demonstrates CD patients, which have classical symptoms (symptomatic CD), such as chronic diarrhea and abdominal pain and show a flat intestinal mucosa.

After following a gluten-free diet, patients show a normal intestinal mucosa. Only a minority of patients show symptomatic CD, whereas silent or potential CD occurs in the majority of patients, which is demonstrated below the water line. Patients with silent CD show atypical, minimal or even missing indications, but develop villous atrophy, are genetically predisposed and show a positive reaction in the tTGA-test. The lower end of the iceberg is formed by CD patients with potential CD. It is described by normal intestinal villi and the lack of classical symptoms. However, these patients are HLA-DQ2/8 positive and show CD typical antibodies. Patients with CD forms below the water line mainly remain undiagnosed and are exposed to long-term consequences such as osteoporosis, infertility, anemia, or malignancy.

1.1.3 Genetics and environmental factors

The development of CD is associated with genetic and environmental risk factors. The predisposition to CD is strongly based on the major histocompatibility complex (MHC) genes, which encode the HLA class II alleles HLA-DQ2 and HLA-DQ8. At least one of these two alleles is expressed in CD patients. About 95% of the CD population are HLA-DQ2/DQ8 positive [Sollid et al., 1989; Sollid and Thorsby, 1993]. Two isoforms of the HLA-DQ2 (DQ2.5 and DQ2.2) are known from literature. The HLA-DQ2.5 heterodimer can be encoded in two forms (*cis* and *trans*), which are both associated with a very high risk for CD. In *cis*-form, two genes (DQA1*0501 and DQB1*0201) are located on the same DR3-DQ2 haplotype, whereas in *trans*-form, these two genes are located on different haplotypes, namely DR5-DQ7 and DR7-DQ2 [Abadie et al., 2011]. In contrast to the HLA-DQ2.5 heterodimer, the DQ2.2 heterodimer is a low risk factor for CD and DQ8 a high risk factor [Sollid and Thorsby, 1993]. About 30% of the general population express HLA-D2/DQ8, but only about 1% of them develop CD [Stokes et al., 1972].

Therefore, the absence of HLA-DQ2/DQ8 is an indicator against CD and HLA-DQ testing can be performed for the exclusion of the disease (see 1.1.5)

Several environmental factors are associated with the development of CD, whereas the main factor is gluten intake and the level of gluten consumption. Other factors seem also to be important for CD development, such as infections [e.g. Stene et al., 2006], microbiota [e.g. Collado et al., 2009], age at gluten introduction, amount of gluten exposure and breastfeeding [e.g. Silano et al., 2010]. According to recent

studies, the age of gluten introduction did not influence CD risk and breastfeeding did not prevent the development of CD [Lebwohl et al., 2016].

1.1.4 Clinical features and pathology

Various symptoms are associated with CD and can be classified into intra- and extraintestinal features. The latter are mainly caused by malabsorption of nutrients. Typical intraintestinal features are diarrhoea, steatorrhoea, vomiting, and abdominal pain. Symptoms such as mineral (iron, magnesium, calcium, copper, zinc, selenium) and vitamin (A, D, E, K, folic acid) deficiencies, anemia, decreased bone mineral density, bone pain and fractures, osteoporosis, dental enamel defects, skin lesions and night blindness are typical extraintestinal features [Richey et al., 2009]. In rare cases, patients show neurological or psychiatric symptoms, such as depression, anxiety, peripheral neuropathy, migraine or epilepsy. Schizophrenia is a psychatric disorder, which is related to CD [Jackson et al., 2012]. Children or adolescents with CD may show slow growth and delayed sexual maturation. In women, CD may have negative impacts on menstrual and reproductive health, such as delayed menarche, early menopause or recurrent miscarriages [Soni and Badawy, 2010].
Associated with CD, lactose intolerance may also occur in patients, because of reduced lactase activity [Ojetti et al., 2005] as well as autoimmune diseases, such as type I diabetis mellitus, autoimmune thyroiditis or autoimmune hepatitis [Kaukinen et al., 2010].

CD is typically characterized by damage of the mucosa of the upper small intestine (duodenum, proximal jejunum), which is accompanied by villous atrophy (see Figure 1.2), crypt hyperplasia, and increased lymphocyte infiltration of the epithelium. Villous atrophy can vary from partial damage to a total absence of villi [Bao et al., 2010]. In order to evaluate the degree of mucosal damage, pathologists use the so-called Marsh-Oberhuber classification, which was developed by Marsh [1992] and modified by Oberhuber et al. [1999]. This classification is based on two paramters (ratio of villous height to crypt depth and the number of intraepithelial lymphocytes (IELs) and distinguishes between several types ranging from normal intestinal mucosa to partial and up to total villous atrophy.

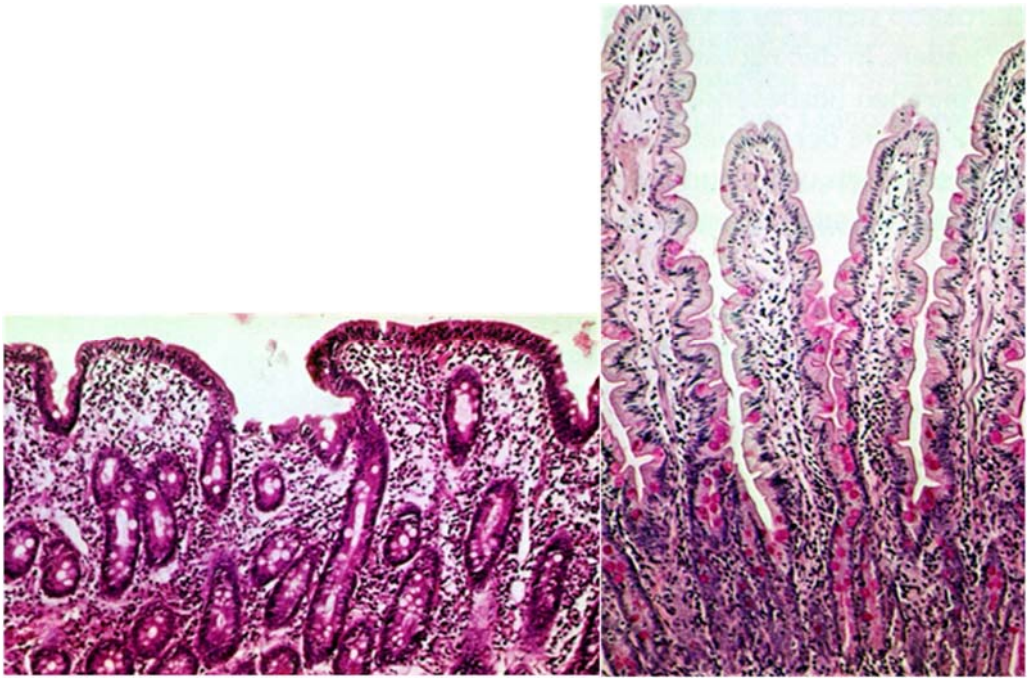

Figure 1.2: Tissue fragment of a coeliac disease damaged intestinal mucosa (left) and an intact intestinal mucosa (right) [German Coeliac Society]

Beside CD, also other forms of intolerances related to the ingestion of gluten were described in the literature, which can be classified into three groups (see Figure 1.3): allergic reaction (wheat allergy), autoimmune-induced reaction (CD, dermatitis herpetiformis, gluten ataxia) and non-autoimmune induced reaction (non-coeliac gluten sensitivity, NCGS) [Ludvigsson et al., 2013; Sapone et al., 2012; Scherf et al., 2016a].

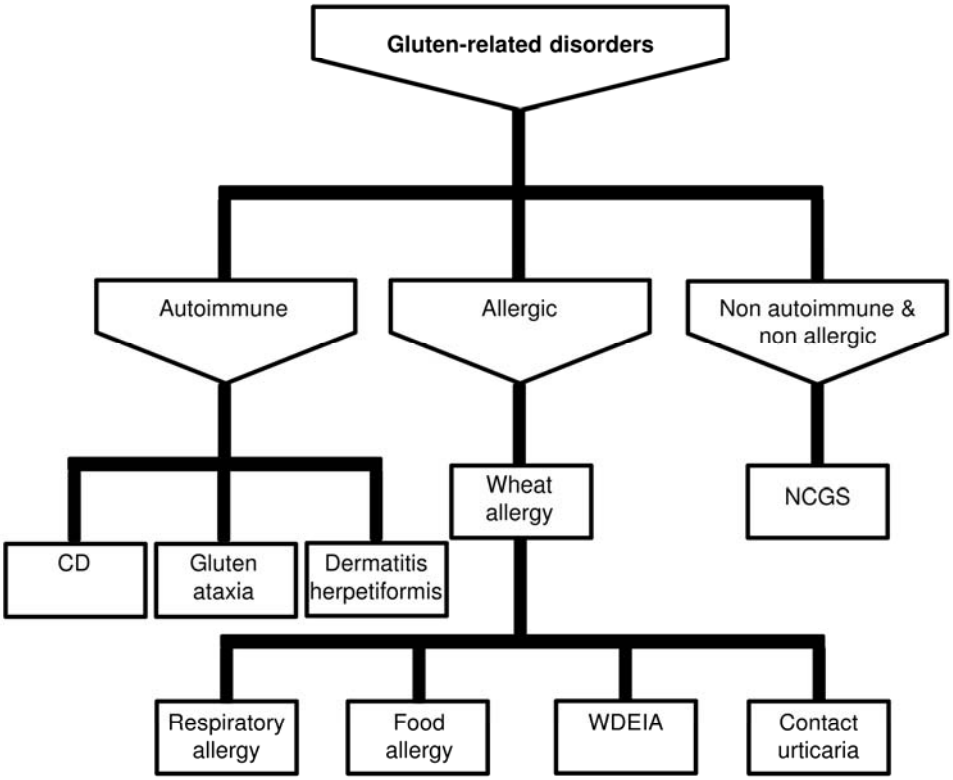

Figure 1.3: Classification of gluten related disorders (CD, coeliac disease; NCGS, non-coeliac gluten sensitivity; WDEIA, wheat-dependent, exercise-induced anaphylaxis) [according to Sapone et al., 2012]

Wheat allergy describes an immunological reaction to wheat proteins, which appears minutes or hours after gluten exposure and can be divided into four forms: immediate food allergy, wheat-dependent, exercise-induced anaphylaxis (WDEIA), respiratory allergy (baker's asthma) and contact urticaria. Immunoglobulin (Ig) E antibodies play a central role in the pathogenesis of these diseases. WDEIA is usually triggered only by specific protein types (ω5-gliadins, HMW-GS), whereas the other allergic responses are induced by a variety of wheat proteins.

Autommune-induced reactions occur weeks to years after gluten exposure. Dermatitis herpetiformis is a skin manifestation of CD presenting with blistering rash. Gluten ataxia is one of the neurological manifestations attributed to CD and can be defined as idiopathic sporadic ataxia with positive serum anti-gliadin antibodies (AGA).

NCGS is neither an allergic reaction nor an autoimmune-induced reaction. Symptoms of NCGS patients are similar to those of CD patients with a prevalence of

extraintestinal symptoms. NCGS patients do not show villous atrophy and serum anti-tTG and anti-deamidated gliadin peptides antibodies nor wheat IgE antibodies, which are indicators for CD or wheat allergy diagnosis. Studies by Carroccio et al. [2013] demonstrated that a basophil activation test with wheat has shown a high sensitivity for NCGS and may be an additional tool for diagnosis. NCGS and irritable bowel syndrome (IBS) are difficult to differentiate, because of an overlap of the intestinal symptoms and more stringent diagnostic criteria are necessary to exclude IBS.

1.1.5 Diagnosis

CD is diagnosed with a combination of three techniques including serological tests, intestinal biopsy and HLA-DQ tests. According to the guidelines of the European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN), the reliable diagnosis depends on the symptoms, serology and histology [Klapp et al., 2013].

Serological tests are based on the screening for the presence of disease-specific antibodies in response to gluten exposure. These tests are evaluated in terms of sensitivity and specificity. Sensitivity of the test describes the capability of the test to correctly identify CD patients [Lalkhen and McCluskey, 2008]. A sensitivity of 100% means that the test is able to correctly identify all patients with CD, whereas a test with 85% sensitivity detects 85% of patients with CD (true positive), but 15% of CD patients are not detected (false negative). Specificity of the test describes the capability of the test to correctly identify patients without CD. A specificity of 100% means that the test is able to correctly identify all patients without CD, whereas a test with 85% specificity correctly identifies 85% of patients without CD (true negative), but CD is incorrectly diagnosed in 15% of patients (false positive).
The screening of IgA- and IgG-AGAs is performed by serological tests and the sensitivity and specificity ranges between 80 and 90% [Leffler and Schuppan, 2010]. However, AGAs can also be detected in non-coeliac disease enteropathies and in 5-10% of healthy humans. The development of anti-endomysial antibody (EMA) tests resulted in higher sensitivity and specificity (> 90%) compared to IgA and IgG-AGA testing. Thus, the EMA test is the favoured serological test for CD [Wong et al., 2008].

Dieterich et al. [1997] identified tTG as CD-specific autoantigen and enzyme-linked immunosorbent assay (ELISA)-based tTGA tests were developed. The application of human recombinant tTG resulted in high specificity and sensitivity (about 98%) [Leffler and Schuppan, 2010].

In the majority of cases, a small intestinal biopsy is taken after serological tests to diagnose the grade of villous atrophy using the Marsh-Oberhuber classification (see 1.1.4) and the number of IELs. For this purpose, at least five samples should be taken including the duodenum and the intestinal bulb. Studies by Gonzalez et al. [2010] demonstrated that the intestinal bulb biopsy can increase the diagnostic yield of CD, because it is the most sensitive part to detect the damage of intestinal mucosa. The detection of increased IELs is more sensitive than the observation of changes in villous structure, because tissue damage can occur locally. Flow cytometry can be used for the measurement of isolated IELs from intestinal biopsies [reviewed by Leon, 2011].

HLA-DQ2 and HLA-DQ8 are CD-specific alleles and about 95% of the CD population are HLA-DQ2/DQ8 positive [Sollid et al., 1989; Sollid and Thorsby, 1993]. HLA-DQ testing is performed by polymerase chain reaction (PCR). HLA-DQ2/DQ8 positivity cannot be used to confirm CD, because DQ2/DQ8 is also very common in the western population. However, the test can be used for the exclusion of CD in high-risk groups (first-degree relatives, diabetes mellitus type I) or in cases of unclear diagnosis by other tests [Hadithi and Pena, 2010].

1.1.6 Pathomechanism

CD is triggered by the ingestion of gluten proteins. Generally, proteins are digested by gastric (pepsin), pancreatic (trypsin, chymotrypsin, elastase, carboxypeptidase) and brush-border enzymes and result in tetra-, tri- or dipeptides or in single amino acids. Gluten proteins typically consist of a high number of proline residues, which are highly resistant towards complete proteolytic digestion. Consequently, relatively large fragments of gluten proteins with a high number of proline and glutamine residues accumulate in the small intestine. Shan et al. [2002] demonstrated by *in vivo* and *in vitro* studies in rats and humans that a large 33-mer peptide from α2-gliadins

(position 56-88) with high proline contents (see 1.1.7.4) remains intact after the digestion with intestinal enzymes.

In general, the gluten digestion of healthy humans and CD patients does not differ. In contrast to healthy humans, CD patients show an intestinal epithelium permeable for macromolecules, such as gluten. The intestinal permeability is controlled by a protein called zonulin, which physiologically modulates the intercellular tight junctions [Fasano, 2011]. Drago et al. [2006] expected that the upregulation of zonulin was the reason for the increased intestinal permeability of CD patients.

In the lamina propria, which is a part of the intestinal lymphatic tissue, gluten peptides can activate two different immune responses: the adaptive and the innate immune response. The mechanism of the adaptive immune response is shown in Figure 1.4 (I-VIII).

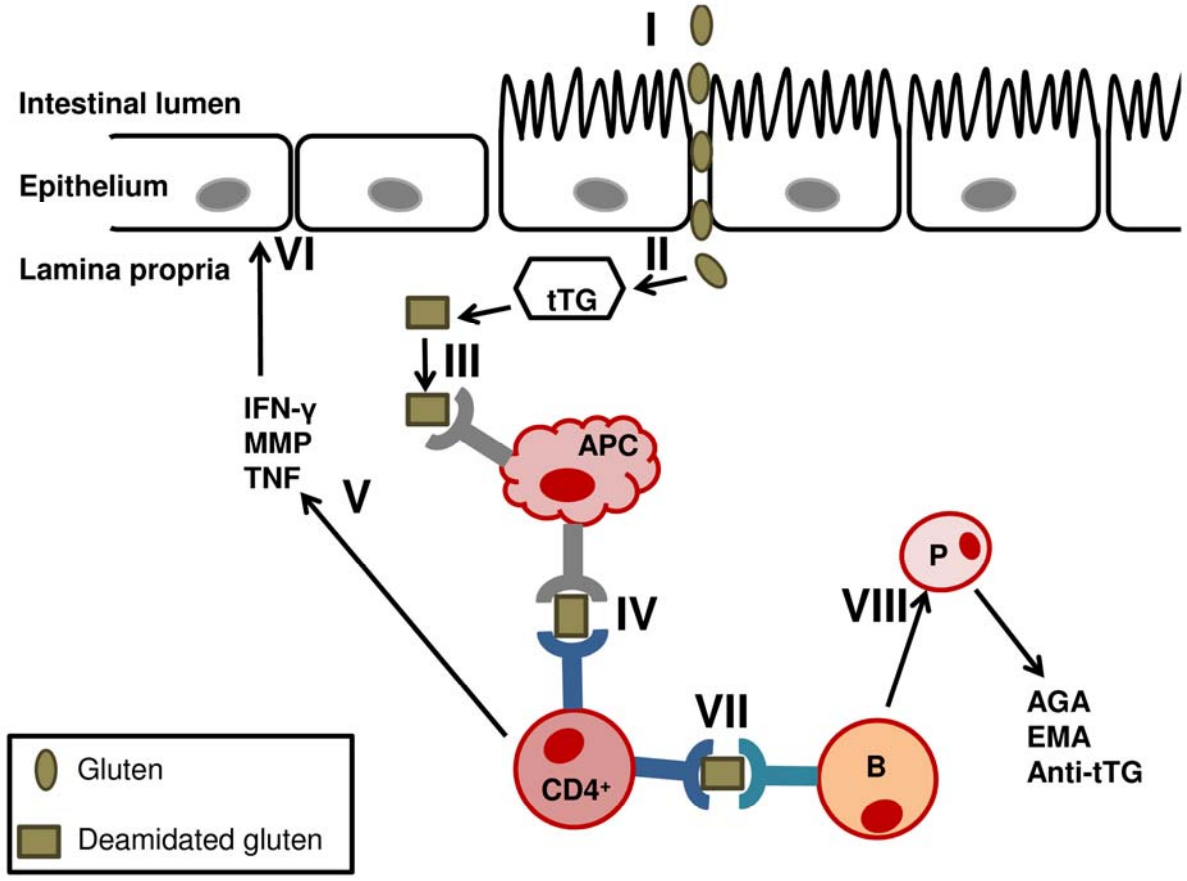

Figure 1.4: Mechanism of the adaptive immune response triggered by gluten intake in CD (AGA; anti-gliadin antibody; Anti-tTG; Anti-tissue transglutaminase antibody; APC, antigen-presenting cell; B, B-cell; $CD4^+$, $CD4^+$-T-cell; EMA, anti-endomysial antibody; IFN-γ, interferon-γ; MMP, matrix metalloproteinase; P, plasma cell; tTG, tissue transglutaminase; TNF, tumor necrosis factor) [modified according to Sollid, 2002 and Dieterich et al., 2003]

Gluten peptides, which pass through the intestinal epithelium (Figure 1.4, I), are deamidated by the calcium-dependent tTG, which converts neutral glutamine residues to negatively charged glutamic acid residues (Figure 1.4, II). In 1997, tTG was identified as major autoantigen in CD [Dieterich et al. [1997]. The negative charges of deamidated gluten peptides result in a high binding affinity of the epitopes to HLA-DQ2/DQ8 heterodimers, which are expressed on the cell surface of antigen-presenting cells (APCs) (Figure 1.4, III) [Molberg et al., 1998]. The DQ-peptide-complex is presented to T-cell receptors and consequently $CD4^+$-T-cells are activated

(Figure 1.4, IV). Gluten peptides must consist of at least nine amino acids to be recognized by $CD4^+$-T-cells [Sollid, 2002]. Activated $CD4^+$-T-cells express a high number of cytokines in the lamina propria, such as interferon (IFN)-γ and tumor necrosis factor (TNF) (Figure 1.4, V) [Dieterich et al., 2003]. As a consequence, matrix metalloproteinases are stimulated, which cause mucosal damage and villous atrophy (Figure 1.4, VI). Furthermore, activated $CD4^+$-T-cells can trigger the generation of B-cells (Figure 1.4, VII) and the expansion of plasma cells, which produce several serum antibodies including IgA and IgG against gluten (AGA), EMA and tTG antibodies (Figure 1.4, VIII) [Schuppan et al., 1998].

Some toxic gluten peptides show only a weak binding to HLA-DQ heterodimers and are not recognized by the adaptive immune response. These peptides can trigger an innnate immune response, which is typically characterized by a high number of IELs and the secretion of the cytokine interleukin (IL)-15, which resulted in increased enterocyte apoptosis and villous atrophy [Dieterich et al., 2003]. IL-15 is suggested to play the key role in the innate immune response and is generated by cells from the epithelium and the lamina propria.
Maiuri et al. [2003] demonstrated that the α-gliadin peptide (position 31-43) LGQQQPFPPQQPY induces an innate immune response. Junker et al. [2012] postulated that amylase-trypsin-inhibitors (ATIs) from cereal flours also activate the innate immune response.
At present, the pathogenisis of CD is not yet completely elucidated and it is unclear, whether the adaptive and the innate immune response occur independently or if both mechanisms induce villous atrophy.

1.1.7 Structures triggering CD

1.1.7.1 Toxicity testing

In general, the identification and evaluation of CD toxicity and immunogenicity of proteins and peptides is performed by *in vivo* challenge of CD patients and *in vitro* tests with tissue and cells of CD patients.
At the beginning, the *in vivo* testing of toxicity was performed by feeding tests, which were based on indicators such as steatorrhea or malabsorption of xylose. A further

development of the technique was the intestinal biopsy. Disadvantages of the *in vivo* studies were that the optimal amount of gluten equivalents used to challenge patients as well as the duration of the challenge were uncertain and large amounts of proteins (10-100 g of gluten equivalent) were required for challenge. The reduction of gluten equivalents (~ 1 g) was carried out by means of an oral challenge by direct instillation into the small intestine followed by biopsy at the beginning and after several hours of the challenge. The tissue was examined for changes in villous height, ratio of villous height to crypt depth and the number of IELs, which were reliable specifications for toxicity evaluation [Fraser et al., 2003; Dewar et al., 2006].

The toxicity or immunogenicity testing by *in vitro* tests enabled testing of only small amounts of peptides (≤ 1 mg of gluten equivalent) and the organ culture test of intestinal tissue of CD patients showed the highest reliability. During the cultivation of tissue in a culture medium, changes in enzyme activity and morphology can be observed, which are missing in the presence of gluten [Browning and Trier, 1969; Shidrawi et al, 1995]. Nowadays, T-cell lines and clones from CD patients are used to measure immunogenicity of peptides and proteins by means of a T-cell proliferation assay. For this purpose, gluten (putative antigen) is incubated with APCs, CD characteristic T-cells and tritiated thymidine followed by determination of the proliferation of T-cells by scintillation, which is a characteristic feature of immunogenic effects. Furthermore, the formation of IFN-γ or IL-4 can be measured. T-cell lines and clones frequently differ in their reaction to antigens and immunogenic effects do not always correspond to toxicity observed in *in vivo* studies. Therefore, *in vitro* tests should be verified by means of *in vivo* tests to assess CD toxicity [Wieser and Koehler, 2008].

1.1.7.2 CD-active cereals

Cereals botanically belong to the grass family (*Poaceae*). The subfamily (*Pooideae*) is divided into two tribes, which are called *Triticeae* and *Aveneae*. Wheat (*Triticum*), rye (*Secale*) and barley (*Hordeum*) are members of the tribe *Triticeae* and closely related, whereas oats (*Avena*) is a distant relative to the *Triticeae* within the *Poaceae* familly [Bouchenak-Khelladi et al., 2008]. According to the taxonomy of plants, they were distinguished between safe (CD-nontoxic) and unsafe (CD-toxic) [Kasarda, 2001]. All wheat species (hexa-, tetra- and diploid), triticale (*Triticum* x *Secale*), rye

and barley were classified as CD-toxic, due to the botantical relationship and similar protein compositions [Wieser, 2000; Gellrich et al., 2003; Lange et al., 2007]. The toxicity of oats is controversally discussed in the literature, but oats should be avoided in a gluten-free diet according to the Codex Stan 118-1979 [2015]. CD-nontoxic cereals (rice, *Oryza*; corn, *Zea*; sorghum, *Sorghum*; millet, *Pennisetum*) belong to other subfamilies of the *Poaceae* and show seperate evolutionary lines within the grass familiy. Seeds outside the grass family (buckwheat, amaranth, and quinoa) are called pseudocereals and have also been used in a gluten-free diet and shown to be safe.

1.1.7.3 CD-active proteins

According to their solubility, cereal proteins can be traditionally divided into the so-called Osborne fractions: albumins (water-soluble), globulins (salt-soluble), prolamins (soluble in aqueous alcohols) and glutelins (soluble in aqueous alcohols in the presence of reducing agents) [Osborne, 1924]. Albumins and globulins are composed of metabolic and protective proteins, such as enzymes and enzyme inhibitors (e.g. ATIs), whereas prolamins and glutelins form the storage proteins. Albumins and globulins do not trigger CD, but other interolerances, such as NCGS, which is probably caused by ATIs [Junker et al., 2012]. CD is triggered by the storage proteins from wheat, rye, barley and possibly oats, which are called gluten in the field of CD.

Different trivial names are related to these gluten proteins: gliadins (prolamins) and glutenins (glutelins) in wheat, secalins in rye, hordeins in barley and avenins in oats.

According to homologous amino acid sequences and similar molecular weights (M_r), the gluten proteins can be classified into the high-molecular-weight (HMW), the medium-molecular-weight (MMW) and the low-molecular-weight (LMW) groups [Shewry and Tatham, 1990; Wieser, 1994] (summarised in Table 1.1).

The HMW group consists of HMW-glutenin subunits (GS) from wheat, HMW-secalins from rye and D-hordeins from barley. The HMW-group is characterized by the repetitive unit QQPGQG and the formation of interchain disulphide bonds, which are responsible for the insolubility in aqueous alcohol without reducing agents.

The MMW-group is composed of the homologous ω1,2-gliadins from wheat, ω-secalins from rye and C-hordeins from barley and the unique ω5-gliadins from wheat,

which show the characteristic repetitive units (Q)QPQQPFP or (Q)QQQFP. This group contains only monomeric proteins, which are soluble in aqueous alcohols.
The LMW-group contains the monomeric α/β- and γ-gliadins from wheat, γ-40k-secalins from rye, γ-hordeins from barley and avenins from oats as well as the polymeric LMW-GS from wheat, γ-75k-secalins from rye and B-hordeins from barley. The protein types γ-gliadins, γ-40k-secalins and γ-hordeins are characterized by the repetitive unit QPQQPFP. The characteristic repetitive unit QPQPFPPQQPYP occurs in α/β-gliadins and PFVQQQQ in avenins. Furthermore, (Q)QQPPFS is the characteristic repetitive unit in LMW-GS and QQPQQPFP in γ-75k-secalins.

The high content of glutamine and proline residues is a structural feature of all CD-toxic proteins. Due to the high content of proline residues, these proteins are resistant to complete proteolytic digestion in humans [Shan et al., 2002].

Table 1.1: Classification of gluten proteins of wheat, rye, barley, and oats (modified according to Wieser et al., 2014)

Group	Wheat	Rye	Barley	Oats
HMW	HMW-GS (p)	HMW-secalins (p)	D-hordeins (p)	-
MMW	ω1,2-gliadins (m) ω5-gliadins (m)	ω-secalins (m)	C-hordeins (m)	-
LMW	LMW-GS (p) γ-gliadins (m) α/β-gliadins (m)	γ-75k-secalins (p) γ-40k-secalins (m)	B-hordeins (p) γ-hordeins (m)	Avenins (m)

HMW, high-molecular-weight; MMW, medium-molecular-weight; LMW, low-molecular-weight; GS, glutenin subunits; p, polymeric; m, monomeric

In 1970, the first study by Hekkens and coworkers demonstrated the toxicity of a well-defined protein fraction (aggregative α/β-type gliadins), which was performed by injection into the small intestine followed by biopsy. Furthermore, *in vivo* and *in vitro* studies elucidated that all gliadin types (α/β-, γ- and ω-gliadins) showed toxic effects

in CD [Ciclitira et al., 1984; Howdle et al., 1984]. Subsequent studies clarified that also glutenin types (HMW- and LMW-GS) induced a CD-specific immune response [Molberg et al., 2003; Dewar et al., 2006; Vader et al., 2002].
In 2010, an *in vivo* oral barley challenge was used to test hordein types for CD-specific immunotoxicity, which showed that all types were immunogenic, but C- and D-hordeins were most active compared to B- and γ-hordeins [Tanner et al., 2010].
In contrast, the different secalin-types were not tested up to now, but based on structural homologies with wheat proteins, it is very likely that they are also immunogenic [Vader et al., 2003].
The toxicity of avenins from oats is controversially discussed in the literature. On the one hand, an *in vitro* study by Kilmartin et al. [2003] did not show T-cell activation in the small intestinal biopsies of CD patients challenged with avenins. The *in vivo* study by Cooper et al. [2012] also demonstrated the lack of oat toxicity to CD patients. On the other hand, some patients have an intestinal T-cell response to oats that can cause mucosal inflammation [Lundin et al., 2003; Arentz-Hansen et al., 2004]. However, oats contain significant amounts of vitamins, minerals and especially dietary fibre, which would improve the nutritional value of a gluten-free diet [Comino et al., 2015]. Therefore, oats specially processed to avoid contamination from wheat, rye and barley could be included in a gluten-free diet under medical supervision [Pulido et al., 2009].
In general, it is recommended that oats should be avoided in a gluten-free diet due to the controversial scientific results mentioned before. Furthermore, oats is often contaminated with wheat, barley or rye during harvest, storage or processing of the cereals. To minimize the potential security risk of gluten-free food products, oats is eliminated as ingredient by the production of these products.

1.1.7.4 CD-active peptides

The toxicity of gluten peptides was also tested by *in vitro* (organ culture test) and *in vivo* studies (instillation), which demonstrated that the toxic amino acid sequences occur in the repetitive N-terminal domain of α-gliadins and generally consist of glutamine, proline and hydrophobic amino acids (leucine, phenylalanine, tyrosine) [Wieser and Koehler, 2008]. However, peptides from ω- and γ-gliadins as well as from secalins, hordeins and avenins have not been tested yet by organ culture tests.

Further studies were focused on CD epitopes from α- and γ-gliadins, secalins, hordeins and avenins that stimulate T-cells of CD patients [van de Wal et al., 1999; Arentz-Hansen et al., 2000a and 2002; Vader et al, 2002 and 2003; Shan et al., 2002]. Most of the peptides were rarely stimulatory to T-cells, but showed an increased T-cell response after treatment with tTG. tTG catalyses the deamidation of specific glutamine residues to glutamic acid, which results in an increased T-cell response and a high affinity to HLA-DQ2 [Johansen et al, 1997; Molberg et al., 1998]. In order to be recognized by T-cells, peptides must consist of at least nine amino acids [Sollid, 2002]. Vader et al. [2002b] demonstrated that the specificity of tTG depends on the presence of proline next to glutamine. The sequence QXP (X = any other amino acid) was a preferred motif for tTG, whereas the glutamine residues of QP or QXXP were not targeted [Vader et al, 2002b].

The stability of gluten peptides towards enzymatic breakdown is reponsible for CD toxicity and results in a high intestinal concentration of potentially immunogenic peptides [Wieser et al., 2014]. The resistance of peptides to gastrointestinal digestion is caused by the high number of proline residues in the repetitive domains [Shan et al., 2002]. A selection of T-cell stimulatory gluten peptides is shown in Table 1.2.

Table 1.2: Amino acid sequences of selected T-cell stimulatory gluten peptides (according to Wieser and Koehler, 2008; Wieser et al., 2014)

Origin (position)	Amino acid sequence[a]
α2 (57–68)	QLQPFPQP**Q**LPY[1]
α2 (56–88)	LQLQPFPQP**Q**LPYPQP**Q**LPYPQP**Q**LPYPQPQPF[2]
α20 (93-106)	PFRP**Q**QPYPQPQPQ[3]
γ5 (66–78)	FP**Q**QP**Q**QPYPQQP[4]
γ5 (102–113)	FSQP**Q**Q**Q**PFPQPQ[4]
γ30 (222–236)	VQG**Q**GIIP**Q**QPAL[3]
LMW 156 (40–50)	QQQQPPFS**Q**QQ**Q**SPFS[3]
LMW 17 (46–60)	QQPPFS**QQ**QQ**Q**QPLPQ [3]
HMW 2 (722–734)	GQ**Q**GYYPTSP**Q**QS[5]
HMW 2 (724–735)	**Q**GYYPTSP**Q**QSG[5]
Sec-α2	QPFPQP**Q**QPFPQSQ[6]
Sec-α9	P**Q**QPFPQP**Q**QPFPQ[6]
Hor-α2	Q**Q**FPQP**Q**QPFPQQP[6]
Hor-α9	P**Q**QPFPQP**Q**QPFRQ [6]
Ave-α9	QYQPYPEQ**Q**EPFVQ[6]
Ave-α9	QYQPYPEQ**Q**QPFVQ[6]

[1] Arentz-Hansen et al., 2000a; [2] Shan et al., 2002; [3] Vader et al., 2002; [4] Arentz-Hansen, et al, 2002; [5] van de Wal et al. 1999; [6] Vader et al., 2003;

[a] One letter code for amino acids; **Q**, glutamine residues deamidated by tissue transglutaminase (tTG)

The immunodominant 33-mer peptide

The large 33-mer peptide from α2-gliadin (positions 56–88, see Table 1.2) is resistant to cleavage by all gastric, pancreatic, and intestinal brushborder membrane peptidases [Shan et al., 2002]. It is widely called the most immunodominant gluten peptide [Shan et al., 2002; Carmarca et al., 2009; Qiao et al., 2005], because it contains three overlapping T-cell epitopes, namely PFPQPQLPY (DQ2.5-glia-α1a, one copy), PYPQPQLPY (DQ2.5-glia-α1b, two copies) and PQPQLPYPQ (DQ2.5-

glia-α2, three copies), which result in the induction of a strong immune response [Sollid et al., 2012]. It was used as a model peptide to study CD mechanisms [Fraser et al., 2003; Dorum et al., 2010] or the efficiency of gluten-degrading enzymes [Shan et al., 2004; Stepniak et al., 2006; Knorr et al., 2016]. Therefore, it plays an important role in the field of CD (see 1.1.8.2). Arentz-Hansen et al. [2000b] were the first to identify the 33-mer in α2-gliadin from the Norwegian common (bread) wheat (*Triticum aestivum*) cultivar Mjølner. DNA-sequencing revealed the entire amino acid sequences of eleven α-gliadins (α1 - α11) of this cultivar, but only α2-gliadin contained the 33-mer [Arentz-Hansen et al., 2000b]. T-cell proliferation assays demonstrated that treatment of the 33-mer with tTG resulted in a high T-cell immune response after specific deamidation of the glutamine residues in positions 65 and 72 [Arentz-Hansen et al., 2002; Arentz-Hansen et al., 2010], followed by strong binding to HLA-DQ2 [Dieterich et al., 1997, Vader et al., 2002b].
Furthermore, two monoclonal antibodies (A1 und G12) were raised against partial sequences of this peptide [Moron et al. 2008b] and are used in commercially available ELISAs for the immunochemical quantitation of gluten (see 1.2.2.2).
From the more than 580 published amino acid sequences of α-gliadins (BLAST Search, UniProtKB database, February 07, 2017), only 20 sequences contain the 33-mer and only one (P18573) has evidence at protein level based on data of the Norwegian wheat cultivar [Arentz-Hansen et al., 2000b]. Studies on the frequency of occurence of the 33-mer in different wheat species and cultivars are not available to date. However, this would be important in order to make a precise assessment of the relevance of the 33-mer in CD toxicity.

1.1.7.5 Approaches to the detoxification of gluten

For the first time, Shan et al. [2005] introduced prolyl-endopeptidases (PEPs) to detoxify gluten proteins. This class of enzyme is able to cleave peptide bonds at the carboxyl side of proline residues. A proline-rich peptide from α-gliadin, the 33-mer (see 1.1.7.4) was often used as model peptide to study the cleavage efficiency of this enzyme class. For example, the breakdown of the 33-mer peptide was catalyzed by PEP from *Flavobacterium meningosepticum* [Shan et al., 2002]. Furthermore, the fungal peptidase from *Aspergillus niger* (AN-PEP) showed a highly efficient degradation and detoxification of the 33-mer [Stepniak et al., 2006]. Walter and

coworkers [2014; 2015] showed that AN-PEP was suitable for the degradation of gluten in wheat bran and bread drink as well as in rye sourdough products. This approach enabled the production of gluten-free foods, e.g. gluten-free bread from originally gluten-containing raw material. Knorr et al. [2016] demonstrated that a peptidase-active barley malt extract is able to degrade the 33-mer into small peptides, which are not CD-active any more. This barley malt extract was used for the production of gluten-free beer (see 1.1.8.1).

Peptidases from lactic acid bacteria (lactobacilli) are also capable of hydrolysing proteins and peptides, which have high proline contents. Di Cagno et al. [2002] used four different strains of lactobacilli for wheat sourdough fermentation and showed that these bacteria were able to hydrolyse albumins, globulins and prolamins. Furthermore, Rizzello et al. [2007] demonstrated that the mixture of lactobacilli and fungal peptidases was capable of degrading gluten during long-time fermentation of wheat flour. The gluten content in sourdough was determinded by ELISA and resulted in 12 mg gluten/kg, which confirmed the detoxification of gluten. These studies offer new opportunities for the production of special sourdough-type breads that have low gluten contents or even that are gluten-free.

These peptidases are also suggested for use as therapeutic agents for oral enzyme therapy (see 1.1.8.2).

1.1.8 Treatment of CD

1.1.8.1 Gluten-free diet by consuming gluten-free foods

Currently, the only effective therapy for CD patients is the strict adherence to a lifelong gluten-free diet by consuming only gluten-free food products. Gluten-free foods are mainly based on raw materials such as corn, rice, sorghum, millet, amaranth, buckwheat and quinoa that do not contain CD-active proteins (see 1.1.7.2).

The production of gluten-free bread and beer is a big challenge for industry, because in general, these products are made from gluten-containing wheat or barley.

Due to the unique properties of wheat proteins (gliadins, glutenins), only wheat flour can form a cohesive dough with gas-holding ability, viscosity and elasticity, which results in bread with high volume and porous crumb [Wieser, 2007]. It is difficult to

mimic these unique properties for the production of gluten-free bread, which is usually produced from gluten-free flours or starches (e.g. potato, rice, corn). Wheat starch can also be used for production, if the gluten content of starch is less than 100 mg gluten/kg and the content of the final product is less than 20 mg gluten/kg. The water absorption capacity in gluten-free flour mixes is achieved by the addition of hydrophilic carbohydrates (e.g. carboxymethyl-cellulose, carrageenan or guar gum). Gluten proteins are replaced by proteins such as caseinates, skim milk powder, maize, egg or soy proteins [summarised by Houben et al., 2012].

According to the German purity law, beer must be only made from water, hops, yeast and malt, typically from gluten-containing barley or wheat. Gluten-free beer surrogates are based on safe cereals (e.g. rice, corn or millet) or pseudocereals (e.g. buckwheat, quinoa and amaranth). Because these raw materials are non-compliant to the German purity law, the finished product may not be called beer. Gluten-free beer surrogates differ in aroma and taste from barley-based beers. Studies by Knorr and coworkers [2016] enabled the development of a gluten-free beer in compliance to the German beer regulation. For this purpose, an enzyme-active malt extract produced from special barley malt with high gluten-specific peptidase activity was applied in the brewing process, which was able to degrade coeliac-active peptides in a beer matrix. Competitive R5 ELISA results showed that the obtained beer was gluten-free. This novel approach offers an opportunity to produce gluten-free barley-based beers with better sensory properties than beer surrogates.

The adherence to a gluten-free diet is often a big challenge for CD patients. Wheat flour, protein or starch is often used as filling material or additive to produce e.g. instant meals. Furthermore, malt extract is often used as flavouring agent in chocolate. Therefore, CD patients have to pay attention to the list of ingredients to avoid products containing so called hidden gluten. In general, eating out in restaurants is difficult for CD patients, because wheat flour is often used as ingredient and a cross contamination of supposedly gluten-free ingredients can not be excluded.

1.1.8.2 Novel approaches for alternative therapies

CD research is focused on several therapeutic approaches for CD treatement as an alternative for the gluten-free diet. Special emphasis is placed on oral enzyme therapy, inhibition of tTG and HLA-DQ blocking.

Oral enzyme therapy

By means of oral enzyme therapy, gluten proteins and peptides have to be degraded into small fragments containing less than nine amino acids already in the stomach, which do not trigger an immune response in the small intestine [Sollid, 2002]. For this purpose, peptidases from germinated cereals, bacteria and fungi can be applied (see 1.1.7.5).
Oral enzyme therapy describes the intake of a peptidase pill as therapeutic agent, which is able to break down CD-triggering proteins and peptides present in the diet. Enzmyes for use as therapeutic agent have to be resistent towards gastric digestive enzymes and active at low pH levels (pH of the stomach: 1-2) and physiological temperature (37 °C).
For example, Shan et al. [2004] evaluated the activity, specificity and acid stability of three different peptidases (PEP from *Flavobacterium meningosepticum*, *Sphingomonas capsulata* and *Myxococcus xanthus*) by *in vivo* and *in vitro* studies. The results demonstrated that all peptidases were relatively resistant to acid, pancreatic peptidases of the small intestinal mucosa, but showed deficencies regarding specificity. In contrast, PEP from *Aspergillus niger* and endoprotease B2 (glutamine-specific protease of germinating barley in combination with PEP from *Sphingomonas capsulata*) were active and stable at acid pH. Therefore, a combination of both was suggested as therapeutical agent and was currently evaluated in clinical studies [Mitea et al., 2008b].

However, oral enzyme therapy will not be able to degrade immunogenic epitopes of a normal daily gluten ingestion (approximately 13 g gluten/day), but it could be used to eliminate the harmful effect of gluten contaminations present in the gluten-free diet [Wieser et al., 2014].

Inhibition of tTG

The concept of inhibition of tTG is suggested as therapy of CD, because tTG plays an important role in the adaptive immune reponse (see 1.1.6). Due to the deamidation of specific glutamine residues by tTG, gluten peptides bind more strongly to HLA-DQ molecules than the unmodified peptides, which results in a strong T-cell stimulation. Presently, reversible, irreversible or competitive tTG inhibitors have been proposed for CD treatment [Siegel and Khosla, 2007]. The structure of competitive inhibitors is typically characterized by a primary amine, e.g. cystamine. The main principle is that the inhibitor competes with natural amine substrates, such as protein-bound lysine residues in the deamidation or transamidation reaction while tTG is still enzymatically active.
However, irreversible inhibitors, such as thiodiazoles, epoxides or dihydroisooxazoles effect a covalent modification, which prevents enzyme activity. Studies by Hausch et al. [2003] demonstrated that gluten peptide analogs containing acivicin or alternatively 6-diazo-5-oxo-norleucine induce the irrevesible and selective inhibition of human tTG, which was confirmed in tTG activity assays.
In contrast, reversible tTG inhibitors, such as thienopyrimidines prevent enzyme activity by blocking substrate access to the active center of the enzyme [Siegel and Khosla, 2007] see above.

HLA-DQ blocking

Gluten peptides bind to HLA-DQ2/8 on the surface of APCs and induce the activation of $CD4^+$-T-cells, which result in the initiation of the adaptive immune response and mucosal damage (see 1.1.6). The approach to blocking the binding site of HLA-DQ2/8 to prevent the presentation procedure has been suggested for CD treatment. An *in vitro* study using small intestinal mucosa from CD patients demonstrated that the decapeptide QQPQDAVPQF from durum wheat acted as an antagonist for gliadin peptides [Silano et al., 2007] by forming a strong interaction with HLA-DQ2/8.
Several peptide blockers, such as cyclic or dimeric peptides were developed, which have a higher affinity to HLA-DQ molecules than native gluten peptides and are not targeted by $CD4^+$-T-cell receptors [Xia et al., 2007].

Further progress on these novel therapies is needed and therefore, the gluten-free diet is still the only effective and safe therapy for CD patients at present.

1.1.9 Legislation

The legal provisions regarding gluten-free products are regulated in various ways in different countries. The guidelines of the Codex Alimentarius serve as a basis for recommendations, but they are not legally binding. In 1963, the Codex Alimentarius was established by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) and contributes to the safety, quality and fairness of the international food trade. The *Codex Standard for Foods for Special Dietary Use for Persons Intolerant to Gluten* includes a definition of gluten (see 1.1.1), the threshold, labelling, and the method of analysis (see 1.2.2.2). The threshold of 20 mg gluten/kg food must not be exceeded, if the food product is declared as “gluten-free”. The labelling “low level gluten” can be used, when “the foods consist of one or more ingredients from wheat, rye, barley, oats or their crossbred varieties, which have been specially processed to reduce the gluten content to a level above 20 up to 100 mg/kg in total” [Codex Stan 118, 2015]. The recommended detection method is the ELISA R5 Mendez Method [Garcia, 2005], which enables the quantitation of the prolamin fraction. Because the prolamin content of gluten is generally taken as 50% according to the Codex, the prolamin fraction is multiplied by a factor of 2 to obtain the gluten content (see 1.2.2.2).

The regulation of the European Union (EU) is in compliance with the Codex Alimentarius regarding definitions, labelling and thresholds and is legally laid down in the European Commission (EC) Regulation No 41/2009. This regulation is repealed by EC Regulation No 609/2013, which lays down the requirements for *“food intended for infants and young children, food for special medical purposes, and total diet replacement for weight control”*. In order to regulate the declaration on prepacked and non-prepacked foods, the EC Regulation No 1169/2011 on *“the provision of food information to consumers”* was passed. The declaration based on this law should help to improve the information of consumers. Therefore, allergens or ingredients triggering intolerances (inter alia wheat, rye, barley, oats, spelt, kamut or their

hybridized strains, and products of these cereals) have to be underlined and are listed in annex II of the regulation.

In the USA, the U.S. Food and Drug Administration (FDA) issued a regulation that defined the term “gluten-free” for food labeling. Gluten is defined as “protein that occurs naturally in wheat, rye, barley, and crossbreeds of these grains”. According to the FDA, gluten-free foods can be labeled in four different variations, namely “gluten-free”, “free of gluten”, “no gluten” or “without gluten”. The specified threshold is 20 mg gluten/kg foods, which is in compliance to the Codex Alimentarius.

In Canada, food safety is regulated by the *Canadian Food and Drugs Act* and the *Canadian Food and Drug Regulations* (FDR). The requirements for gluten-free foods are specified in the regulation “*Foods for Special Dietary Use*”. Accoridng to this provision, gluten is defined as “any gluten protein from the grain of any of the following cereals or the grain of a hybridized strain created from at least one of the following cereals: barley, oats, rye, triticale, wheat” (a) or “any modified gluten protein, including any gluten protein fraction, that is derived from the grain of any of the cereals referred to in paragraph (a) or the grain of a hybridized strain referred to in that paragraph”. Section B.24.018 of the regulation specifies that it “is prohibited to label, package, sell or advertise a food in a manner likely to create an impression that it is a gluten-free food, if the food contains any gluten protein or modified gluten protein, including any gluten protein fraction, referred to in the definition “gluten”. A gluten threshold was not defined in this regulation and, therefore, Health Canada considers that “gluten-free foods, prepared under good manufacturing practices, which contain levels of gluten not exceeding 20 parts per million as a result of cross-contamination, meet the health and safety intent of section B.24.018 when a gluten-free claim is made” based on available scientific evidence.

In Australia and New Zealand, the terms related to gluten are regulated by Food Standards Australia and New Zealand (FSANZ) in the *New Zealand and Australian Food Standard Codes* (FSC). The Standards 1.2.7 (*Nutrition, Health and Related Claims* of 30 October 2014) and 2.9.5 (*Food for Special Medical Purposes* of 30 October 2014) govern the requirements for gluten-free foods and were revoked on 1 March 2016 by the new Standard 5.1.1. The label “gluten-free” can only be applied to

foods, which contain “no detectable gluten; or oats and their products; or cereals containing gluten that have been malted, or their products”. In this case “gluten-free” means that no gluten is detected by the current available techniques, e.g. R5 ELISA, which has a limit of detection (LOD) of 3 mg/kg (see 1.2.2.2). Due to this more stringent regulation compared to the Codex Stan 118 [2015], it is difficult to declare foods as “gluten-free”.

1.2 Gluten analysis

To ensure the safety of gluten-free products, it is essential that appropriate analytical methods with high specificity and sensitivity are available. Wieser [2008], Haraszi et al. [2011] and Wieser et al. [2014] summarised the main points about gluten analysis. The analytical procedure is generally divided into three levels. Firstly, the complete extraction of gluten proteins or peptides from the matrix is performed, which should be suitable for raw and processed food products. Secondly, an accepted reference material is applied for calibration. The third step involves the quantitation of the extracted proteins or peptides.
Most of the currently applied methods aim for the quantitation of the prolamin fraction of gluten and the glutelin fraction is not targeted although both fractions contain immunogenic epitopes [van de Wal et al., 1999]. Presently, the focus is on immunological methods (ELISA) to detect trace levels of gluten proteins or peptides in raw and processed food products. Due to several drawbacks of ELISA, it is important to find independent non-immunochemical methods to control the ELISA results. PCR, chromatographic and several mass spectrometric techniques have been suggested as promising methods to detect gluten proteins.

1.2.1 Gluten extraction

The first level of gluten analysis is the complete extraction of gluten proteins from raw materials or processed food products. Native gluten proteins are characterized by a high complexity of the structure including high molecular weights and inter- and intramolecular disulphide bonds [Haraszi et al., 2011], which are responsible for their insolubility in water or salt solutions. Only albumins and globulins are soluble in water

or salt solution and were most often preextracted before gluten extraction. However, partially hydrolysed gluten (e.g. in beer) is extractable with water, salt or aqueous alcohol solutions.

Prolamins are extracted with aqueous alcohols (ethanol, propanol) and according to Codex Stan 118 [2015] the prolamin extraction solvent has been defined as 40-70% ethanol. The extraction with 60% ethanol or 50% propanol is recommended according to a previous study, which demonstrated that the optimal extraction of gliadins from wheat flour was achieved at this concentration [Wieser et al., 1994]. In processed materials, aqueous alcohols are inadequate to solubilise the prolamin fraction, because gliadins and glutenins form insoluble aggregates by interchain disulphide bonds through heat treatment [Kieffer et al., 2007]. Therefore, the extraction of prolamins in processed foods as well as glutelins from raw and processed materials is performed using the combination of aqueous alcohols, reducing agents (e.g. 2-mercaptoethanol, tris(2-carboxyethyl)-phosphine (TCEP), dithiothreithol (DTT)) and disaggregating agents (e.g. guanidine, sodium dodecyl sulphate (SDS), urea). The sandwich R5 ELISA method [Koehler et al., 2013a] involves the extraction by the so-called cocktail, which is composed of 2-mercaptoethanol (reducing agent) and guanidine (disaggregating agent) in a phospate buffer. The cocktail enables the complete extraction of gluten from raw and heat-processed materials in one step and an incubation for 40 min at 50 °C is recommended [Garcia et al., 2005]. The utilisation of 2-mercaptoethanol is associated with several drawbacks due to the weak reducing power, toxicity and unpleasant odor. Therefore, it was replaced by TCEP and studies by Gessendorfer et al. [2010] showed that the extraction of gluten proteins was as effective as the commercial cocktail solution. The combination of TCEP and N-lauroyl-sarcosine in phosphate buffer was called UPEX (universal prolamin and glutelin extraction solvent) and was shown to be suitable to extract gluten proteins and peptides from various food products [Mena et al., 2012]. In cereal chemistry, DTT is usually used as a nontoxic and effective agent for the reduction of disulphide bonds [e.g. van den Broeck et al., 2009].

In summary, various extraction procedures were described for gluten analysis and their compatibility with the subsequent analytical method needs to be verified for each approach.

1.2.2 Reference materials and calibration

Reference materials are widely used for the calibration of measuring instruments and procedures. In accordance to the ISO Guide 30 [2015], the term reference material is defined as “material, which is sufficiently homogeneous and stable with respect to one or more specified properties and has been established to be fit for its intended use in the measurement process”.

In order to enable the quality assurance and the comparability of analytical results between different methods and laboratories as well as the validation of measurements (calibration and traceability), it is important to establish standardized and well-characterized reference materials [Lauwaars and Anklam, 2004].

Gluten is a complex mixture of different proteins with various molecular weights and is characterized by a high biological variability, such as modifications in amino acid composition caused by exchange or deletion (isoforms) of amino acids, post-translational modifications and varying protein contents. Furthermore, gluten is not intact but partially degraded in fermented food products, such as beer and sourdough. Due to these high variabilities, the standardization of a gluten reference material is more difficult compared to well-characterized chemical entities like mycotoxins [Lacorn et al., 2013].

To date, the only well-characterized reference material is the so-called PWG gliadin, which was developed by the Working Group on Prolamin Analysis and Toxicity in 2006 [van Eckert et al., 2006]. PWG gliadin is a reference material, which was isolated from a mixture of 28 wheat cultivars representative of the three main European wheat producing countries, France, UK and Germany. However, PWG gliadin represents only the alcohole-soluble fraction of total gluten and can only be applied in analytical methods such as ELISA, which target the prolamin fraction. The calibration of the sandwich R5 ELISA is performed with PWG gliadin, whereas other ELISA kits utilize different reference materials for calibration, e.g. vital wheat gluten (AgraQuant® ELISA Gluten G12).

Currently, the development of a new standardized reference material for gluten analysis is implemented by the MoniQA Association (International Association for Monitoring and Quality Assurance in the Total Food Supply Chain) [Poms, 2013]. Hajas et al. [2017] in cooperation with MoniQA developed several criteria for the

selection of wheat cultivars, which would be suitable as a basis for reference material production.

1.2.2.1 Chromatographic methods

For gluten separation, characterization and quantitation, high-performance liquid chromatography (HPLC) has long been used. Gel-permation (GP) and reversed-phase (RP) chromatography are most commonly used for gluten separation according to different molecular weights (GP) or different hydrophobicities (RP). Wieser et al. [1998] established a combined extraction-RP-HPLC procedure to quantitate the amounts of different wheat protein types in flour. The flour proteins were separated into three fractions, namely the water-/salt-soluble albumins/globulins, the alcohol-soluble prolamins and the glutelins, which were only soluble after reduction of the disulphide bonds. The calibration was performed with the reference material PWG gliadin [van Eckert et al., 2006] (see 1.2.2). UV absorbance at 200-220 nm was applied to detect the proteins eluted from the column. At these wavelengths, a strong correlation between the absorbance units and the protein quantity was observed [Wieser et al., 1998].

Scherf et al. [2016] developed a novel method for the quantitation of gluten in wheat starch by GP-HPLC-FLD. The sensitivity of RP-HPLC with UV detection was not sufficient to detect gluten below 300 mg/kg. Therefore, FLD was used, because the sensitivity was about 100-fold higher compared to UV [Chan et al., 2011]. GP-HPLC-FLD allowed the sensitive detection of gluten with a limit of quantitation (LOQ) of 17.2 mg/kg, which was calculated as the sum of gliadins and glutenins. By this means, both fractions of gluten were determined. Low selectivity is the limitating factor of the described chromatographic methods and, therefore, they are limited to the detection of gluten in raw materials.

1.2.2.2 Immunological methods

Enzyme-linked immunosorbent assay (ELISA)

ELISAs are based on the specific reaction of antibodies with antigens (CD-toxic proteins or peptides). The antibodies were produced by immunisation of animals (usually mice or rabbits) by injection of the corresponding immunogens. Antibodies or antigens are covalently linked to an enzyme, such as horseradish peroxidase or alkaline phosphatase, which forms a coloured product for spectrophotometric measurement [Wieser and Koehler, 2008]. In general, two types of antibodies are used: monoclonal antibodies, which recognize only one main epitope and polyclonal antibodies, which recognize many epitopes on a single protein.

The determination of gluten by ELISA is an appropriate method for routine analysis, because ELISA is relatively easy to perform, often cheaper than other techniques (e.g. liquid chromatography mass spectrometry, LC-MS), supplies rapid results, showes high sensitivity and selectivity, and can even be perfomed by small laboratories.

In general, two types of ELISA systems can be distinguished: the sandwich ELISA and the competitive ELISA (Figure 1.5) [Wieser et al., 2014]. The sandwich ELISA is used to detect intact proteins in raw materials (e.g. flour, spices) and processed food products like pasta, bakery products and ice cream. The antigen needs to have two spatially separated binding sites and therefore the ELISA system is only used to detect large antigens, such as gluten proteins. In contrast, the competitive system is applied for the analysis of fermented or hydrolysed food products like beer, sourdough, starch sirup, and malt extract due to the requirement of only one binding site.

In sandwich ELISA, a known amount of the capture antibody is immobilized on a microtiter plate. In step 1 (Figure 1.5) the gluten-containing sample (antigen) is applied, resulting in the formation of the antibody-antigen-complex. After washing to remove the excess antigen, the enzyme-labelled detection antibody is added, which binds to the second binding site of the antigen (step 2). By this means, the antigen is “sandwiched” between two antibodies and unbound detection antibodies are washed out. After addition of the substrate, a coloured product is formed and the absorbance is measured spectrophotometrically (step 3). Based on the calibration curve of a

gluten reference material, the antigen concentration can be calculated because it is directly proportional to absorbance in the sample.

In competitive ELISA, the microtiter plate is coated with capture-antibodies. In step 1, a limited and constant quantity of enzyme-labelled antigens and unlabelled antigens from the sample compete for the limited number of antibody binding sites. Unbound antigens are removed by washing, the substrate is added and the generated coloured product is determined. In contrast to sandwich ELISA, the absorbance is inversely proportional to the quantity of antigen in the sample.

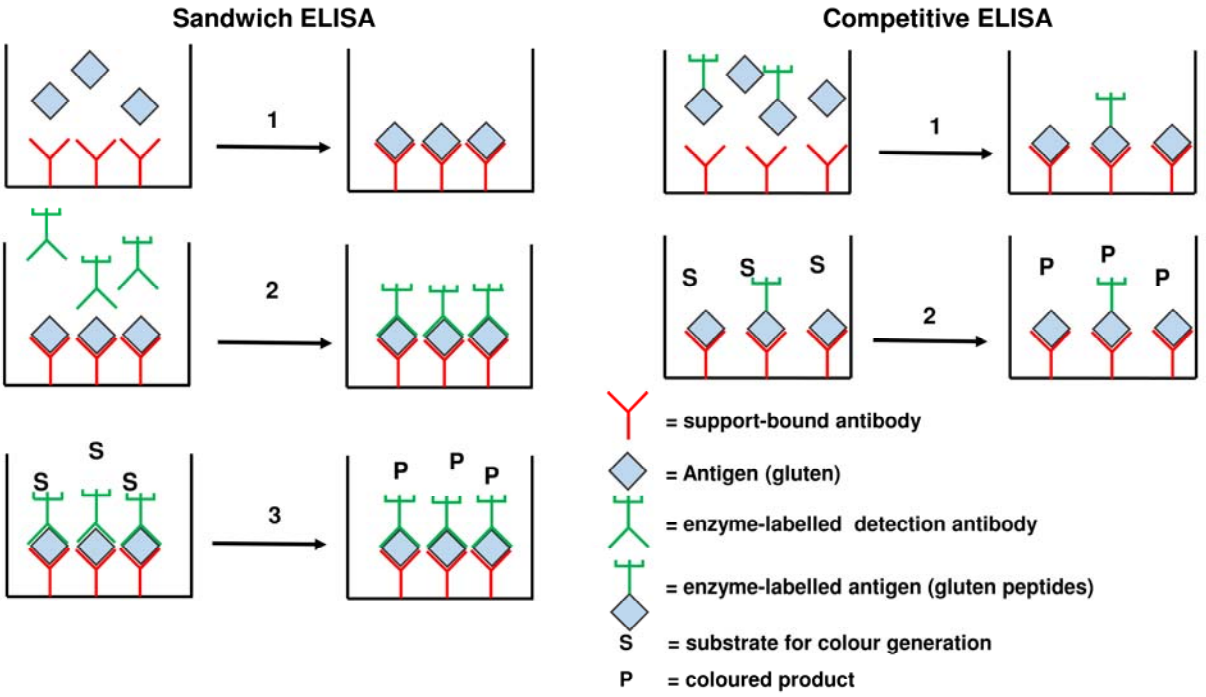

Figure 1.5: Illustration of the main principles of sandwich and competitive ELISAs [according to Wieser, 2008]

Several ELISA test kits, which are based on different mono- or polyclonal antibodies are commercially available on the market. In 1990, Skeritt and Hill developed a sandwich ELISA based on the monoclonal antibody 401.21, which was raised against heat-stable ω-gliadins and reacts with the epitope QQGYYP [Colgrave et al., 2012]. This assay was developed for gluten quantitation in raw and processed foods with a sensitivity of 20 to 160 mg gluten/kg. Particular drawbacks of the 401.21

monoclonal antibody are that barley prolamins are poorly recognized and the results are strongly cultivar-dependent because of different proportions of ω-gliadins and their homolgs [Wieser et al., 1994]. This ELISA has been well evaluated in collaborative studies, approved by the Association of Official Analytical Chemists (AOAC, International), patented and sold by different companies (e.g. BioKits Gluten Assay Kit, Neogen ®, Ayr, Scotland and Gluten Aller-Tek, ELISA Technologies ®, Gainesville, FL, USA).

In 2003, Valdes et al. established a sandwich ELISA based on the monoclonal R5 antibody. The R5 antibody was raised against ω-secalins from rye and recognizes the epitopes QQPFP, QQQPF, LQPFP, and QLPFP in prolamins from wheat, rye, and barley [Kahlenberg et al., 2006], but shows limited reactivity towards the glutelin fractions. Tanner et al. [2013b] demonstrated that the reactivity of the R5 antibody is different depending on the hordein type, because C-hordeins were recognized more sensitively than γ-, B-, and D-hordeins. The determination of intact gluten by sandwich R5 ELISA together with cocktail extraction [Garcia et al.; 2005] is endorsed as type I method by the Codex Alimentarius Commission and was validated by two collaborative studies [Mendez et al., 2005; Koehler et al., 2013a] followed by the adoption as AACCI Approved Method 38-50.01. The R5 sandwich assay is commercially available on the market from different manufacturers (e.g. RIDASCREEN ® Gliadin, R-Biopharm, Darmstadt, Germany, Veratox ® for Gliadin R5, Neogen, Lansing, USA) and enables the detection of gliadin with an LOD of 1.5 mg gliadin/kg and an LOQ of 2.5 mg gliadin/kg. The sandwich test kit is calibrated with the reference material PWG gliadin. Furthermore, the R5 ELISA can be obtained as a competitve system, which is calibrated with a mixture of peptic-tryptic hydrolysates from wheat, rye, and barley prolamins for the quantitation of prolamin peptides in fermented products with an LOD of 2.3 mg prolamin/kg and an LOQ of 6.7 mg prolamin/kg [Gessendorfer et al., 2009]. The competitive R5 ELISA was also validated by a collaborative study and is adopted as AACCI Approved Method 38-55.01 [Koehler et al., 2013b].

Two monoclonal antibodies, termed G12 and A1 were raised against the immunodominat 33-mer peptide (see 1.1.7.4) from α2-gliadin [Shan et al., 2002]. The antibodies recognize the epitopes QPQLPY, QPQLPF, QPQLPL, QPQQPY (G12) and QLPFPQP, QQPFPQP, QLPYPQP, QQPYPQP, QQPYPQE (A1) in decreasing order of affinity. Both antibodies are the basis of a sandwich test kit as well as a

competitive system [Moron et al., 2008a; 2008b]. The assay uses an ethanolic extract of vital wheat gluten for calibration. The sandwich ELISA allows the detection of wheat, rye, and barley prolamins with an LOD of 2 mg gluten/kg and an LOQ of 4 mg gluten/kg. By G12 ELISA, glutelin fractions of wheat, rye, and barley showed lower gluten recovery compared to the prolamin fraction, which was demonstrated by Rallabhandi et al. [2015]. Studies by Comino et al. [2011] showed that the G12 monoclonal antibody had a low affinity to oats, which resulted in a higher LOD for oat prolamins. It was postulated that the reactivity was proportional to the potential immunotoxicity of oat cultivars. In 2014, a collaborative study of the G12/A1 sandwich test kit resulted in the adoption as AACCI Approved Method 38-52.01 [Don et al., 2014].

In 2008, Mitea et al. characterized the specificity of the monoclonal antibody α20, which detects the epitope RPQQPY from α-gliadins [Mitea et al., 2008a]. The competitive ELISA based on the α20 antibody is commercially available (Gluten-Tec® ELISA, EuroProxima, Arnheim, Netherlands) and was tested in a collaborative study [Mujico et al., 2012]. The assay is calibrated with a synthetic peptide (GPFRPQQPYPB) and consequently the obtained peptide concentration (ng/mL peptide) must be converted into ng gliadin/g, by a conversion factor of 100 (from peptide to gliadin) and a factor of 2 (from gliadin to gluten). The assay showed an LOD of 2.5 mg gliadin/kg. Sajic et al. [2017] used the synthetic DQ2.5-glia-α3 peptide (QPFRPQQPYPQPQ) for assay calibration and the obtained peptide concentrations were converted into gliadin contents using a multiplication factor of 250 and a factor of 2 (from gliadin to gluten). The LOD of the assay was 2.9 mg gliadin/kg.

All ELISA kits are mainly based on the detection of the prolamin fraction, which represents only one fraction of total gluten. To determine the gluten content of a sample, the prolamin content has to be multiplied by a factor of 2, because the prolamin content of gluten is taken as 50 % according to Codex Stan 118-1979 [2015]. However, Wieser and Koehler [2009] demonstrated that the ratio of prolamin/glutelin was generally higher than 1 and strongly influenced by the cereal species. In cases of barley, oats and especially rye, the ratio was higher than 1. In cases of wheat starch the ratio of prolamin/glutelin was below 1 and lay between 0.19 and 0.52 [Scherf et al., 2016b]. As a consequence, the gluten contents will be either over- or underestimated by the multiplication with the factor 2, which carries a high level of risk to ensure the safety of gluten-free products in case of underestimation.

Lexhaller et al. [2016] compared five different ELISA test kits based on the R5, G12, α20 monoclonal antibodies and two polyclonal antibodies for their sensitivity and specificity using isolated prolamins and glutelins from wheat, rye, and barley. The study demonstrated that various gluten fractions gave variable results within one test kit and furthermore different ELISAs gave variable results within one gluten fraction. In conlusion, the antibody sensitivities and specificities depend on the source of gluten and the gluten fraction. Due to this fact, it is not suprising that different ELISA kits resulted in different gluten contents when analysing the same wheat starch sample [Scherf, 2017].

Different commercial ELISA test kits can hardly be compared, because of different calibration standards, different sample extraction solutions and especially the application of various antibodies, which differ in the specificity and sensitivity.

Immunological assays are also available as dipstick or lateral flow tests for rapid and qualitative determination of gluten, which indicate only the absence or presence of gluten proteins. These assays are currently available based on the R5, G12, and Skeritt monoclonal antibodies and can be used as swab tests of potentially contaminated surfaces [Scherf and Poms, 2016].

Immunosensors

Immunosensors are analytical devices, in which the immunochemical reaction (antibody/antigen) is coupled to a physicochemical transducer. The methodology of an immunosensor is similar to that of an immunoassay, which is based on the specific antigen/antibody interaction [Luppa et al., 2001]. Immunosensors can be classified into different detection principles including electrochemical, optical, gravimetrical, magnetical and calorimetrical immunosensors [Scherf et al., 2016c].

Nassef et al. [2008] developed an electrochemical immunosensor to detect gliadins in gluten-free and gluten-containing raw and processed food products. The immuosensor was based on an antibody, which was raised against the CD-immunodominant epitope LQLQPFPQPQLPYPQPQLPY (α56-75) [Fraser et al., 2003]. The obtained results were compared to those by ELISA based on a polyclonal antibody and a strong correlation was observed. The detection of gliadins or gliadin fragments in raw and processed food samples (e.g. beer and skimmed-milk) was achieved by a competitive magneto immunosensor based on gliadin immobilized to

tosyl-activated magnetic beads [Laube et al., 2011]. Both immunosensor systems guaranteed the sensitve detection of gliadins with an LOD of around 10 ng gliadin/mL. In 2016, Manfredi et al. developed the first competitive electrochemical immunosensor based on gliadin-functionalized carbon/nanogold screen-printed electrodes. This new immunosensor enabled the rapid and sensitive detection of gliadins with an LOD of 8 ng gliadin/mL.

Immunosensors are simple, rapid, user-friendly and cost-effective analytical tools for gluten detection in foods and therefore a promising alternative to established immunochemical methods like ELISA [Scherf et al., 2015].

1.2.2.3 Polymerase chain reaction

PCR is used for the amplification of specific DNA segments. As a consequence, PCR does not target gluten proteins themselves, but DNA, which indicates the presence of gluten. The PCR cycle involves three repetitive steps: 1) denaturation by heat, 2) hybridisation, and 3) polymerization. Firstly, a double-stranded DNA sequence is denaturated at 95 °C to separate the strands. Secondly, characteristic oligonucleotide sequences (primers) are added, which are complementary to the target DNA segment and the temperature is reduced to 55 °C so that the primers can bind to the corresponding ends of the DNA strands. Thirdly, polymerase is inserted to synthesise a new DNA strand and the temperature is increased to 72 °C, which is the enzyme optimum. This procedure is repeated multiple times and thousands to millions of copies are generated in a short time [Lottspeich and Zorbas, 1998].
Gluten analysis by PCR was firstly applied by the group of Lüthy in Berne (Switzerland) [Wieser and Koehler, 2008]. A PCR assay was established to detect wheat contamination in foods and a highly repetitive and characteristic genomic wheat DNA fragment was used as primer for amplification. The assay was tested in 35 various food samples, which included bakery additives as well as heated and processed foods [Allmann et al., 1993]. The results showed that wheat starch had a strong positive reaction and pure gluten additives were not detected because of the lack of genomic DNA.
Furthermore, Sandberg et al. [2003] developed a real-time PCR assay for the specific discrimination of wheat, barley, rye, and oats in gluten-free foods. A specific primer

was used, which targeted cereal prolamin genes. The results showed good correlations with the results obtained from the ELISA with the Skeritt antibody.
Further investigations in this field resulted in the detection of wheat, spelt, kamut, rye, barley, and oats by real-time PCR. Primers, which were characteristic for HMW subunits were used to detect wheat, spelt, kamut, and rye with an LOD of 5 mg/kg, respectively. The gene Hor3 was chosen for the detection of barley and the gene encoding the 12S seed storage protein was selected to detect oats with a sensitivity of 10 mg/kg for each of them [Zeltner et al., 2009].
Scharf et al. [2013] evaluated the performance of PCR and ELISA methods for the determination of wheat by proficiency testing over a period of 6 years. In this study 45 laboratories submitted PCR results and 170 laboratories submitted ELISA results. The PCR method demonstrated no false-negative results and ELISA methods showed only 2% false-negative results in pastry and sausage meat, which consist of complex matrices.

All studies demonstrated that PCR can be used as a sensitive screening method to detect the presence of gluten from different types of grains. The disadvantage of PCR is that is not possible to detect gluten in processed and hydrolysed samples such as malt extracts and beer because of the extreme degradation of DNA.

1.2.2.4 Mass spectrometry

Main principle

Several approaches to the analysis of proteins or peptides by different mass spectrometric techniques were developed in recent years. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was mainly used for protein characterization [e.g. Camafeita et al., 1998; Koenig et al.; 2015] to determine the relative molecular mass of proteins. Untargeted LC-MS/MS was performed for the identification of peptides [e.g. Rombouts et al., 2013] and targeted LC-MS/MS in the multiple reaction monitoring (MRM) mode for the quantitation of selected peptides [e.g. Sealey-Voyksner et al., 2010]. The quantitation of gluten-specific peptides by targeted LC-MS/MS was mainly based on an external calibration procedure by spiking peptides, gluten or gluten-containing flour into a gluten-free

matrix [e.g. Fiedler et al., 2014; van den Broeck et al., 2015]. Alternatively, the addition of an internal standard prior to sample preparation and the quantitation based on a specific peptide response factor can be attempted. The use of an isotopically labelled peptide as internal standard offers the advantage that analyte and standard have the same chemical properties and behaviour during sample preparation. Therefore, the loss of analyte during sample preparation can be compensated, which results in a very precise determination of the peptide concentration. Presently, this approach was not attempted so far in the field of gluten peptide analysis. However, in other scientific fields, such as biomedicine, this strategy was already undertaken frequently [Gillette and Carr, 2013].

The main principle of a targeted LC-MS/MS approach in the MRM mode and the application of an isotopically labelled peptide, which was used as internal peptide standard for quantitation, are illustrated in Figure 1.6.

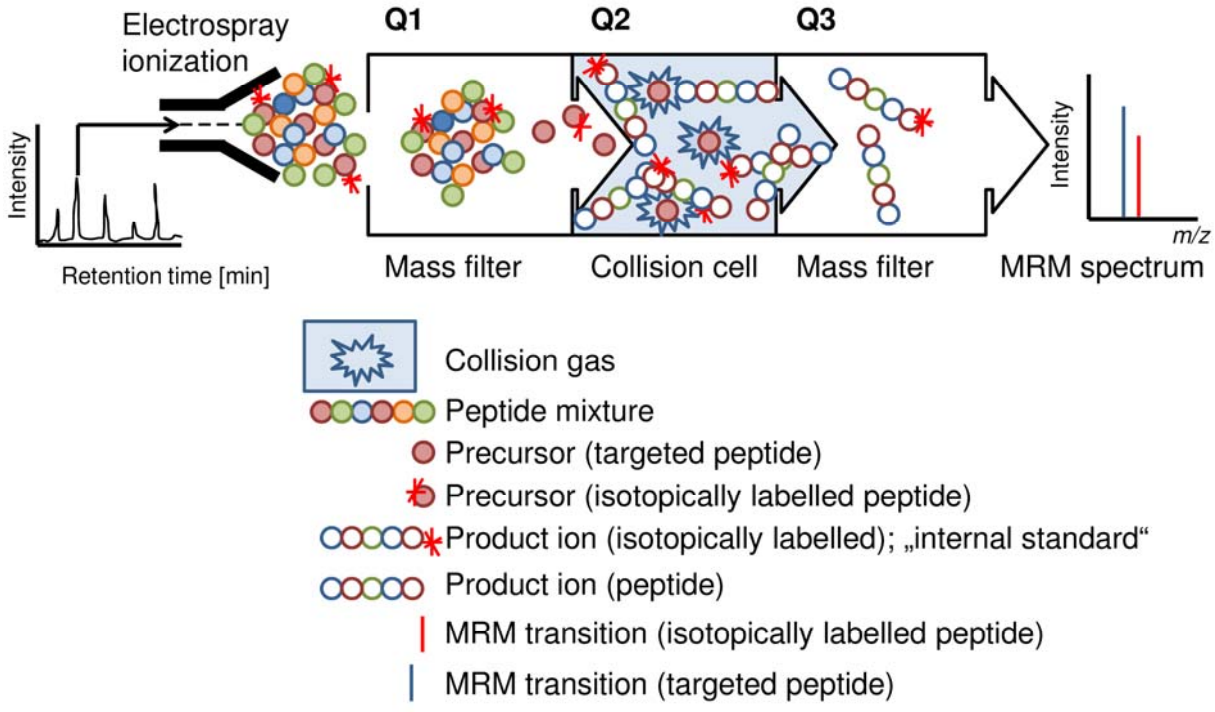

Figure 1.6: The main principle of a targeted LC-MS/MS approach in the MRM mode and the application of an isotopically labelled peptide as internal standard [modified according to Gillette and Carr, 2013]

For peptide quantitation, a triple quadrupole mass spectrometer in the MRM mode is most commonly used. The first quadruple (Q1) is used as mass filter and the precursor ion of the targeted peptide is selected followed by fragmentation of the precursor ion by collision with gas atoms (N_2, He, or Ar) in the second quadrupole (collision cell; Q2). The fragmentation is carried out with the product ion-specific collision energy, which can vary between 10 and 40 volts to induce the highest intensity [Rychlik and Asam, 2008]. In the third quadrupole (Q3), the specific peptide product ions are selected followed by detection of the MRM transition (from precursor to the specific product ion). Usually, peptides are detected in different charge states and the most intensive one is selected in Q1 and fragmented in Q2. Two to three peptide-specific product ions are selected in Q3, because it is recommended to analyse two to three MRM transitions for safe identification of the targeted peptide in different matrices. The most intensive transition is then used for quantitation (so-called quantifier) and the other transitions are used for qualification (so-called qualifier).

The approach in Figure 1.6 demonstrats also the application of an isotopically labelled peptide as internal standard. The heavy labelled internal standard is fragmented identically to the targeted peptide, but is distinguished in the MS and MS/MS spectra by the increased masses of precursor and product ions containing the heavy labelled amino acids. The internal standard is usually heavy labelled with ^{2}H, ^{13}C or ^{15}N. Using the peak areas of analyte and standard and the known concentration of the heavy labelled peptide, the concentration of the targeted peptide can be determined [Gillette and Carr, 2013].

Gluten analysis by MALDI-TOF-MS

MALDI-TOF-MS was the first approach to the quantitation of wheat gliadins in native and processed food samples based on the direct observation of the specific gliadin mass pattern by an epitope-independent method [Camafeita et al., 1997]. Samples were simultaneously analysed by MALDI-TOF-MS and a laboratory sandwich ELISA and a good correlation between these two techniques was observed. Camafeita et al. [1998] demonstrated that gliadins, secalins, hordeins, and avenins from up to 40 wheat, rye, barley, and oat cultivars each showed characteristic mass profiles within the range of M_r 20 000 to 40 000. Due to the characteristic patterns, MALDI-TOF-MS

allowed the discrimination of cereal species as well as the identification of prolamins in food. Further investigations enabled the determination of small amounts of gliadins in maize- and rice-based foods by MALDI-TOF-MS after a two-step extraction procedure with 60 % ethanol followed by acetic acid. Prolamins of maize and rice remained insoluble in acetic acid, which was confirmed by MALDI-TOF-MS of the acidic extract. The results showed a typical mass fingerprint ranging from M_r 30 000 to 45 000 which corresponded to gliadin components. This approach enabled the detection of gliadins with an LOD of 100 mg gliadin/kg [Hernando et al. 2003], which is not sensitive enough to detect gliadins in concentrations near the threshhold of 20 mg gluten/kg.

Gluten analysis by LC-MS/MS

In 2016, Scherf and Poms summarised the principal workflow of the identification and quantitation of gluten peptides by LC-MS/MS. The workflow involves: 1) the appropriate extraction of gluten proteins and peptides (see 1.2.1); 2) the choice of a suitable enzyme for gluten digestion; 3) the selection of specific gluten marker peptides; and 4) the calibration with a representive reference material to enable the quantitation of gluten contents based on marker peptide concentrations.

In order to generate and detect gluten peptides, gluten proteins have to be digested with a suitable enzyme. Usually, pepsin, trypsin or chymotrypsin or a combination of those are used for gluten hydrolysis.
Trypsin is often used in proteomic approaches, because of its reliability and specificity [Salplachta et al., 2005]. It cleaves peptides at the carboxylic side of the amino acids lysine or arginine. In gluten, only low amounts of these amino acids occur, which mainly results in only few and large gluten peptides.
However, chymotrypsin has been previously reported to be an effective enzyme for the study of gluten proteins, because it specifically cleaves peptide bonds on the C-terminal side of tyrosine, phenylalanine and tryptophan, which frequently occur in gluten proteins [Salplachta et al., 2005; Vensel et al., 2011]. Consequently, a variety of peptides are generated, which mainly contain 10 to 20 amino acids and are well detectable by LC-MS/MS.

Several approaches to the quantitation of gluten marker peptides by targeted LC-MS/MS were published in recent years. In 2009, Weber et al. developed a LC-MS/MS method to identify the source of gluten (barley and wheat) in tryptic digested beer samples. The results were compared with those generated by R5 ELISA. It was possible to detect barley in two beer samples even though they gave a low response with ELISA, but LC-MS/MS did not allow the quantitation of the barley content.

Studies by Sealey-Voyksner et al. [2010] used a novel LC-MS/MS method to detect six CD-immunogenic wheat marker peptides from α- and γ-gliadins in a range of 0.01 to 100 mg/kg. Without prior extraction, native as well as processed food samples were digested with pepsin, trypsin and chymotrypsin to simulate the gastric and duodenal protein digestion in humans. The study was focused on the detection and quantitation of marker peptides, which were representive of various cultivars of wheat. However, the quantitation of the gluten content and the quantitation of peptides derived from wheat glutenins were not attempted.

The analysis of 60 tryptically hydrolysed beers by untargeted LC-MS/MS in the MRM mode was undertaken by Tanner et al. [2013a]. This method enabled the relative quantitation of hordein peptides. The relative hordein concentrations determined by LC-MS/MS were compared to the content obtained by sandwich ELISA using the Skerritt monoclonal antibody, which was calibrated against a total hordein preparation [Tanner et al., 2013b]. The results demonstrated the underestimation of hordeins by ELISA, because several beers gave low or zero readings, but near average hordein contents by MS. Further investigations illustrated that those beers with high contents of B-hordein fragments gave near zero values by ELISA caused by a dose-dependent suppression of the ELISA response by gluten peptides [Colgrave et al., 2014].

Studies by Fiedler et al. [2014] demonstrated the development of a targeted LC-MS/MS approach to the detection of two wheat marker peptides from α-gliadins (RPQQPYPQPQPQY and LQLQPFPQPQLPY) to detect wheat contamination in gluten-free oats. To obtain peptide mixtures, the prolamin extract from the wheat/oat flour mixtures (1 – 100 000 mg/kg) were reduced, alkylated and digested with chymotrypsin. In this way, targeted LC-MS/MS enabled the detection of these two marker peptides at the concentration level of down to 10 mg/kg wheat flour in oat flour which corresponds to 1 mg gluten/kg.

Furthermore, a novel approach was focused on the quantitation of only harmful epitopes in different wheat species, which stimulate the development of CD. Nine immunogenic peptides from α-gliadins encoded by the A- and D- genome were quantitated by targeted LC-MS/MS. The quantitation of the peptides in two hexaploid and one tetraploid wheat varieties will enable the selection of varieties with low amounts of immunogenic epitopes to prevent CD [van den Broeck et al., 2015].
A further approach enabled proteomic profiling of 16 cereal grains and the quantitation of four wheat marker peptides down to 15 mg/kg in wheat-contaminated soy flour [Colgrave et al., 2015].
Martinez-Esteso et al. [2016] applied a comprehensive proteomic approach to define the wheat gluten peptide fingerprint. This study showed a strategy to define a selection of specific wheat marker peptides, which was based on two criteria: 1) peptides which are unique to a single gluten protein sequence, and 2) peptides which contain CD-immunogenic sequences. Several peptides proved to be ideal candidates for gluten quantitation but the quantitation was not achieved so far.
The main focus of the described studies was on the quantitation of marker peptides from only one specific gluten-containing type of grain. In contrast, Manfredi et al. [2015] developed a LC-MS/MS method to quantitate 14 marker peptides derived from wheat, barley, rye, and oats with LODs of 2-18 mg/kg. The new method allowed to assess the presence of gluten-containing cereals in gluten-containing or gluten-free raw materials as well as processed food products.
The identification of barley-specific peptides in gluten-enriched fractions derived from 12 barley cultivars by LC-MS/MS was performed by Colgrave et al. [2016]. This study was applied to detect barley contamination in flour as well as the detection of barley in processed food products like breakfast cereals.

Quantitation of gluten-specific peptides by LC-MS/MS is a promising non-immunochemical approach for gluten quantitation in native and processed food products. The limiting factor is that peptide concentrations provide no further information on the gluten content of a sample. To declare food products as “gluten-free”, the threshold of 20 mg gluten/kg has to be adhered and as a consequence peptide concentrations must be converted into protein concentrations to comply with gluten legislation.

So far, only marker peptides were quantitated, but the calculation of protein or gluten concentrations based on the obtained peptide concentrations was not attempted. In order to establish a link between peptide and gluten concentrations, applicable, well-characterized reference proteins are necessery for method calibration. Presently, the only well-characterized reference material is PWG gliadin, which presents only one protein fraction of wheat (see 1.2.2). In order to convert peptide into gluten concentrations, well-defined reference proteins including prolamins and glutelins from wheat, barley, rye and oats are essential and necessary for calibration.

Peptide yields from the respective reference gluten proteins have to be known to attempt the calculation of gluten contents based on peptide concentrations.

Currently, LC-MS/MS is only recommended as additional application in case of contradictory or questionable results by ELISA.

At this time, ELISA is the only accepted method for gluten quantitation in spite of all the described drawbacks.

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2 Aim of the work

CD is an inflammatory disorder of the upper small intestine in genetically predisposed individuals, which is caused by the ingestion of gluten proteins from wheat (gliadins, glutenins), rye (secalins), barley (hordeins), and in rare cases oats (avenins).
The only effective therapy for CD patients is a strict gluten-free diet by consuming gluten-free foods, which contain less than 20 mg gluten/kg. To ensure the safety of gluten-free products for CD patients, it is essential that appropriate analytical methods with high specificity and sensitivity are available.
The most commonly used method for gluten analysis is ELISA. Due to several drawbacks of this method concerning the over- or underestimation based on the gluten calculation by the factor of 2 and the poor comparability of several ELISA kits based on different antibodies, sample extraction and calibration standards, LC-MS/MS has been suggested as a promising alternative.
Several studies on the quantitation of gluten marker peptides by LC-MS/MS were performed in recent years, but most of these studies were only focused on the quantitation of peptides derived from the prolamin fraction of gluten. To improve the analysis of gluten, both the prolamin and glutelin fractions should be detected by LC-MS/MS. Furthermore, the conversion of the obtained marker peptide concentrations into gluten contents was not attempted so far. As requested by legislation, food products must be labelled. Therefore, the conversion of peptide into protein concentrations is essential to enable gluten quantitation by LC-MS/MS as an alternative to currently used ELISA methods.

Consequently, the aim of this study was the development of a targeted LC-MS/MS method for the quantitation of several gluten marker peptides derived from all protein fractions and types from wheat, rye, barley and oats, which should be converted into gluten concentrations.
To achieve this, the first part of the work involved the isolation and characterization of gluten protein types from wheat, barley, rye and oats, which were used as reference materials. The chymotryptically hydrolysed, well-defined gluten reference protein types should be used for the identification of suitable gluten marker peptides. The second part of the study included the development of a targeted LC-MS/MS method

to quantitate selected gluten marker peptides using different isotopically labelled peptides as internal standards.
The quantitation of each marker peptide in the chymotryptic digest of a defined amount of the respective reference gluten protein type should result in peptide-specific yields, which enable the conversion of peptide into protein concentrations.
This new method should be applied to determine gluten contents based on peptide concentrations in wheat-, barley-, rye- or oat-based food products and the obtained results should be compared to ELISA and HPLC (RP-HPLC-UV or GP-HPLC-FLD).

Furthermore, the immunodominant gluten-specific 33-mer peptide from α-gliadin (LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF) should be quantitated by SIDA combined with targeted LC-MS/MS. The 33-mer has played an important role in numerous studies due to its high resistance to proteolytic digestion by intestinal peptidases. It contains three overlapping T-cell epitopes (PFPQPQLPY, one copy; PYPQPQLPY, two copies; PQPQLPYPQ, three copies), which result in the initiation of a strong immune response. Moreover, these epitopes are substrates for tissue transglutaminase, which catalyses the deamidation of specific glutamine residues followed by strong binding to HLA-DQ2. However, the 33-mer was found only in a few entries of the UniProtKB database. Therefore, the aim of this part of the study was the quantitation of the 33-mer in samples of different wheat species from around the world, including hexaploid common wheat (*T. aestivum*) and spelt (*T. aestivum* ssp. *spelta*), tetraploid durum wheat (*T. turgidum durum*) and emmer (*T. turgidum dicoccum*), and diploid einkorn (*T. monococcum*). This part of the work should demonstrate whether the special focus of some studies on this most immunodominant peptide is justified or not and to enable a precise assessment of the importance of this peptide in CD research.

3 Results

3.1 Isolation and characterization of gluten protein types from wheat, rye, barley and oats for use as reference materials

Gluten protein types from wheat, rye, barley and oats were isolated and analytically characterized, which were used as reference materials for the quantitation of gluten marker peptides by LC-MS/MS (see 3.2 and 3.3). Kathrin Schalk partly designed the experiments, performed the experiments, collected and interpreted the data and contributed to the manuscript.

First, gluten protein fractions (prolamins and glutelins) from wheat, rye, barley and oats were isolated from defatted flours (a mixture of 4 cultivars of each grain) according to the modified Osborne procedure. Kathrin Schalk developed a preparative RP-HPLC-UV method, which enabled the separation of gluten protein fractions into the respective gluten protein types (ω5-gliadins, ω1,2-gliadins, α-gliadins, γ-gliadins and HMW- and LMW-GS from wheat, ω-secalins, γ-75k-secalins, γ-40k-secalins and HMW-secalins from rye, C-hordeins, γ-hordeins, B-hordeins and D-hordeins from barley and avenins from oats). Gluten protein types were collected according to their characteristic retention times from several runs.
Kathrin Schalk fully characterized all isolated gluten protein fractions and types using analytical RP-HPLC, SDS polyacrylamide gel electrophoresis (SDS-PAGE), N-terminal sequencing, electrospray-ionization quadrupole time-of-flight mass spectrometry (LC-ESI-QTOF-MS) and untargeted LC-MS/MS of chymotryptic hydrolysates of each single gluten protein type.
In this way, the identity and purity of the isolated gluten protein fractions and types were evaluated by five independent analytical methods.
The results showed that it was possible to isolate well-defined highly purified protein fractions and types from all gluten-containing grains, which can be used as reference materials for LC-MS/MS.

PLOS ONE

RESEARCH ARTICLE

Isolation and characterization of gluten protein types from wheat, rye, barley and oats for use as reference materials

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Abstract

Gluten proteins from wheat, rye, barley and, in rare cases, oats, are responsible for triggering hypersensitivity reactions such as celiac disease, non-celiac gluten sensitivity and wheat allergy. Well-defined reference materials (RM) are essential for clinical studies, diagnostics, elucidation of disease mechanisms and food analyses to ensure the safety of gluten-free foods. Various RM are currently used, but a thorough characterization of the gluten source, content and composition is often missing. However, this characterization is essential due to the complexity and heterogeneity of gluten to avoid ambiguous results caused by differences in the RM used. A comprehensive strategy to isolate gluten protein fractions and gluten protein types (GPT) from wheat, rye, barley and oat flours was developed to obtain well-defined RM for clinical assays and gluten-free compliance testing. All isolated GPT (ω5-gliadins, ω1,2-gliadins, α-gliadins, γ-gliadins and high- and low-molecular-weight glutenin subunits from wheat, ω-secalins, γ-75k-secalins, γ-40k-secalins and high-molecular-weight secalins from rye, C-hordeins, γ-hordeins, B-hordeins and D-hordeins from barley and avenins from oats) were fully characterized using analytical reversed-phase high-performance liquid chromatography (RP-HPLC), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), N-terminal sequencing, electrospray-ionization quadrupole time-of-flight mass spectrometry (LC-ESI-QTOF-MS) and untargeted LC-MS/MS of chymotryptic hydrolyzates of the single GPT. Taken together, the analytical methods confirmed that all GPT were reproducibly isolated in high purity from the flours and were suitable to be used as RM, e.g., for calibration of LC-MS/MS methods or enzyme-linked immunosorbent assays (ELISAs).

Introduction

Wheat is the third most important cereal in terms of production worldwide (729×10^6 t in 2014) [1], but the consumption of wheat and closely related cereals (rye, barley and, in rare cases, oats) may be harmful to predisposed individuals, because specific proteins are responsible for triggering hypersensitivities such as wheat allergy, celiac disease (CD) and non-celiac gluten sensitivity (NCGS) [2–4]. The major causative agents are the storage proteins (gluten)

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of the aforementioned grains, but other proteins such as lipid-transfer-proteins, puroindolines and amylase-trypsin-inhibitors (ATIs) also have the potential to cause harmful effects [5,6]. Cereal grains contain hundreds of different protein components which are traditionally classified into four so-called Osborne fractions: albumins soluble in water, globulins soluble in salt solution, prolamins soluble in aqueous alcohol and insoluble glutelins, which are only alcohol-soluble in the presence of reducing agents. Albumins and globulins (ALGL, ≈ 20–25% of grain proteins) mainly comprise metabolic and protective proteins such as enzymes and enzyme inhibitors whereas prolamins and glutelins (≈ 75–80% of grain proteins) serve as storage proteins. The common names of these closely related gluten proteins are gliadins (prolamins) and glutenins (glutelins) of wheat, secalins of rye, hordeins of barley and avenins of oats. Based on homologous amino acid sequences and similar molecular weights (M_r), the gluten proteins can be divided into the high-molecular-weight (HMW), the medium-molecular-weight (MMW) and the low-molecular-weight (LMW) group [7]. Each group contains numerous related gluten protein types (GPT) with different numbers of single proteins within each type, e.g., HMW-glutenin subunits (GS) with 3–5 proteins and α-gliadins and LMW-GS with more than 20 proteins [8]. Modifications of amino acid sequences caused by nucleotide insertion, deletion or exchange are responsible for the heterogeneity within each type.

Numerous research papers have been published concerning identification and characterization of proteins that trigger wheat hypersensitivities [9–11]. Well-defined proteins are essential for clinical studies [12,13], diagnostic purposes and as reference materials (RM) for food analysis [14], such as the Prolamin Working Group (PWG)-gliadin [15]. Different RM have been used in these papers, but a thorough characterization of the protein source, content and composition often is either missing or proprietary material is used. Gluten and gliadin preparations frequently used for both clinical and analytical purposes were shown to be strongly different in protein content and proportions of ALGL, prolamin and glutelin fractions [16]. Considering the additional lack of reproducible RM production, the quality of assays for diagnosis and food analysis is variable and may lead to questionable and contradictory conclusions. Defined single recombinant proteins were applied in a few cases, e.g., a panel of 11 α-gliadins for CD-specific T-cell proliferation assays [17,18], γ1-gliadin for CD diagnosis [19], HMW-GS 1Dy10 for the investigation of CD serology [20] or HMW-GS 1Ax2 and ω5-gliadin for WDEIA diagnosis [11,21]. However, a single recombinant protein may not be representative for the corresponding GPT, because each GPT consists of several proteins. Using the complete protein mixture isolated from the natural source may therefore improve the accuracy of clinical and food analytical assays.

The aim of the present study was to develop and apply a comprehensive strategy to isolate well-defined gluten protein fractions and GPT from wheat, rye, barley and oat flours suitable as RM for clinical assays and gluten-free compliance testing, e.g., by enzyme-linked immunosorbent assays (ELISAs) or liquid chromatography-mass spectrometry (LC-MS). All isolated GPT were extensively characterized using analytical reversed-phase high-performance liquid chromatography (RP-HPLC), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), N-terminal sequencing, electrospray-ionization quadrupole time-of-flight MS (LC-ESI-QTOF-MS) and untargeted LC-MS/MS of chymotryptic hydrolyzates of the single GPT.

Material and methods

Chemicals and flours

All chemicals and solvents were at least pro analysi or HPLC grade. Water for HPLC was purified using a Milli-Q Gradient A10 system (Millipore, Schwalbach, Germany). PWG-gliadin

[15] used for calibration was provided by Prof. Dr. Peter Koehler, chairman of the PWG. Grains of four cultivars (cv.) each of wheat (cv. Akteur, I.G. Pflanzenzucht, Munich, Germany; cv. Julius, KWS Lochow, Bergen, Germany; cv. Pamier, Lantmännen SW Seed, JK Bergen op Zoom, The Netherlands; cv. Tommi, Nordsaat Saatzucht, Langenstein, Germany), rye (cv. Brasetto, cv. Conduct, cv. Palazzo, cv. Visello, KWS Lochow), barley (cv. Grace, cv. Marthe, Nordsaat Saatzucht; cv. Lomerit, KWS Lochow; cv. Sandra, I.G. Pflanzenzucht) and oats (cv. Aragon, cv. Ivory, cv. Scorpion, Nordsaat Saatzucht; cv. Flämingsgold, KWS Lochow), all harvested in 2013, were mixed in a 1+1+1+1 mass ratio and shaken overhead (Turbula, Willy A. Bachofen Maschinenfabrik, Muttenz, Switzerland) for 24 h to obtain homogeneous grain mixtures. The mixed wheat, rye and barley grains were milled into white flour using a Quadrumat Junior Mill (Brabender, Duisburg, Germany) followed by sieving (mesh size 200 μm). Oat grains were milled with a laboratory grinder (A10, IKA-Werke, Staufen, Germany) and sifted.

Analytical characterization of the flours

The moisture and ash contents were determined according to International Association for Cereal Science and Technology (ICC) Standards 110/1 [22] and 104/1 [23]. The nitrogen contents were determined by the Dumas combustion method using a TruSpec nitrogen analyzer (Leco, Moenchengladbach, Germany) and converted to crude protein (CP) contents by multiplying with a factor of 5.7 according to ICC Standard 167 [24]. The quantities of ALGL, prolamin and glutelin fractions as well as GPT were determined according to the modified Osborne procedure [25,26]. The flours (100 mg) were extracted sequentially with (a) salt solution (2 × 1.0 mL; 0.4 mol/L NaCl with 0.067 mol/L Na_2HPO_4/KH_2PO_4, pH 7.6) for 10 min at 22˚C (ALGL); (b) with ethanol/water (60/40, v/v) (3 × 0.5 mL) for 10 min at 22˚C (prolamins); and (c) glutelin solution (2 × 1.0 mL; 2-propanol/water (50/50, v/v)/0.1 mol/l Tris-HCl, pH 7.5, containing 2 mol/L (w/v) urea and 0.06 mol/L (w/v) dithiothreitol (DTT)) for 30 min at 60˚C under nitrogen (glutelins). The suspensions were centrifuged (3750 × *g*, 20 min, 22˚C), the corresponding supernatants combined, made up to 2.0 mL with the respective extraction solvent and filtered (0.45 μm). Aliquots of the rye and barley prolamin fractions, respectively, were additionally analyzed after reduction (addition of 1% (w/v) DTT, 60˚C, 30 min) [27]. All fractions were analyzed by analytical RP-HPLC [28]: instrument: Jasco XLC (Jasco, Gross-Umstadt, Germany); column: Acclaim™ 300 C_{18} (3 μm, 30 nm, 2.1 × 150 mm, Thermo Fisher Scientific, Braunschweig, Germany); temperature: 60˚C; injection volume: 20 μL of ALGL and glutelin extracts; 10 μL of prolamin extracts; elution solvents: (A) water/trifluoroacetic acid (TFA) (999/1, v/v), (B) acetonitrile/TFA (999/1, v/v); gradient for ALGL: 0 min 0% B, 0.5 min 20% B, 7 min 60% B, 7.1–11 min 90% B, 11.1–17 min 0% B; gradient for prolamins and glutelins: 0 min 0% B, 0.5 min 24% B, 20 min 56% B, 20.1–24.1 min 90% B, 24.2–30 min 0% B; flow rate: 0.2 mL/min; detection: UV absorbance at 210 nm; software: Chrompass (Jasco). PWG-gliadin [15] dissolved in ethanol/water was used for external calibration in the range of 11.6 to 46.6 μg to calculate the protein contents of the ALGL, prolamin and glutelin fractions. The amounts of GPT were calculated from the absorbance area of each GPT relative to the total absorbance area of the respective prolamin or glutelin fraction. All determinations were done in triplicates.

Defatting of the flours

100 g of flour each were stirred three times at 22˚C for 30 min with 250 mL *n*-pentane/ethanol (95/5, v/v) followed by stirring once with 250 mL *n*-pentane [29]. The suspensions were centrifuged (3750 × *g*, 15 min, 22˚C) and the solvent discarded. After the last extraction step the defatted flour residue was vacuum-dried overnight on a filter sheet and homogenized carefully.

Preparation of gluten protein fractions

Defatted flours (2 × 50 g) were extracted three times each with 200 mL of (a) salt solution by homogenizing with an Ultra Turrax blender (16 000 rpm, IKA-Werke, Staufen, Germany) in a centrifuge vessel for 5 min at 22˚C. The suspensions were centrifuged (3750 × g, 25 min, 22˚C) and the supernatants discarded (→ ALGL fraction). The sediments were extracted three times with 200 mL of (b) ethanol/water as described for the ALGL fraction. The resulting supernatants were combined, concentrated under reduced pressure, dialyzed (M_r cut-off: 12 000–14 000, Medicell Membranes, London, UK) and lyophilized (→ prolamin fraction). Then, the sediments were extracted three times under nitrogen with 200 mL of (c) glutelin solution (see above) by homogenizing with an Ultra Turrax blender for 5 min, stirring for 30 min at 60˚C, cooling and centrifugation as described. The supernatants were combined, concentrated, dialyzed and lyophilized (→ glutelin fraction). For oat flour, the extraction was stopped after the prolamin fraction (M_r cut-off for dialysis: 7 000, Medicell Membranes), because oat glutelins are mainly composed of polymeric 12S globulins [30]. The CP contents of the dried prolamin and glutelin fractions were determined according to ICC Standard 167 (n = 3) [24].

Preparation of gluten protein types

The wheat, rye and barley prolamin fractions (100 mg) were dissolved in 10 mL of ethanol/water. The rye prolamin and the wheat, rye and barley glutelin fractions (100 mg) were dissolved in 10 mL of glutelin solution. All solutions were filtered (0.45 μm) and the following conditions were set for the preparative RP-HPLC method: pump: PU-2087 Plus (Jasco); autosampler: AS-2055 Plus (Jasco); column: Jupiter C_{18} (5 μm, 30 nm, 10 × 250 mm, Phenomenex, Aschaffenburg, Germany); temperature: 50˚C; injection volume: 400 μL of prolamins, 700 μL of glutelins; elution solvent: (A) water/TFA (999/1, v/v), (B) acetonitrile/TFA (999/1, v/v); gradient: 0–2 min 0% B, 4 min 24% B, 52 min 56% B, 53 – 58 min 90% B, 65–69 min 0% B; flow rate: 2.0 mL/min; UV detector: UV-2075 Plus (Jasco); detection: UV absorbance at 210 nm; fraction collector: CHF-122SC (Advantec MFS, Dublin, CA, USA); software: Galaxie chromatography data system, version 1.10.0.5590 (Jasco). The GPT were separated according to their characteristic retention times (Figs 1 and 2), collected from several runs, pooled, concentrated under reduced pressure and lyophilized. Oat prolamins (avenins) were not further fractionated.

Characterization of gluten protein types

Protein content. Due to the availability of only small amounts (mg) of GPT, 1 mg of the lyophilized GPT were dissolved in 1 mL of ethanol/water (GPT isolated from prolamin fractions) or glutelin solution (GPT isolated from glutelin fractions), filtered (0.45 μm), injected (20 μL) into the analytical RP-HPLC system (prolamin and glutelin gradient) and the protein concentrations were calculated from external calibration with PWG-gliadin (2.9–46.6 μg) as described above. This re-chromatography also allowed verifying the purity and identity of the isolated GPT by comparing their retention times with those determined previously during the analyses of the corresponding prolamin and glutelin fractions.

SDS-PAGE. SDS-PAGE was carried out according to Lagrain et al. [31] using a homogeneous NuPAGE 10% polyacrylamide - Bis-Tris gel (Invitrogen, Carlsbad, CA, USA) and a MOPS-Tris running buffer (pH 7.7) containing DTT (5 mmol/L) added to the inside chamber. The isolated protein fractions or GPT (1.5 mg) were dissolved in 1 mL of extraction buffer under reducing conditions (DTT, 50 mmol/L), incubated for 24 h, heated to 60˚C for 10 min while shaking and centrifuged (5000 × *g*, 5 min, 22˚C). Per sample, 2–5 μL were applied to the slots. A mixture of seven proteins (M_r 6 500–200 000) was used as marker. The running time

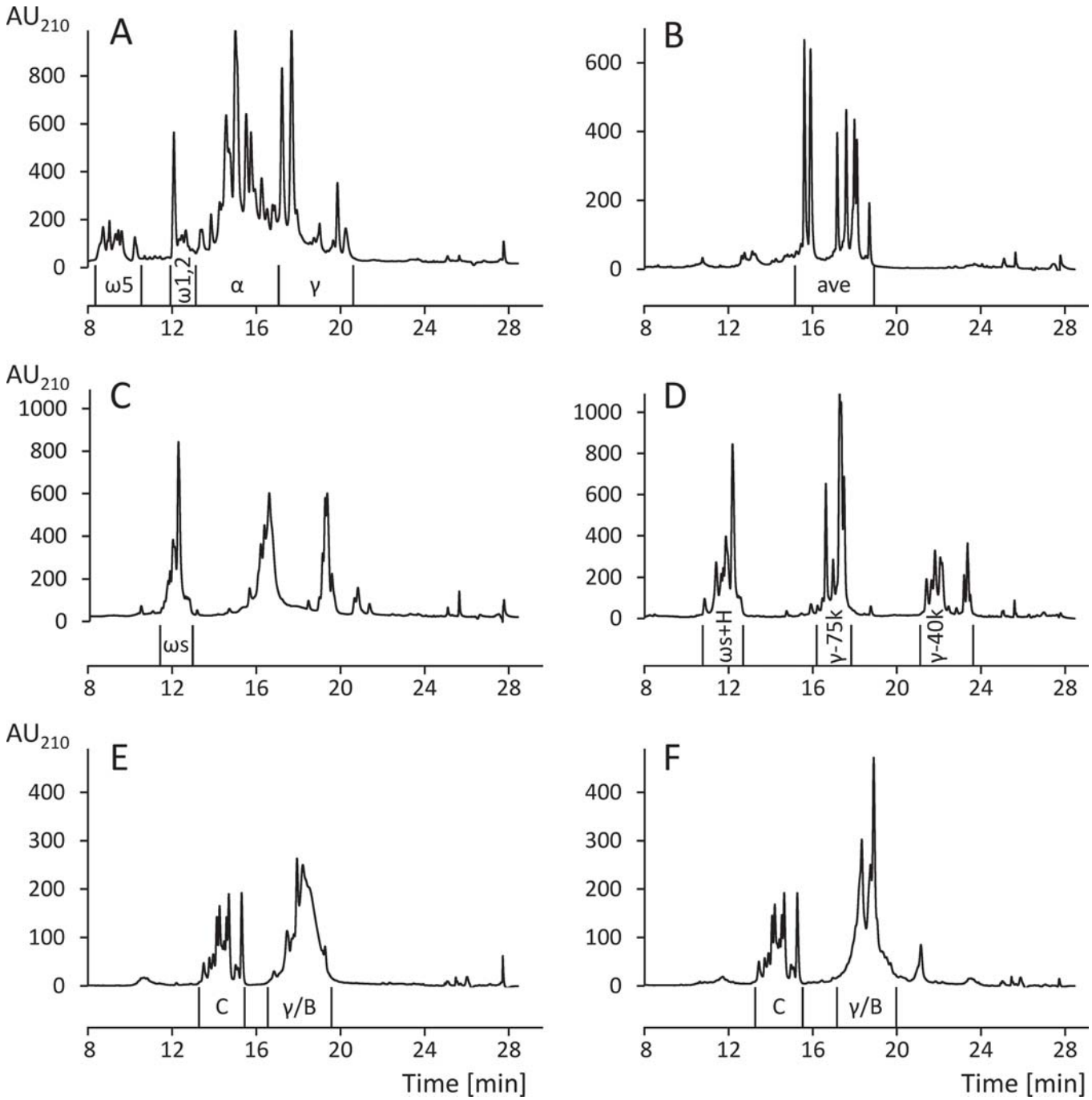

Fig 1. RP-HPLC chromatograms of the prolamin fractions. (A) Wheat prolamins, (B) oat prolamins, (C) rye prolamins, unreduced, (D) rye prolamins, reduced with 1% (w/v) DTT, (E) barley prolamins, unreduced, (F) barley prolamins, reduced with 1% (w/v) DTT. AU, absorbance units at 210 nm, ω5, ω5-gliadins, ω1,2, ω1,2-gliadins, α, α-gliadins, γ, γ-gliadins, ave, avenins, ωs, ω-secalins, ωs+H, ω- and high-molecular-weight (HMW)-secalins, γ-75k, γ-75k-secalins, γ-40k, γ-40k-secalins, C, C-hordeins, γ/B, γ-hordeins and B-hordeins.

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was 40 min at 200 V and 115 mA. After the run, the proteins were fixed for 30 min in 12% trichloroacetic acid, stained for 30 min with Coomassie Brilliant Blue R-250 and destained twice

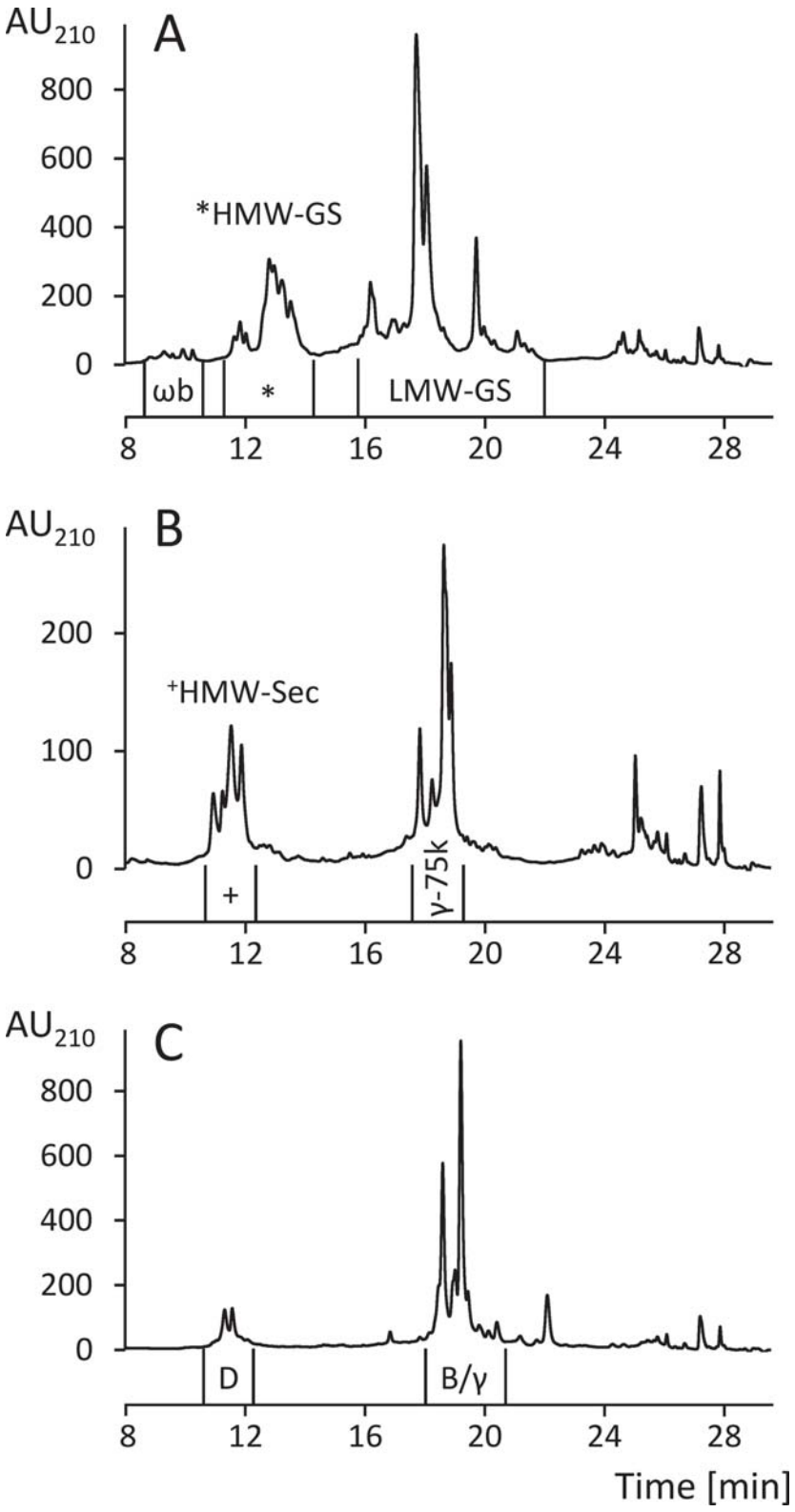
AU210
A
800
600
400
200
0
*HMW-GS
ωb
*
LMW-GS
8
12
16
20
24
28
AU210
B
200
100
0
+HMW-Sec
+
γ-75k
8
12
16
20
24
28
AU210
C
800
600
400
200
0
D
B/γ
8
12
16
20
24
28
Time [min]

Fig 2. RP-HPLC chromatograms of the glutelin fractions. (A) Wheat glutelins, (B) rye glutelins, (C) barley glutelins, all reduced with 1% (w/v) DTT. AU, absorbance units at 210 nm, ωb, ωb-gliadins, HMW-GS, high-molecular-weight glutenin subunits, LMW-GS, low-molecular-weight glutenin subunits, HMW-Sec, HMW-secalins, γ-75k, γ-75k-secalins, γ-40k, γ-40k-secalins, D, D-hordeins, B/γ, B-hordeins and γ-hordeins.

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[31]. The gels were scanned, the images converted to grayscale, the lanes of interest plotted as x/y-diagrams and the peaks integrated using ImageJ open source software (National Institute of Mental Health, Bethesda, MD, USA) [32].

N-terminal sequence analysis. Isolated GPT were dissolved in acetonitrile/water (30/70, v/v) containing 0.1% (v/v) TFA. The amount of protein applied onto the polyvinylidene difluoride membrane was between 50 and 100 pmol. Sequencing was carried out by automated Edman degradation on a protein sequencer Procise 492 (Applied Biosystems, Carlsbad, CA, USA) running in the pulsed-liquid mode with ten degradation cycles [33].

LC-ESI-QTOF-MS. An ESI-QTOF-MS (microTOF-Q, Bruker Daltonics, Bremen, Germany) coupled with an UltiMate 3000 HPLC system (Dionex, Idstein, Germany) was used for LC-MS experiments [34]. The stationary phase was an XBridge Protein BEH C_4 column (3.5 μm, 30 nm, 2.1 × 150 mm, Waters, Milford, MA, USA). The mobile phase was (A) water/TFA (999/1, v/v) and (B) acetonitrile/TFA (999/1, v/v) with a linear elution gradient from 0–0.4 min 0% B, 0.5 min 24% B, 20 min 56% B, 20.1–32 min 90% B and 32–33 min 0% B at a flow rate of 0.2 mL/min and a temperature of 30˚C. Isolated GPT (2–5 mg) were dissolved in 1 mL acetonitrile/water (30/70, v/v) acetonitrile containing 0.1% (v/v) TFA and 20 μL were injected. The MS was operated in the positive ionization mode (capillary voltage: -4000 V, end plate offset: -500 V). Nitrogen was used as drying (8.0 L/min, 180˚C) and nebulizing gas (0.13 MPa). The scan range was *m/z* 750–3200 (quadrupole ion energy: 5.0 eV). Analysis of the LC-MS data was performed using the software DataAnalysis 3.4 (Bruker Daltonics). M_r were calculated with related-ion deconvolution (mass range: 5000–100 000, maximum charge: 100, envelope cut-off: 75%, M_r agreement: 0.05%) and maximum entropy deconvolution (mass range: 5000–100 000, instrument resolution power: 10 000).

Untargeted LC-MS/MS of chymotryptic GPT hydrolyzates. The isolated GPT (1 mg) were reconstituted in 1 mL of Tris-HCl buffer (0.1 mol/L, pH 7.8, 2 mol urea/L) containing α-chymotrypsin (TLCK treated, ≥ 40 unit/mg protein, Sigma-Aldrich, Steinheim, Germany) at an enzyme/substrate ratio of 1/200 (w/w). After incubation for 24 h at 37˚C, the digestion was stopped by addition of 3 μL TFA. The resulting peptide mixtures were subjected to solid phase extraction on Supelco DSC-C_{18} tubes (Sigma-Aldrich). The tubes were conditioned with methanol (1 mL) and equilibrated with TFA (0.1%, v/v, 1 mL). After loading the peptide mixtures, the tubes were washed with water containing TFA (0.1%, v/v, 5 x 1 mL) and the peptides were eluted with methanol (2 mL). The eluate was dried using a vacuum centrifuge (40˚C, 6 h, 800 Pa), reconstituted in 500 μL formic acid (FA) (0.1%, v/v), filtered (0.45 μm) and analyzed by ion trap LC-MS/MS [35]. An UltiMate 3000 HPLC system (Dionex) was coupled to an HCTultra PTM ion trap MS (Bruker Daltonics) with collision-induced dissociation (CID). The peptides were separated on an Aeris PEPTIDE XB-C_{18} column (3.6 μm, 10 nm, 2.1 × 150 mm, Phenomenex) and water/FA (999/1, v/v) (A) and acetonitrile/FA (999/1, v/v) (B) as solvents with a flow rate of 0.2 mL/min, a column temperature of 30˚C, an injection volume of 10 μL and a linear gradient: 0–5 min 0% B, 45 min 30% B, 55–60 min 90% B, 62–77 min 0% B. The ESI interface was operated using the following parameters: mode: positive, capillary voltage: -4000 V, capillary exit voltage: -1500 V, skimmer voltage: 40 V, drying gas: nitrogen (8.0 L/min, 325˚C), nebulizing gas: nitrogen (207 kPa). The MS instrument settings were: scan: standard enhanced, *m/z* range: 500–2000, scan speed: 8.1 *m/z*/s, smart target value: 300 000, maximum acquisition time: 100 ms, MS/MS setting: Auto-MS(n), collision gas: helium,

absolute threshold: 10 000, relative threshold: 0.5%, fragmentation amplitude: 0.4 V. Data analysis was carried out with the software DataAnalysis 3.4 and BioTools 3.2 (Bruker Daltonics). A Mascot generic file (*.mgf) was generated from the MS/MS data file, which was exported to the MS/MS ions search module of the Mascot software (Matrix Science, London, UK) using the National Center for Biotechnology Information non-redundant (NCBI) database (U.S. National Library of Medicine, Bethesda, MD, USA) of February 2014. Peptides were searched within the taxonomy *Viridiplantae* with peptide mass tolerance: ± 5 Da, fragment mass tolerance: ± 0.5 Da, mass value: monoisotopic, peptide charges: +1, +2, +3, enzyme: chymotrypsin, maximum number of missed cleavages: 2 and variable modification: ammonia-loss. Peptide ion scores were calculated by the software as -10 × log(P), with P: probability for the observed match being a random event. Peptide scores > 40 were considered to indicate identity or extensive homology ($p < 0.05$) [36] and scores between 15 and 40 were additionally verified manually [37]. Protein scores (maximum number of protein hits: 30) were derived from peptide scores as sum of the highest ions score for each particular protein sequence, excluding the scores of duplicate matches.

Results and discussion

The wheat, rye, barley and oat flours were mixtures of four cultivars each to account for the genetic variability between different cultivars [38]. The cultivars were selected based on their production yields relative to the total production of winter wheat, rye, winter and summer barley, and oats in the year 2012 in Germany to include the most relevant cultivars (cumulative production share for wheat: 16%, rye: 53%, barley: 35%, oats: 41%) [39]. For wheat, additional criteria were that the mixture contained flours of three different German baking performance classes (E: elite, A: high, B: bread quality) and covered the most important HMW-GS (cv. Akteur: Ax1, Dx5, Bx7, By9, Dy10; cv. Julius: Ax1, Dx2, Bx6, By8, Dy12; cv. Pamier: Dx5, Bx7, By9, Dy10; cv. Tommi: Dx2, Bx7, By9, Dy12). For rye, three hybrid (cv. Brasetto, cv. Palazzo and cv. Visello) and one population (cv. Conduct) cultivars were chosen. For barley, the selection included two winter (cv. Lomerit, six-row, and cv. Sandra, two-row) and two summer (cv. Grace and cv. Marthe, both two-row) barley cultivars. The contents of water, ash, CP, the Osborne fractions ALGL, prolamins and glutelins as well as gluten were determined for the wheat, rye, barley and oat flours (Table 1) and the quantitative values were in good agreement with earlier studies [26,27,33,40,41].

The qualitative RP-HPLC profiles also corresponded well to those reported in the literature [25,27,40], so that all GPT could be assigned within the prolamin and glutelin fractions (Figs 1 and 2). Prolamins were separated into the following GPT: ω5-, ω1,2-, α- and γ-gliadins of wheat (Fig 1A), avenins of oats (Fig 1B), ω-secalins and a minor amount of HMW-secalins, γ-75k- and γ-40k-secalins of rye (Fig 1C and 1D) as well as C- and γ/B-hordeins of barley (Fig 1E and 1F). Glutelins were subdivided into ωb-gliadins, HMW-GS and LMW-GS of wheat (Fig 2A), HMW-, γ-75k- and γ-40k-secalins of rye (Fig 2B) and D- and B/γ-hordeins of barley (Fig 2C).

The separation of rye gluten proteins into prolamins and glutelins according to solubility in ethanol/water (60/40, v/v) was less clear-cut than for wheat [27], so that γ-75k-secalins and a minor part of HMW-secalins appeared in both fractions. Therefore, the content of ω-secalins was calculated from the chromatogram of the unreduced prolamin fraction (Fig 1C). The first peak in the chromatogram of the reduced prolamin fraction contained ω-secalins and a minor amount of HMW-secalins (ωs+H, Fig 1D), so that the difference between ωs+H and ω-secalins alone was due to HMW-secalins. The contents of γ-75k- and γ-40k-secalins were calculated from the respective peak areas in the chromatogram of the reduced prolamin fraction. The percentages given for HMW-, ω-, γ-75k- and γ-40k-secalins (Table 2) are the sum of each rye

Table 1. Analytical characterization of the flours. Contents of water, ash, crude protein (CP) and the Osborne fractions albumins/globulins (ALGL), prolamins and glutelins of wheat, rye, barley and oat flours (mixture of four cultivars each).

g/100 g of flour	Wheat	Rye	Barley	Oats
Water	13.23 ± 0.17	11.30 ± 0.09	12.85 ± 0.09	11.8 ± 0.16
Ash[a]	0.49 ± 0.01	1.14 ± 0.01	0.87 ± 0.00	1.03 ± 0.00
CP	11.28 ± 0.08	7.13 ± 0.09	7.66 ± 0.10	8.07 ± 0.04
ALGL	1.22 ± 0.01	1.84 ± 0.09	1.24 ± 0.03	2.37 ± 0.04
Prolamins	5.94 ± 0.07	2.53 ± 0.03	3.13 ± 0.06	1.29 ± 0.03
Glutelins	2.98 ± 0.04	0.55 ± 0.01	1.10 ± 0.02	1.01 ± 0.05
Gluten[b]	8.92 ± 0.11	3.08 ± 0.04	4.23 ± 0.08	1.29 ± 0.03[c]
Insoluble residue[d]	1.16 ± 0.05	2.18 ± 0.07	2.23 ± 0.06	3.43 ± 0.04

Values are given as mean ± standard deviation (n = 3) on an as-is basis unless specified

[a]based on dry mass

[b]sum of prolamin and glutelin fractions

[c]only the oat prolamin fraction is considered as oat gluten, because oat glutelins mostly contain 12S globulins [30]

[d]difference between CP and the sum of ALGL, prolamin and glutelin contents quantified by RP-HPLC.

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GPT considering both fractions [27]. The same separation issue was true for barley gluten proteins, because γ- and B-hordeins appeared in both the prolamin and glutelin fractions. Most γ-hordeins are alcohol-soluble monomers, but some form alcohol-insoluble polymers linked by interchain disulfide bonds. The opposite is the case for B-hordeins, some of which are alcohol-soluble monomers, but the majority of which are polymeric [42,43]. It was evident from the chromatogram of the reduced barley prolamins (Fig 1F) that the peak shape of γ/B-hordeins changed after reduction (Fig 1E), while that of the monomeric C-hordeins remained the same. This confirmed that γ/B-hordeins were, at least partly, present as oligomers or polymers linked by disulfide bonds. Unfortunately, the RP-HPLC method applied here did not allow a separation of γ-hordeins from B-hordeins, because there was no separate peak visible in the unreduced prolamin fraction that remained at the same retention time in the reduced prolamin fraction. Due to this limitation, γ- and B-hordeins could only be analyzed and collected together from both fractions. The GPT collected from the prolamin fraction was designated as γ/B-hordeins and that from the glutelin fraction as B/γ-hordeins in the following. Earlier reports found that γ-hordeins are minor components, constituting less than 5% of total hordeins [44–46], which is why this limitation seemed to be acceptable.

Strategy to isolate gluten protein fractions and types

The analytical characterization of wheat, rye, barley and oat flours was performed with non-defatted flour. For preparative isolation of gluten protein fractions and types it is advisable to use defatted flour [29], especially in the case of oats. A schematic overview of the strategy to prepare defined protein fractions and GPT from wheat, rye and barley flours is presented in Fig 3. Avenins, the prolamin fraction of oats, was not further subdivided. Rye γ-75k- and γ-40k-secalins were only prepared from the reduced prolamin fraction, because the quantities of these GPT were higher in the reduced prolamin fraction than in the glutelin fraction. This procedure is applicable to flours made of pure cultivars as well as to mixtures of cultivars, e.g., as done here with a mixture of four cultivars each, or as described before for the preparation of the PWG-gliadin RM from 28 wheat cultivars [15].

All gluten protein fractions and GPT isolated following this strategy (Fig 3) with yields ranging from 4–7 mg (minor GPT such as ω5- and ω1,2-gliadins and HMW-GS, HMW-secalins and D-hordeins) to 16–36 mg (major GPT such as α-gliadins, LMW-GS, γ-75k-secalins

Table 2. Analytical characterization of the isolated gluten protein types (GPT). Proportions of each GPT in wheat, rye, barley and oat flours, protein content of each isolated GPT, their N-terminal sequence(s), molecular weight ranges (M_r) determined by LC-ESI-QTOF-MS and the M_r of the most appropriate reference sequence found in the NCBI database given with its specific accession.

	Proportion in gluten [%][a]	Protein content of isolated GPT [%][a]	N-terminal sequence	M_r (LC-ESI-QTOF-MS)	M_r (of NCBI accession)[b]	NCBI accession
Wheat[c]						
HMW-GS[A]	9.3 ± 0.2	94.8 ± 2.5	EGEASGQLQC	83 696[d]	87 643	AHZ62762.1
			EGEASEQLQC		87 256	AHN66476.1
			EGEASRQLQC		68 154	AAU04841.1
ω5-gliadins[B]	5.7 ± 0.2	94.4 ± 3.7	SRLLSPRGKE	48 576 - 54 968	50 927	BAE20328.1
ω1,2-gliadins[C]	7.5 ± 1.0	100.8 ± 1.1	ARELNPSNKE	39 104 - 41 875	39 651	ADA67917.1
α-gliadins[B]	32.6 ± 3.4	88.1 ± 0.7	VRVPVPQLQP	29 994 - 33 979	30 487	AHN85627.1
γ-gliadins[D]	20.8 ± 1.7	92.9 ± 1.1	NMQVDPSGQV	30 295 - 35 212	32 307	P21292.1
LMW-GS[E]	22.3 ± 0.2	81.3 ± 2.1	SHIPGLERPS	32 449 - 41 544	39 478	ACA63857.1
			METSHIPGLE		39 637	ACY08820.1
			METSRVPGLE		37 232	AAP44991.1
Rye						
HMW-secalins[A]	5.5 ± 0.3	74.3 ± 3.7	EGEASGQLQC	78 173 - 85 154	78 156	CAC40680.1
γ-75k-secalins[E]	48.5 ± 0.8	94.7 ± 2.7	NMQVNPSGQV	52 313 - 60 476	52 513	ADP95479.1
ω-secalins[C]	18.8 ± 0.6	96.9 ± 4.8	RQLNPSEQEL	39 004 - 39 457	39 359	ACQ83628.1
γ-40k-secalins[D]	27.2 ± 1.3	95.1 ± 4.1	NMQVGPSGQV	32 141 - 32 446	21 377	AEW46799.1
Barley						
D-hordeins[A]	7.6 ± 0.2	98.8 ± 3.8	EREINGNNIF	n.d.[d]	72 882	BAA11642.1
C-hordeins[C]	22.7 ± 0.1	95.0 ± 1.0	RQLNPSSQEL	44 786 - 46 722	34 287	AAB28161.1
γ/B-hordeins[D]	51.3 ± 1.4	99.7 ± 1.6	ITTTTMQFNP	31 458 - 34 707	33 168	P80198.1
B/γ-hordeins[E]	18.4 ± 0.5	85.3 ± 6.3	QQQPFPQQPI	31 429 - 34 706	31 444	P06470.1
Oats						
avenins		79.2 ± 0.6[e]	TTTVQYNPSE	22 439 - 28 795	23 524	AAA32716.1
			TTTVQYDPSE		23 818	AGB56858.1

[a]Mean ± standard deviation (n = 3) determined by RP-HPLC

[b]monoisotopic mass without signal peptide

[c]proportions of GPT for wheat only add up to 98.2% (not 100%), because ωb-gliadins (1.8%) were not isolated and therefore not included here

[d]only one mass or no masses (n.d.) were detected, because HMW-GS and D-hordeins were difficult to solubilize and ionize

[e]crude protein content (Dumas) of the avenin fraction

[A]homologous high-molecular-weight gluten proteins

[B]unique to wheat

[C]homologous medium-molecular-weight gluten proteins

[D,E]homologous low-molecular-weight gluten proteins. HMW-GS, high-molecular-weight glutenin subunits, LMW-GS, low-molecular-weight glutenin subunits, HMW-secalins, high-molecular-weight secalins.

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and γ/B-hordeins) per 10 HPLC runs were characterized by determination of the protein content, analytical RP-HPLC, SDS-PAGE, N-terminal sequencing, LC-ESI-QTOF-MS and untargeted LC-MS/MS of chymotryptic GPT hydrolyzates.

Isolation and characterization of gluten protein fractions and types

Wheat (gliadins and glutenins). The CP contents of the lyophilized wheat fractions were 93.5 ± 0.4% for gliadins and 82.8 ± 0.2% for glutenins, showing that the extraction procedure

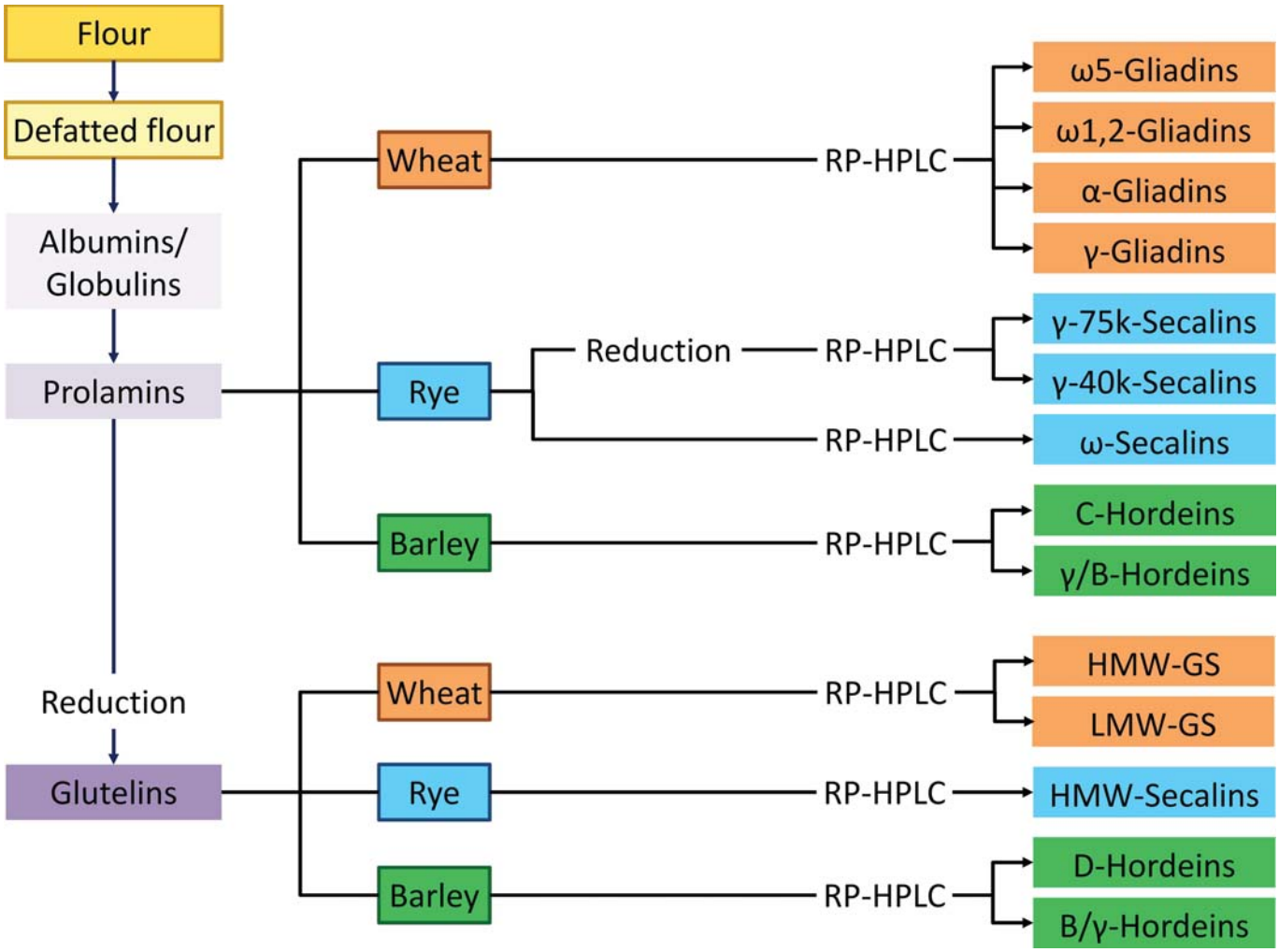

Fig 3. Overview of the preparative strategy. This strategy allows the isolation of well-defined gluten protein fractions and types from wheat, rye, barley and oat flours. HMW-GS, high-molecular-weight glutenin subunits, LMW-GS, low-molecular-weight glutenin subunits.

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from the flour followed by dialysis and lyophilization yielded gluten fractions with very high protein contents comparable to that of PWG-gliadin [15]. The isolated GPT (ω5-, ω1,2-, α- and γ-gliadins and HMW- und LMW-GS) separated from the fractions by preparative RP-HPLC also had very high protein contents ranging from 81.3 ± 2.1% for LMW-GS to 100.8 ± 1.1% for ω1,2-gliadins (Table 2). Re-chromatography of the isolated GPT by analytical RP-HPLC confirmed the identity of each GPT (S1 Fig), because the characteristic retention times matched those in Figs 1A and 2A and there were essentially no impurities visible at 210 nm. SDS-PAGE of the wheat flour, gliadin and glutenin fractions and wheat GPT revealed that all GPT had been obtained in high purity (Fig 4A). The characteristic bands for each GPT were observed at the corresponding M_r ranges of 80 000–120 000 for HMW-GS, 60 000–68 000 for ω5-gliadins, 43 000–60 000 for ω1,2-gliadins and 32 000–45 000 for α- and γ-gliadins and LMW-GS, as reported before [31]. Minor traces of HMW-GS (≈ 2.8%, determined by semiquantitative image analysis of the SDS-PAGE gel using ImageJ) were observed in the wheat gliadin fraction, but these disappeared in the HPLC-purified GPT.

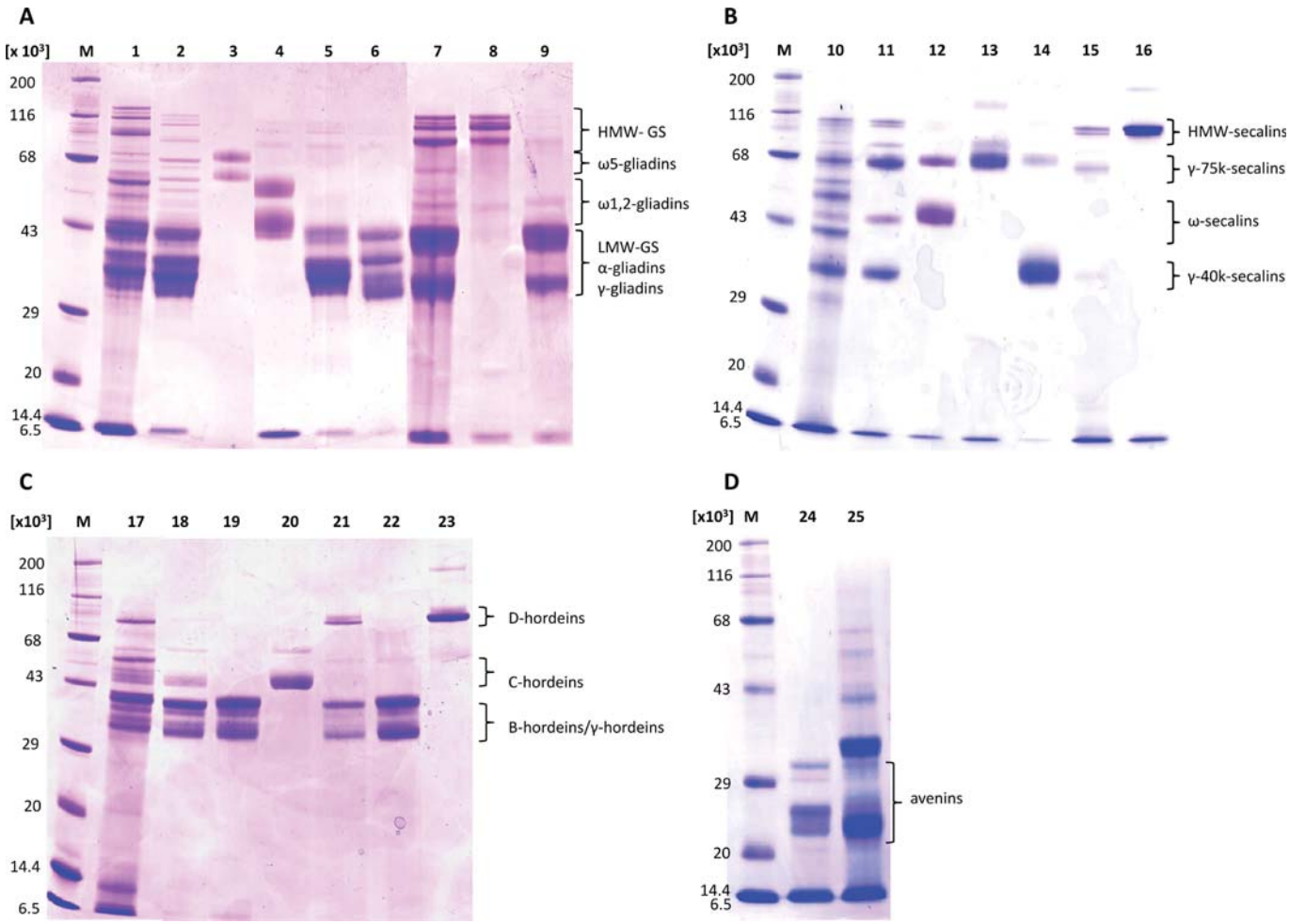

Fig 4. SDS-PAGE of flours, prolamin and glutelin fractions and isolated gluten protein types. (A) Wheat. M: marker, 1: wheat flour, 2: wheat prolamin fraction, 3: ω5-gliadins, 4: ω1,2-gliadins, 5: α-gliadins, 6: γ-gliadins, 7: wheat glutelin fraction, 8: high-molecular-weight glutenin subunits (HMW-GS), 9: low-molecular-weight glutenin subunits (LMW-GS). (B) Rye. M: marker, 10: rye flour, 11: rye prolamin fraction, 12: ω-secalins, 13: γ-75k-secalins, 14: γ-40k-secalins, 15: rye glutelin fraction, 16: HMW-secalins. (C) Barley. M: marker, 17: barley flour, 18: barley prolamin fraction, 19: γ/B-hordeins, 20: C-hordeins, 21: barley glutelins, 22: B/γ-hordeins, 23: D-hordeins. (D) Oats. 24: oat prolamin fraction (avenins), 25: oat flour.

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N-terminal sequencing of the wheat GPT was used as an additional confirmation of the purity and identity of the isolates (Table 2). Typical N-terminal sequences were determined for all wheat GPT [17,47,48,49], including EGEASGQLQC characteristic of HMW-GS Ax1, Bx7 and Bx6, EGEASEQLQC characteristic of HMW-GS Dx2 and Dx5, and EGEASRQLQC characteristic of HMW-GS By9, Dy10 and Dy12 [31], all of which were present in the wheat flour mixture. For LMW-GS, both the s-type and the m-types [50] were detected by N-terminal sequencing. The M_r of the GPT determined by LC-ESI-QTOF-MS were in good agreement with reference sequences in the NCBI database (Table 2). Only one mass signal was detected for HMW-GS, because this GPT was very hard to solubilize. Compared to SDS-PAGE, the M_r obtained by LC-ESI-QTOF-MS for the isolated GPT were about 30% lower. This overestimation of M_r by SDS-PAGE, which was observed for all GPT studied here, is ascribed to a stretched conformation of the proline-rich sequence domains in the presence of SDS and has frequently been reported before [27,31,51]. Untargeted LC-MS/MS of chymotryptic digests

of the single wheat GPT resulted in the identification of 157 characteristic peptides in total. In the hydrolysates of each GPT from wheat, 6 peptides were identified in ω5-gliadins, 24 in ω1,2-gliadins, 31 in α-gliadins, 11 in γ-gliadins, 43 in HMW-GS and 42 in LMW-GS (S1 Table). These peptides matched 12 protein sequences for ω5-gliadins in the NCBI database, 25 for ω1,2-gliadins, 63 for α-gliadins, 28 for γ-gliadins, 64 for HMW-GS and 82 for LMW-GS, all of which had a protein score above 63 (S5 Table), which is the threshold calculated by the Mascot software for a protein identification to be significant. The NCBI accession given in Table 2 as reference sequence for each GPT is the best match considering correct N-terminal sequence, a M_r within the range detected by LC-ESI-QTOF-MS and the highest protein score calculated by the Mascot software after untargeted LC-MS/MS analysis considering the type and number of identified peptides. As shown in S5 Table, other protein sequences with higher scores were also assigned to the pool of detected peptides from each GPT, but these either had alternative N-terminal sequences from the main one(s) determined by N-terminal sequencing (e.g., NIQVDPSGQV in AFX69682.1 for γ-gliadins or MENSHIPGLE in ACA63873.1 for LMW-GS) or the sequences in the database were only fragments and not complete protein sequences (e.g., AGK83348.1, AGK83148.1 and AGK83270.1 for LMW-GS). The analysis of the chymotryptic GPT hydrolysates was important to confirm the identities of the isolated GPT, identify characteristic peptides and check for possible impurities. No peptides from other wheat GPT were detected in HMW-GS, ω5-gliadins and ω1,2-gliadins, reconfirming the results of analytical RP-HPLC, SDS-PAGE and N-terminal sequencing. Four and 3 peptides from LMW-GS were detected within the isolated α-gliadins and γ-gliadins, which were assigned to 5 and 9 LMW-GS protein sequences, respectively. Vice-versa, 5 α-gliadin peptides were detected within the LMW-GS isolate that corresponded to 3 α-gliadin accessions (S1 and S5 Tables). Due to their similar M_r and RP-HPLC retention times (15 – 20 min, S1C and S1F Fig), α-gliadins and LMW-GS can only be separated according to solubility during sequential extraction of wheat flour. Three extraction steps were shown to yield ≈ 95% of the gliadins [25], but the co-extraction of alcohol-soluble oligomeric HMW gliadins (13–20% of total gliadins) could not be avoided. HMW gliadins consist of ≈ 50% LMW-GS, so that 7–10% of total gliadins are estimated to actually be LMW-GS [32]. To avoid this slight impurity, further pre-fractionation by gel-permeation HPLC would be necessary prior to RP-HPLC, a step which was deemed expendable in the present study after thoroughly weighing benefits (obtaining α-gliadins with > 95% purity as opposed to ≈ 90%) and costs (labor-, material- and time-intensive). Untargeted LC-MS/MS also revealed some additional information, e.g., that LMW-GS of the i-type were also present (e.g., BAB78763.1), which had not been detected by N-terminal sequencing, probably because the i-type occurs in smaller amounts compared to the s- and m-types [50].

Rye (secalins). The lyophilized rye prolamin and glutelin fractions had a CP content of 89.4 ± 0.1% and 53.7 ± 0.8%, respectively. The HPLC-purified GPT (HMW-, ω-, γ-75k- and γ-40k-secalins) contained 74.3 ± 3.7% (HMW-secalins) to 96.9 ± 4.8% (ω-secalins) protein (Table 2) and had their identities and purities confirmed by re-chromatography on the analytical RP-HPLC system (S2 Fig) and comparison to Figs 1C and 1D and 2B. The typical M_r ranges determined by SDS-PAGE (Fig 4B) were 95 000–105 000 for HMW-secalins, 68 000–75 000 for γ-75k-secalins, 43 000–50 000 for ω-secalins and 35 000–40 000 for γ-40k-secalins, which corresponds well to earlier studies [27]. As already seen with RP-HPLC, the prolamin fraction contained all four secalin types, whereas ω- and γ-40k-secalins were missing from the glutelin fraction. The N-terminal sequence of HMW-secalins was identical to one of the two of wheat x-type HMW-GS, because of the close botanical relationship of wheat and rye [49]. All N-terminal sequences (Table 2) matched those reported in the literature [24,49,51]. LC-ESI-Q-TOF-MS revealed the M_r ranges of all rye GPT (Table 2) and these agreed well with reference sequences for HMW-, γ-75k- and ω-secalins. Fig 5A shows the *m/z*-scans within the peak

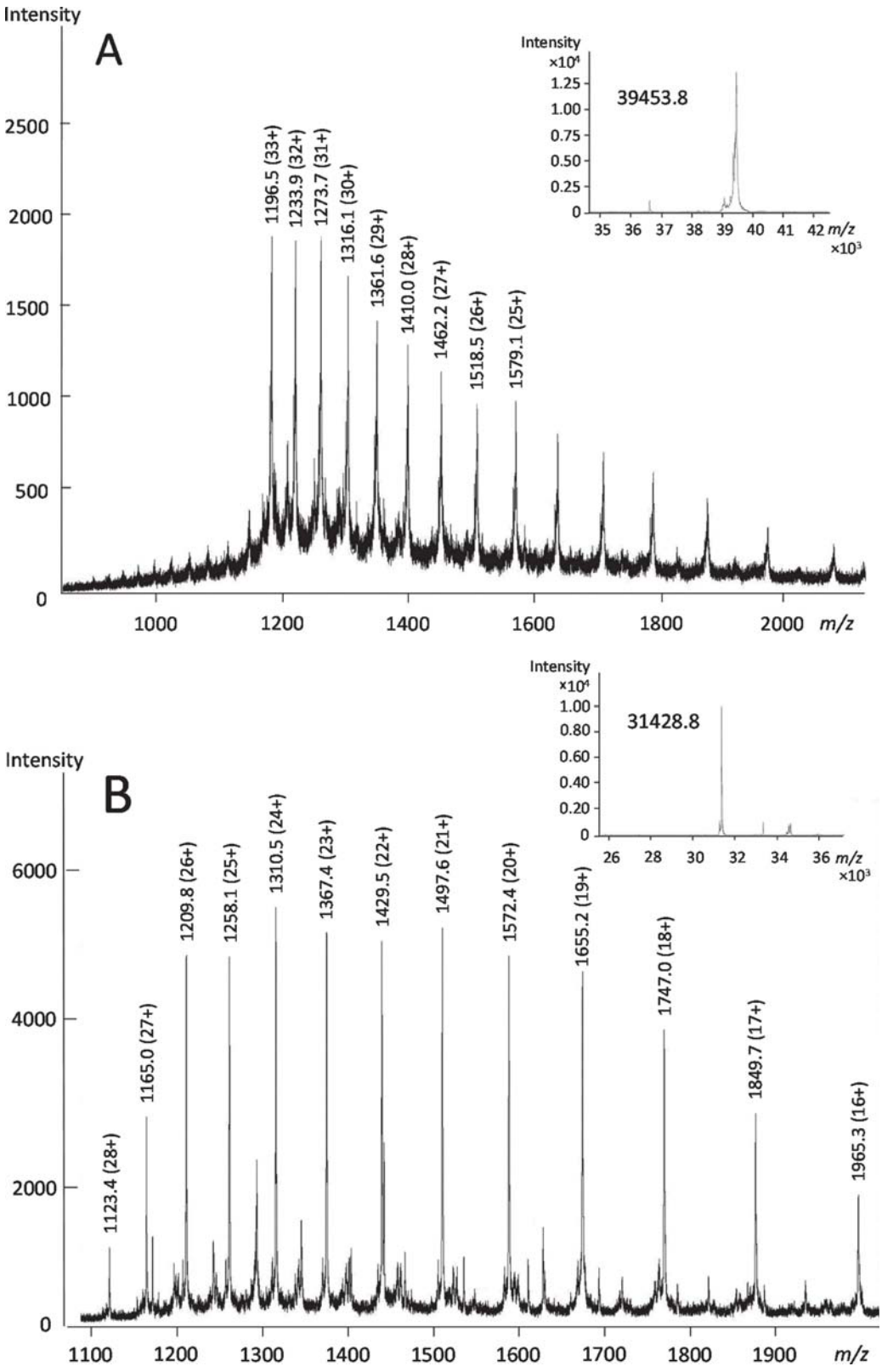
A
Intensity
2500
2000
1500
1000
500
0
1000
1200
1400
1600
1800
2000
m/z
1196.5 (33+)
1233.9 (32+)
1273.7 (31+)
1316.1 (30+)
1361.6 (29+)
1410.0 (28+)
1462.2 (27+)
1518.5 (26+)
1579.1 (25+)
Intensity
×10⁴
1.25
1.00
0.75
0.50
0.25
0
39453.8
35 36 37 38 39 40 41 42 m/z
×10³
B
Intensity
6000
4000
2000
0
1100
1200
1300
1400
1500
1600
1700
1800
1900
m/z
1123.4 (28+)
1165.0 (27+)
1209.8 (26+)
1258.1 (25+)
1310.5 (24+)
1367.4 (23+)
1429.5 (22+)
1497.6 (21+)
1572.4 (20+)
1655.2 (19+)
1747.0 (18+)
1849.7 (17+)
1965.3 (16+)
Intensity
×10⁴
1.00
0.80
0.60
0.40
0.20
0
31428.8
26 28 30 32 34 36 m/z
×10³

Fig 5. Mass spectra of isolated (A) ω-secalins and (B) γ-hordeins. The spectra show the average of scans under the peak with retention times (A) 8.9 min and (B) 12.5 min from the respective base peak MS chromatograms after LC-ESI-QTOF-MS analysis of the isolated ω-secalins and γ-hordeins, respectively. The insets show the mass spectra simulated by maximum entropy deconvolution.

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eluting at 8.9 min that were used for maximum entropy deconvolution to calculate the M_r of 39 453.8 of this specific ω-secalin.

In case of γ-40k-secalins, there are only 4 reference sequences in the whole NCBI database (S6 Table), one of which is complete (AEW46799.1), whereas the other 3 are fragments. Even this complete sequence is only predicted and, to the best of our knowledge, there is no reliable reference sequence available, neither in the NCBI nor the UniProtKB database, because the curation status of rye gluten proteins is generally low and there are no reviewed entries (as of December 12, 2016). Some database entries (e.g., ADP95517.1, 75k gamma secalin from *T. aestivum*) also appeared to be somewhat imprecisely named, because gluten proteins from *T. aestivum* are called gliadins or glutenins, but not secalins. The M_r of the γ-40k-secalin entry (21 377, Table 2) was too low compared to the M_r determined by LC-ESI-QTOF-MS (≈ 32 300), but this sequence identified by untargeted LC-MS/MS of the chymotryptic γ-40k-secalin digest was the only database match available and identified based on 7 characteristic peptides (γ40k.1, .2, .5, .9, .11, .13 and .15, S2 Table). In total, 78 characteristic rye gluten peptides were identified in the chymotryptic hydrolysates of the single isolated rye GPT, 34 of which were from HMW-secalins, 11 from γ-75k-secalins, 18 from ω-secalins and 16 from γ-40k-secalins (S2 Table). These peptides allowed the identification of 14 protein sequences for HMW-secalins, 65 for γ-75k-secalins, 16 for ω-secalins and 4 for γ-40k-secalins (S6 Table). The extensive homology of wheat and rye gluten proteins [27,49] was again evident from the fact that 3 peptides occurred in both HMW-GS and HMW-secalins (designated HG+HS) and 12 peptides in both ω1,2-gliadins and ω-secalins (designated ωg+ωs). In contrast, the γ-75k- and γ-40k-secalin peptides appeared to be unique to rye. As described above and expected from Gellrich et al. [27], 3 peptides from HMW-secalins (corresponding to 4 protein sequences) were detected in the ω-secalin isolate, but the amount of HMW-secalins is expected to be negligible, because SDS-PAGE of ω-secalins revealed no visible band with an M_r of ≈ 100 000 (Fig 4B). No peptides from other rye GPT were detected within HMW-secalins and γ-75k-secalins, but 2 peptides from γ-75k-secalins were detected within the γ-40k-secalin isolate. Due to the virtual lack of reference sequences for γ-40k-secalins, it was impossible to determine whether these 2 peptides (SQLEVVRSL and ASIVTGIVGH) were truly from γ-75k-secalins (which is unlikely, because the RP-HPLC retention times were clearly separated, S2C and S2D Fig), or whether these could also occur in γ-40k-secalins themselves, because both types share the same evolutionary origin [52]. The alignment of both protein sequences (AEW46799.1 and ADP95479.1) using the "Align" tool (UniProtKB) revealed an identity of 30.7%, with homologous sections close to the C-terminus of this γ-75k-secalin sequence. Two very similar peptides (**A**QLE-V**I**RSL and AS**T**V**A**GI**GGQ**) also occur in this γ-40k-secalin sequence, substantiating the assumption that these peptides could also be present in yet unidentified sequences of γ-40k-secalins, because single to multiple amino acid substitutions occur very frequently within gluten proteins.

Barley (hordeins). The lyophilized barley fractions contained 87.3 ± 0.4% CP (prolamins) and 62.0 ± 0.5% CP (glutelins). The barley GPT (D-, C-, γ- and B-hordeins) isolated by preparative RP-HPLC had protein contents in the range from 85.3 ± 6.3% (B/γ-hordeins) to 99.7 ± 1.6% (γ/B-hordeins) (Table 2). The identities and purities of the GPT were again confirmed by re-chromatography (S3 Fig) and comparison to Figs 1E and 1F and 2C. The separation of the barley prolamin and glutelin fractions and types by SDS-PAGE showed the

following $M_r \approx 100\,000$ for D-hordeins, 45 000–65 000 for C-hordeins and 32 000–40 000 for γ- and B-hordeins, which matched earlier investigations [41,53]. The major N-terminal sequences (Table 2) again corresponded to earlier reports [45,47,54] and more specifically matched γ3-hordein and B1-hordein. The M_r for γ-hordeins (Fig 5B) and B-hordeins determined by LC-ESI-QTOF-MS were in agreement with the reference sequences from the database, but the M_r for C-hordeins (≈ 45 000) was higher than any of the 6 protein sequences that were identified after chymotryptic digestion of the C-hordein isolate and untargeted LC-MS/MS (S3 and S7 Tables). Of those 6 sequences from the NCBI database, 3 were only fragments (P02864.1, P17991.1, AAA32942.1) and the one given as reference sequence (AAB28161.1) already had the highest M_r of the remaining 3 entries. Only 9 sequences in total are available for C-hordeins, 5 of which are fragments with a length of 105 amino acids or less (UniProtKB, as of December 12, 2016). The issue of incomplete proteomes within the *Poaceae*, and especially within *Hordeum* sp. and *Secale* sp. has been noted before and often results in unmatched peptide/protein identifications [55]. Overall, the reference sequences identified here for all barley GPT were very similar to those reported by Colgrave et al. [46]. Untargeted LC-MS/MS analyses led to the identification of 45 barley peptides in total, with 9 in the D-hordein, 11 in the C-hordein and 25 in the γ/B- and B/γ-hordein hydrolysates combined, of which 4 were specific for γ-hordeins (S3 Table). One peptide within D-hordeins was also identified in HMW-secalins (KVAKAQQL) and one within B-hordeins also in LMW-GS (LQPHQIAQL). All peptides identified within the C- and D-hordein hydrolysates were specific for that barley GPT, but, as discussed before, the isolation strategy applied here did not allow a separation of γ- and B-hordeins, because both GPT were present in γ/B- and B/γ-hordeins.

Oats (avenins). Oat avenins were extracted from defatted oat flour with 60% ethanol and not further fractionated by preparative HPLC, because this fraction only contained 6 major protein peaks (Fig 1B). Furthermore, only the oat prolamin fraction is considered as oat gluten (avenins), because oat glutelins mostly contain 12S globulins [30] that are not considered as gluten. The CP content of the isolated avenins was 79.2 ± 0.6% and the N-terminal sequence TTTVQYDPSE (Table 2) was found to be similar to ones reported as avenins 5–7 by Anderson [56]. One alternative N-terminal sequence (TTTVQYNPSE) was also detected. The M_r range of the avenin fraction was 25 000–32 000 by SDS-PAGE (Fig 4D) and again lower (≈ 22 000–29 000) by LC-ESI-QTOF-MS. The two characteristic bands of α-globulins (≈ 35 000) and β-globulins (≈ 23 000) [30] seen in the oat flour on the SDS-PAGE gel were missing in the avenin fraction. A total of 37 avenin-specific peptides were detected in the chymotryptic hydrolysate by untargeted LC-MS/MS and assigned to 49 avenin protein sequences (S4 and S8 Tables). Globulin-specific peptides were not detected indicating the high purity of the avenin fraction.

Conclusion

The preparative strategy was suitable to isolate well-defined gluten protein fractions and types from wheat, rye, barley and oat flours in high purity as confirmed by five independent protein analytical methods. The study also highlighted the need for an improvement of the curation status of protein databases within the taxonomy *Poaceae*. Some peptides, especially from C-hordeins, γ-hordeins and γ-40k-secalins, could hardly be matched to corresponding protein sequences or no reference sequence could be found that matched all analytical results, especially considering M_r and specific peptide sequences. The isolated GPT may be used as well-defined RM for analytical studies, e.g., for gluten quantitation using targeted LC-MS/MS or for studies on reactivities of antibodies used in ELISA test kits. They may also be applied for clinical studies, e.g., for basophil activation tests in case of wheat allergy, or for a whole variety of other *in vitro* cell- and tissue-based assays to study the mechanisms of CD, NCGS and wheat allergy.

Supporting information

S1 Fig. RP-HPLC chromatograms of the isolated wheat gluten protein types. (A) ω5-gliadins, (B) ω1,2-gliadins, (C) α-gliadins, (D) γ-gliadins, (E) high-molecular-weight glutenin subunits (HMW-GS), (F) low-molecular-weight glutenin subunits (LMW-GS).
(TIF)

S2 Fig. RP-HPLC chromatograms of the isolated rye gluten protein types. (A) ω-secalins, (B) high-molecular-weight (HMW)-secalins, (C) γ-75k-secalins, (D) γ-40k-secalins.
(TIF)

S3 Fig. RP-HPLC chromatograms of the isolated barley gluten protein types. (A) C-hordeins, (B) γ/B-hordeins, (C) D-hordeins, (D) B/γ-hordeins.
(TIF)

S1 Table. Peptides identified in each isolated wheat gluten protein type. Peptide sequences, their scores, *m/z* ratios, charge states and relative molecular weights (M_r). For corresponding protein sequences, see S5 Table.
(PDF)

S2 Table. Peptides identified in each isolated rye gluten protein type. Peptide sequences, their scores, *m/z* ratios, charge states and relative molecular weights (M_r). For corresponding protein sequences, see S6 Table.
(PDF)

S3 Table. Peptides identified in each isolated barley gluten protein type. Peptide sequences, their scores, *m/z* ratios, charge states and relative molecular weights (M_r). For corresponding protein sequences, see S7 Table.
(PDF)

S4 Table. Peptides identified in the oat avenin fraction. Peptide sequences, their scores, *m/z* ratios, charge states and relative molecular weights (M_r). For corresponding protein sequences, see S8 Table.
(PDF)

S5 Table. Protein sequences (protein score > 63) identified in each isolated wheat gluten protein type (GPT). The isolated wheat GPT were digested with chymotrypsin, analyzed by untargeted LC-MS/MS and the MS/MS files searched using the Mascot software and the NCBI Protein database (taxonomy *Viridiplantae*).
(PDF)

S6 Table. Protein sequences (protein score > 63) identified in each isolated rye gluten protein type (GPT). The isolated rye GPT were digested with chymotrypsin, analyzed by untargeted LC-MS/MS and the MS/MS files searched using the Mascot software and the NCBI Protein database (taxonomy *Viridiplantae*).
(PDF)

S7 Table. Protein sequences (protein score > 63) identified in each isolated barley gluten protein type (GPT). The isolated barley GPT were digested with chymotrypsin, analyzed by untargeted LC-MS/MS and the MS/MS files searched using the Mascot software and the NCBI Protein database (taxonomy *Viridiplantae*).
(PDF)

S8 Table. Protein sequences (protein score > 63) identified in oat avenins. The isolated avenin fraction was digested with chymotrypsin, analyzed by untargeted LC-MS/MS and the MS/

MS files were searched using the Mascot software and the NCBI Protein database (taxonomy *Viridiplantae*).
(PDF)

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Resources: PK.

Supervision: KAS PK.

Visualization: KS BL.

Writing – original draft: KS.

Writing – review & editing: BL KAS PK.

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3.2 Targeted liquid chromatography tandem mass spectrometry to quantitate wheat gluten using well-defined reference proteins

Isolated wheat gluten reference proteins including protein fractions (gliadins and glutenins) and types (ω5-, ω1,2-, α-, γ-gliadins, HMW- and LMW-GS from chapter 3.1 were used in an approach to define wheat gluten marker peptides and to used them to quantitate the gluten content of wheat starches. Untargeted LC-MS/MS analysis of chymotryptic digested reference proteins yielded a number of wheat-specific potential marker peptides, which fulfilled predefined criteria regarding specificity for protein types and species, peptide length and the absence of cysteine residues. Kathrin Schalk defined the criteria for gluten marker peptides, performed the experiments, and selected marker peptides, which were used for gluten quantitation. In total, 16 wheat marker peptides were chosen. Kathrin Schalk developed a targeted LC-MS/MS method in the MRM mode for the quantitative determination of the selected 16 wheat marker peptides using an isotopically labelled peptide as internal standard. These marker peptides were quantitated in the chymotryptic digest of a defined amount of the respective isolated wheat gluten reference protein type to obtain peptide-specific yields. This enabled the conversion of peptide into protein type concentrations and a strong correlation between gluten contents and peptide concentrations was observed. Gluten contents were expressed as sum of all protein type concentrations. This new method was applied to quantitate gluten contents in several wheat starches with different gluten contents. The obtained results were compared to those determined by R5 ELISA (sandwich) and GP-HPLC-FLD (provided by her co-author) and resulted in a strong correlation between LC-MS/MS and the other two methods.

Additionally, Kathrin Schalk wrote the manuscript and revised it according to the comments of the reviewers.

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RESEARCH ARTICLE

Targeted liquid chromatography tandem mass spectrometry to quantitate wheat gluten using well-defined reference proteins

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Abstract

Celiac disease (CD) is an inflammatory disorder of the upper small intestine caused by the ingestion of storage proteins (prolamins and glutelins) from wheat, barley, rye, and, in rare cases, oats. CD patients need to follow a gluten-free diet by consuming gluten-free products with gluten contents of less than 20 mg/kg. Currently, the recommended method for the quantitative determination of gluten is an enzyme-linked immunosorbent assay (ELISA) based on the R5 monoclonal antibody. Because the R5 ELISA mostly detects the prolamin fraction of gluten, a new independent method is required to detect prolamins as well as glutelins. This paper presents the development of a method to quantitate 16 wheat marker peptides derived from all wheat gluten protein types by liquid chromatography tandem mass spectrometry (LC-MS/MS) in the multiple reaction monitoring mode. The quantitation of each marker peptide in the chymotryptic digest of a defined amount of the respective reference wheat protein type resulted in peptide-specific yields. This enabled the conversion of peptide into protein type concentrations. Gluten contents were expressed as sum of all determined protein type concentrations. This new method was applied to quantitate gluten in wheat starches and compared to R5 ELISA and gel-permeation high-performance liquid chromatography with fluorescence detection (GP-HPLC-FLD), which resulted in a strong correlation between LC-MS/MS and the other two methods.

Introduction

Celiac disease (CD) is an inflammatory disorder of the upper small intestine in genetically predisposed individuals. It is triggered by the ingestion of storage proteins from wheat (gliadins, glutenins), rye (secalins), barley (hordeins), and possibly oats (avenins) that are called gluten in the field of CD. Typically, CD patients develop a flat intestinal mucosa (villous atrophy) resulting in malabsorption of nutrients together with extra- and intraintestinal symptoms [1]. Consequently, the only effective therapy for CD patients is to follow a strict gluten-free diet to prevent long-term consequences such as anemia, edema, osteoporosis, infertility, T-cell lymphoma, and other malignancies. The daily intake of gluten may not exceed 20 mg [2] and, therefore, CD patients need to consume gluten-free products which contain less than 20 mg

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gluten/kg according to Codex Standard 118–1979 [3]. To ensure the safety of gluten-free products, it is essential that appropriate analytical methods with high specificity and sensitivity are available. Enzyme-linked immunosorbent assays (ELISAs) are most frequently used by e.g. food manufacturers or control authorities to verify the gluten content in food products. Several ELISA kits for gluten detection are established on the market and the majority is based on the Skerritt (401.21) [4], R5 [5], G12 [6], and α20 [7] monoclonal antibodies. Currently, the ELISA based on the R5 monoclonal antibody is endorsed by legislation as Codex Alimentarius type 1 method [8]. Most of the antibodies are assumed to detect only prolamins, the gluten fraction soluble in aqueous alcohols. As a consequence, the gluten content is calculated by multiplying the prolamin content by a factor of 2, because the prolamin content of gluten is taken as 50% [3]. Several studies demonstrated that this calculation of the gluten content resulted in an over- or underestimation of gluten [9] which is mostly caused by different prolamin/glutelin ratios depending on the type of grain and the degree of food processing [10,11]. Due to this over- or underestimation of the gluten content by ELISA, new independent methods are urgently needed to verify the results determined by ELISA and to identify the source of gluten.

Currently, gluten analysis by mass spectrometry is the most promising non-immunochemical approach to ensure the safety of gluten-free products. Several approaches to the quantitation of gluten marker peptides by targeted liquid chromatography tandem mass spectrometry (LC-MS/MS) were published in recent years [12–16]. Sealey-Voyksner et al. (2010) developed an LC-MS/MS method to detect six CD-immunogenic wheat marker peptides in a range of 0.01 to 100 mg/kg in native and processed food samples. The method was calibrated by spiking a cocktail of six target peptides into proteolyzed corn flour at different concentrations [12]. Studies by Fiedler et al. (2014) demonstrated the development of a targeted LC-MS/MS approach based on two wheat marker peptides from α-gliadins to detect wheat contamination in oats. For this purpose, wheat flour was spiked into gluten-free oat flour to produce flour mixtures containing 10000 to 1 μg/g of wheat [13]. A further approach enabled proteomic profiling of 16 cereal grains and the quantitation of four wheat marker peptides down to 15 mg gluten/kg in wheat-contaminated soy flour [14]. Furthermore, nine CD-immunogenic peptides from α-gliadins were quantitated by van den Broeck et al. (2015) using LC-MS/MS. The calibration was performed by spiking a cocktail of nine marker peptides into a tryptic digest of a wheat gluten extract or of bovine serum albumin [15]. Although many studies reported the quantitation of gluten marker peptides [16], the calculation of gluten contents based on the obtained peptide concentrations was not attempted or achieved so far. All the illustrated LC-MS methods described an external calibration procedure by spiking peptides, gluten or gluten-containing flour into a gluten-free matrix. The quantitation was neither performed based on the addition of an internal peptide standard nor were defined gluten reference proteins used.

This paper demonstrates a novel strategy to define wheat gluten marker peptides as well as the development of a targeted LC-MS/MS method for the quantitative determination of 16 wheat marker peptides, which were specific for each wheat gluten protein type. The quantitation of marker peptides in well-characterized wheat reference proteins enabled the conversion of peptide into protein concentrations to quantitate gluten concentrations using an independent non-immunochemical method.

Materials and methods

Chemicals

The quality of all chemicals was of analytical grade or stated otherwise. Water for high-performance liquid chromatography (HPLC) was purified using an Arium 611VF water purification

Table 1. Selected wheat marker peptides. Amino acid sequences of the 16 peptides (P1-16), their specificity for wheat gluten protein types, and the detected peptide scores in the flour.

Peptide	Amino acid sequence	Specificity (protein type)	Score[1]	NCBI Accession[2]
P1	QQQPLPPQQTFPQQPL	LMW-GS	41	ABD72601.1
P2	GQQPQQQQL	LMW-GS	33	AGK83348.1
P3	VQQQIPVVQPSIL	LMW-GS	30	ACF93464.1
P4	SIILQEQQQGF	LMW-GS	71	ACA63873.1
P5	LQPGQGQQGY	HMW-GS	49	CAI72574.1
P6	TASLQQPGQGQQGHYPASL	HMW-GS	42	CAA43361.1
P7	HVSVEHQAASL	HMW-GS	36	AHZ62762.1
P8	ASIVAGIGGQ	γ-gliadins	28	AGZ20271.1
P9	NIQVDPSGQVQW	γ-gliadins	57	AAF42989.1
P10	LQPQQPQQSFPQQQQPL	γ-gliadins	63	ACJ03470.1
P11	LQLQPFPQPQLPYPQPQPF	α-gliadins	63	AAZ94421.1
P12	FQPSQQNPQAQGF	α-gliadins	64	BAM08452.1
P13	RPQQPYPQPQPQY	α-gliadins	48	AHN85627.1
P14	QQYPQQQPSGSDVISISGL	ω5-gliadins	53	BAE20328.1
P15	GSSLTSIGGQ	ω1,2-gliadins	43	BAN29067.1
P16	FPHQSQQPF	ω1,2-gliadins	26	ADF58069.1

[1] Individual peptide ion scores >40 are considered to indicate identity or extensive similarity ($p < 0.05$) and scores 15–40 were validated manually.

[2] Accession number of the best match in the database National Center for Biotechnology Information (NCBInr) database.

HMW-GS, high-molecular-weight glutenin subunits; LMW-GS, low-molecular-weight glutenin subunits; underlined sequences are known to be CD-active

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system (Sartorius, Goettingen, Germany). Disodium hydrogen phosphate dihydrate, ethanol, formic acid (FA; 98–100%), hydrochloric acid (32%), *n*-pentane, 1-propanol, potassium dihydrogen phosphate, sodium chloride, tris(hydroxymethyl)-aminomethane (TRIS), and urea were purchased from Merck (Darmstadt, Germany). α-Chymotrypsin (from bovine pancreas, TLCK-treated, ≥ 40 U/mg protein) and triflouroacetic acid (TFA; 99%) were obtained from Sigma-Aldrich (Steinheim, Germany. Acetonitrile (LC-MS-grade) was purchased from CLN (Freising, Germany). The wheat marker peptides (P1-16) and the isotopically labelled peptide LQLQPFPQPQLPYPQPQP*F* (*P11) with P*: L-[$^{13}C_5$][^{15}N]-proline and F*: L-[$^{13}C_9$][^{15}N]-phenylalanine (Table 1), were purchased from Genscript (Hongkong, PR China) with a purity of > 90%.

Grain samples

Grains of four common wheat cultivars (cv.) (cv. Akteur, I.G. Pflanzenzucht, Munich, Germany; cv. Julius, KWS Lochow, Bergen, Germany; cv. Pamier, Lantmännen SW Seed, JK Bergen op Zoom, The Netherlands; cv. Tommi, Nordsaat Saatzucht, Langenstein, Germany), all harvested in 2013, were mixed in the ratio 1/1/1/1 (w/w/w/w) and shaken overhead (Turbula, Willy A. Bachofen Maschinenfabrik, Muttenz, Switzerland) for 24 h to obtain a homogeneous grain mixture. The wheat grain mixture was milled on a Quadrumat Junior mill (Brabender, Duisburg, Germany) and sieved to a particle size of 0.2 mm (wheat flour mixture).

Analytical characterization of the wheat flour mixture

The crude protein content (nitrogen content x 5.7) of the wheat flour mixture was determined by the Dumas combustion method according to International Association for Cereal Science and Technology (ICC) Standard Method 167 [17] using a TruSpec Nitrogen Analyzer (Leco,

Kirchheim, Germany). The moisture and ash contents were determined according to ICC Standards 110/1 [18] and 104/1 [19]. Extraction of the wheat flour mixture followed by quantitative determination of the Osborne fractions by reversed-phase (RP)-HPLC was carried out as reported earlier [20,21]. The gluten content was calculated as sum of gliadins and glutenins. The gluten protein types were calculated from the RP-HPLC absorbance area (210 nm) of each gluten protein type relative to the total absorbance area of the respective gliadin or glutenin fraction. All determinations were done in triplicates.

Preparation of gluten reference proteins

Preparative isolation of reference gluten protein fractions and types as well as the characterization of the obtained proteins was performed as described in detail by Schalk et al. (2017) [22]. Reference gluten protein fractions (gliadins and glutenins) were isolated by modified Osborne fractionation followed by preparative RP-HPLC to isolate reference gluten protein types (ω5-, ω1,2-, α-, and γ-gliadins, high-molecular-weight (HMW), and low-molecular-weight (LMW) glutenin subunits (GS)).

Digestion of gluten reference proteins and the quantitation of marker peptides in each reference protein type

First, the wheat flour mixture (200 mg) was defatted with *n*-pentane/ethanol (95/5, v/v; 2 x 2.0 mL) [23]. Each gluten protein type isolated from the wheat flour mixture (ω5-, ω1,2-, α-, and γ-gliadins, HMW-GS and LMW-GS; 5 mg), each gluten fraction (gliadins and glutenins; 5 mg) as well as the defatted wheat flour mixture (50 mg) were suspended in a TRIS-HCl-buffer (2.0 mL, 0.1 mol/L TRIS-HCl, pH 7.8, urea 120 mg/mL) and hydrolysed with α-chymotrypsin (enzyme-to-protein ratio of 1/200, w/w) for 24 h at 37˚C. To stop the digestion, TFA (5 μL) was added [24]. The obtained peptide mixtures were purified by solid phase extraction (SPE) on Supelco DSC-C_{18} tubes (100 mg, Supelco, Steinheim, Germany). The C_{18} cartridges were conditioned with methanol (1 mL) and equilibrated with TFA (0.1%, v/v, 1 mL). After loading the peptide mixtures, the cartridges were washed with water containing TFA (0.1%, v/v, 5 x 1 mL) and the peptides were eluted stepwise with different concentrations of aqueous methanol (gluten protein types and fractions: 50% and 100%, v/v, 1 mL; wheat flour mixture: 20%, 40%, 60%, and 100%, v/v, 1 mL). The eluates were dried separately in a vacuum centrifuge (40˚C, 6 h, 800 Pa) and analysed by untargeted LC-MS/MS.

For the quantitation of marker peptides, all reference gluten protein types of the wheat flour mixture were hydrolysed as described above. The labelled standard *P11 was added (75 μL; 100 μg/mL) prior to the digestion. The obtained unpurified peptide mixtures were analysed by targeted LC-MS/MS.

Untargeted LC-MS/MS

For untargeted LC-MS/MS, an HCT-Ultra PTM iontrap MS (Bruker Daltonics, Bremen, Germany) with collision-induced dissociation (CID), was used. The MS was coupled with an UltiMate 3000 HPLC (Dionex, Idstein, Germany) system and peptide separation was performed on an Aeris Peptide 3.6 μm XB-C_{18} column (2.1 × 150, 10 nm × 2.1 mm; Phenomenex, Aschaffenburg, Germany). The MS contained a spherical iontrap with an electrospray ionization (ESI) interface running in the positive mode (capillary voltage, -4000 V; capillary exit voltage, -1500 V; skimmer voltage, 40 V). Nitrogen was used as drying (8.0 L/min, 325˚C) and nebulizing gas (0.2 MPa). The LC conditions were set as follows: solvent A, FA (0.1%, v/v) in water, solvent B, FA (0.1%, v/v) in acetonitrile; gradient 0–5 min isocratic 0% B, 5–45 min linear 0–30% B, 45–55 min linear 30–50% B; 55–60 min linear 50–90% B, 60–62 min isocratic 90%

B, 62–65 min linear 90–0% B, 65–72 min, isocratic 0% B; flow rate, 0.2 mL/min; injection volume, 15 μL; column temperature, 22˚C. Peptides were scanned in the standard enhanced mode, the scan range was *m/z* 300 to 1500 with 13000 *m/z*/s (smart target value, 300000; target mass, 900 *m/z*; maximum acquisition time: 100 ms), and CID-MS/MS scan steps were performed on precursor ions using the AutoMS/MS mode (fragmentation amplitude, 1.0 V; collision gas, helium).

Peptide identification

MS/MS data were converted into a Mascot generic file (*.mgf) and evaluated by means of the DataAnalysis 3.4 software (Bruker Daltonics) using the MS/MS ions search module of the Mascot software (Matrix Science, London, UK) based on the NCBI database (National Library of Medicine, Bethesda, MD, USA) (taxonomic category, *Viridiplantae*; peptide mass tolerance, ± 5 amu; product ion mass tolerance, ± 0.5 amu; peptide charges, 1+, 2+ and 3+; monoisotopic ions; variable modification, ammonia loss; enzyme, chymotrypsin; maximum missed cleavage sites, 2). Individual peptide ion scores > 40 were considered to indicate identity or extensive similarity ($p < 0.05$). All peptide identifications with peptide ion scores between 15 and 40 were manually validated according to Chen et al. [25].

Identification of marker peptides

All identified peptides had to fulfill the following criteria to be acceptable as suitable marker peptides for gluten quantitation: sequence specificity for each protein type, number of amino acids (8–20), and no cysteine present in the amino acid sequence [26]. Only peptides, which fulfilled all criteria and had the highest peptide scores within one protein type, were defined as ideal candidates. For each protein type, two to three marker peptides were defined.

Targeted LC-MS/MS

The quantitation of the wheat marker peptides P1-16 was performed on a triple-stage quadrupole mass spectrometer (TSQ Vantage, Thermo Fisher Scientific, Dreieich, Germany). For peptide separation, an UltiMate 3000 HPLC system (Dionex) was coupled to the mass spectrometer and an XBridge Peptide 3.5 μm BEH-C_{18} column (1.0 x 150 mm, 13 nm; Waters, Eschborn, Germany) was used. The LC conditions were set as follows: solvent A, FA (0.1%, v/v) in water, solvent B, FA (0.1%, v/v) in acetonitrile; gradient 0–5 min isocratic 5% B, 5–25 min linear 5–55% B, 25–30 min isocratic 90% B; 30–35 min linear 90–5% B, 35–45 min isocratic 5% B, flow rate, 0.1 mL/min; injection volume, 10 μL, column temperature, 22˚C. The ion source was operated in the ESI positive mode (source parameters: spray voltage, 4500 V; vaporizer temperature, 50˚C; sheath gas pressure, 40 arbitrary units (au); aux gas pressure, 5 au; capillary temperature, 300˚C). The MS was operated in the timed multiple reaction monitoring (MRM) mode (retention time ± 3 min). Two MRM transitions for each marker peptide were monitored and used as quantifier (most abundant MRM transition) and qualifier. A declustering voltage of -10 V was set for all transitions. The transitions from the precursor ions of P1-16 and *P11 to the respective product ions (b- and y-fragments) and the optimised collision energies are shown in Table 2. All peptides were dissolved in FA (0.1%, v/v, 10 μg/mL). These 17 stock solutions were mixed in molar ratios n (*P11)/n (P1-16) (1+9, 1+4, 1+1, 4+1, 9+1) for calibration.

Table 2. Optimized LC-MS/MS parameters for the 16 wheat marker peptides. Multiple reaction monitoring (MRM) parameters of P1-16 and the isotopically labelled peptide standard (*P11) as well as the corresponding response factors (*RF*), each referred to *P11.

Peptide	Precursor ion	Product ions[1]	Collision energy	Retention time	Response factor
	[*m/z*] (charge state)	[*m/z*]	[V]	[min]	(*RF*)
P1	938.78 (2+)	595.83 (b5)[2]	12	16.7	1.721
		585.55 (y5)[3]	14		
P2	527.97 (2+)	314.01 (b3)[2]	10	12.9	1.646
		186.00 (b2)[3]	14		
P3	725.07 (2+)	852.44(y8)[2]	10	17.7	0.294
		429.22(y4)[3]	16		
P4	645.63 (2+)	313.92 (b3)[2]	14	16.7	2.341
		736.19 (y6)[3]	10		
P5	538.63 (2+)	238.97 (y2)[2]	10	13.1	2.221
		182.01 (y1)[3]	16		
P6	657.06 (2+)	172.96 (b2)[2]	24	15.1	2.714
		219.21 (y2)[3]	10		
P7	589.56 (2+)	237.05 (b2)[2]	18	13.6	0.981
		444.91 ($b8^{2+}$)[3]	16		
P8	872.70 (2+)	431.19 (y5)[2]	24	15.5	1.502
		502.23 (y6)[3]	24		
P9	685.88 (2+)	315.52 (y2-NH_3)[2]	20	17.1	3.159
		356.09 (b3)[3]	16		
P10	1011.42 (2+)	839.02 (y7)	18	15.5	1.126
		228.96 (y2)[3]	20		
P11	755.20 (3+)	262.96(y2)[2]	14	19.0	1.277
		973.64 (y8)[3]	10		
*P11	760.50 (3+)	278.96 (y2)[2]	14	19.0	-
		989.64 (y8)[3]	10		
P12	739.15 (2+)	647.39 (y6)[2]	12	15.0	0.582
		176.01 (b2)[3]	18		
P13	814.24 (2+)	407.12 (y3)[2]	20	14.1	0.517
		770.48 (b6)[3]	18		
P14	1016.85 (2+)	901.58 (b7)[2]	16	17.7	2.712
		476.32 (y4)[3]	14		
P15	906.72 (2+)	461.28 (y5)[2]	24	14.5	3.582
		562.32 (y6)[3]	24		
P16	558.72 (2+)	853.60 (b7)[2]	12	14.9	0.367
		262.96 (y2)[3]	24		

[1] Charge state: 1+

[2] Precursor to product ion transition was used as quantifier

[3] Precursor to product ion transition was used as qualifier

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Matrix calibration

The defatted wheat flour mixture was spiked with commercially available potato flour (RUF Lebensmittelwerk KG, Quakenbrück, Germany) in different ratios (1+1, 1+3, 1+9, 1+19, 1+39, 1+200) to obtain different gluten contents. The defatted wheat flour mixture (500 mg) and all spiked samples (500 mg) were extracted with a buffered salt solution (2 x 2.0 mL 0.067 mol/L K_2HPO_4/KH_2PO_4-buffer, 0.4 mol/L NaCl, pH = 7.6) at 22˚C to obtain albumins and globulins

(ALGL), which were discarded. The residue was extracted with gluten extraction solvent (3 x 2 mL; 50% (v/v) 1-propanol, 0.1 mol/L TRIS-HCl, pH 7.5, 0.06 mol/l (w/v) dithiothreitol) at 60˚C under nitrogen. After addition of the respective solvent, each flour suspension was vortexed for 2 min and stirred for 10 min (ALGL) or 30 min (gluten). The gluten suspensions were centrifuged for 20 min at 3550 *g* and 22˚C, the supernatants were dried using a vacuum centrifuge (40˚C, 6 h, 800 Pa), and re-suspended in TRIS-HCl-buffer. The standard *P11 was added (100 μL; 100 μg/mL) to the samples, followed by hydrolysis with α-chymotrypsin as described above and analysed by targeted LC-MS/MS.

Limit of detection (LOD) and limit of quantitation (LOQ) of the MS method

The LOD and LOQ of the quantitation method for the wheat marker peptides P1-16 were determined using potato flour (RUF Lebensmittelwerk KG) as blank. The extraction procedure and chymotryptic hydrolysis were performed as described above. To determine the LOD and LOQ of the LC-MS/MS method, the gluten extract was spiked at 7 different concentrations (0.01–100 mg/kg) of each marker peptide and the samples were hydrolysed by α-chymotrypsin followed by targeted LC-MS/MS analysis. The LOD was calculated based on a signal-to noise-ratio (S/N) of 3, and the LOQ on an S/N of 10 according to Schalk et al. [24]. The noise was defined as interfering peak next to the analyte, which could have an influence on the detection of the marker peptide.

Quantitation of marker peptides in wheat starch

The extraction and chymotryptic hydrolysis of wheat starches were performed as described above. After stopping the hydrolysis with TFA (5 μL), the samples were purified by centrifugation with a membrane filter (Amicon Ultra-4, PLGC Ultracel-PL membrane, cut-off 10 kDa; Merck Millipore, Darmstadt, Germany) to remove gelatinized starch. The peptide-containing eluates were dried using a vacuum centrifuge (40˚C, 6 h, 800 Pa), dissolved in FA (0.1%, v/v, 750 μL) and analysed by targeted LC-MS/MS. The results were compared to those obtained by R5 ELISA and gel-permeation high-performance liquid chromatography with fluorescence detection (GP-HPLC-FLD) [11].

Statistics

Pearson's product moment correlations were calculated between contents of each peptide (P1-16) and the gluten content of the wheat flour mixture and the spiked samples. Correlation coefficients (r) were defined according to Thanhaeuser et al. [20] ($r > 0.78$, strong correlation; 0.67–0.78, medium correlation; 0.54–0.66, weak correlation; $r < 0.54$, no correlation). Statistically significant differences between the gluten contents analysed by LC-MS/MS, R5 ELISA and GP-HPLC-FLD were determined by one-way analysis of variance (ANOVA) with Tukey's test as all pairwise multiple comparison procedure at a significance level of $p < 0.05$ using SigmaPlot 12.0 (Systat Software, San José, CA, USA). Furthermore, Pearson's product moment correlations were determined between the gluten contents obtained by LC-MS/MS, R5 ELISA and GP-HPLC-FLD.

Results and discussion

Analytical characterization of the wheat flour mixture and preparation of reference proteins

To select marker peptides from wheat, a wheat flour mixture of four cultivars widely grown in Germany was used to include genetic variability between different cultivars [22,27]. The

cultivars were selected based on their production yields relative to the total production of winter wheat, in the year 2012 in Germany to include the most relevant cultivars (cumulative production share for wheat: 16%) [28]. Additionally, the wheat mixture contained flours of three different German baking performance classes (E: elite, A: high, B: bread quality) and covered the most important HMW-GS (cv. Akteur: Ax1, Dx5, Bx7, By9, Dy10; cv. Julius: Ax1, Dx2, Bx6, By8, Dy12; cv. Pamier: Dx5, Bx7, By9, Dy10; cv. Tommi: Dx2, Bx7, By9, Dy12). The crude protein content of the wheat flour mixture was 11.3 ± 0.1%, the moisture content was 13.2 ± 0.2%, and the ash content was 0.49 ± 0.01%. The sum of gliadins (5.9 ± 0.1%) and glutenins (3.0 ± 0.0%) resulted in 8.9 ± 0.1% of gluten in the wheat flour mixture and agreed with earlier findings [20]. Well-defined reference proteins were obtained by isolation of gluten protein fractions and types from the wheat flour mixture followed by characterization according to Schalk et al. [22].

Identification of wheat marker peptides

The reference gluten protein fractions (gliadins and glutenins), types (ω5-, ω1,2-, α-, and γ-gliadins, HMW-GS and LMW-GS) and the wheat flour mixture were chymotryptically hydrolysed. The obtained peptide mixtures were used to identify wheat gluten-specific peptides (wheat marker peptides) suitable for gluten quantitation (Fig 1A). The selection of suitable marker peptides was based on several criteria. The first requirement was the specificity of the peptides, i.e., that the amino acid sequences had to be characteristic for each protein type and the peptide sequences did not occur in other gluten protein types or other proteins. Secondly, the marker peptides had to consist of 8 to 20 amino acids, because shorter peptides were not specific enough and peptides longer than 20 amino acids were rather unsuitable for LC-MS/MS quantitation due to the large number of fragments and the resultant high complexity of the MS/MS spectra. Thirdly, the marker peptides should not contain cysteine residues, because of their tendency to oxidation [26]. The selection of marker peptides was not necessarily based on CD-epitope-containing peptides [29], but on peptides, which are gluten-specific and occur as widely as possible.

In the first step of identification, the isolated wheat protein types (ω5-, ω1,2-, α-, and γ-gliadins, HMW-GS and LMW-GS) were hydrolysed with α-chymotrypsin and analysed by untargeted LC-MS/MS using an iontrap MS. In total, 157 peptides were identified in all isolated wheat protein types. In each protein type the following number of peptides were identified: (ω5) 6, (ω1,2) 24, (α) 31, (γ) 11, (HMW-GS) 43, and (LMW-GS) 42. Of these, 84 peptides were potential marker peptides based on the three criteria described above. This resulted in the following number of potential marker peptides for each protein type: (ω5) 2, (ω1,2) 9, (α) 12, (γ) 10, (HMW-GS) 27, and (LMW-GS) 24. A large number of the peptides identified in ω1,2- and α-gliadins consisted of 24 to 33 amino acids and consequently did not fulfill the second criterion.

The second step of marker peptide identification was to verify this selection of 84 potential marker peptides. For this purpose, hydrolysed gliadin and glutenin fractions as well as the hydrolysed wheat flour mixture were analysed accordingly. Only peptides which were identified in hydrolysed protein types, fractions and the wheat flour mixture were suitable for gluten quantitation. 26 wheat-specific peptides were identified throughout all three stages which resulted in the following number of specific peptides for each wheat protein type: (ω5) 1, (ω1,2) 2, (α) 7, (γ) 4, (HMW-GS) 3, and (LMW-GS) 9. Based on this verified selection of peptides, two to three peptides which were detected with the highest peptide ion score in flour were defined as wheat marker peptides for each protein type. One marker peptide for each protein type was not satisfactory for gluten quantitation, because amino acids could be

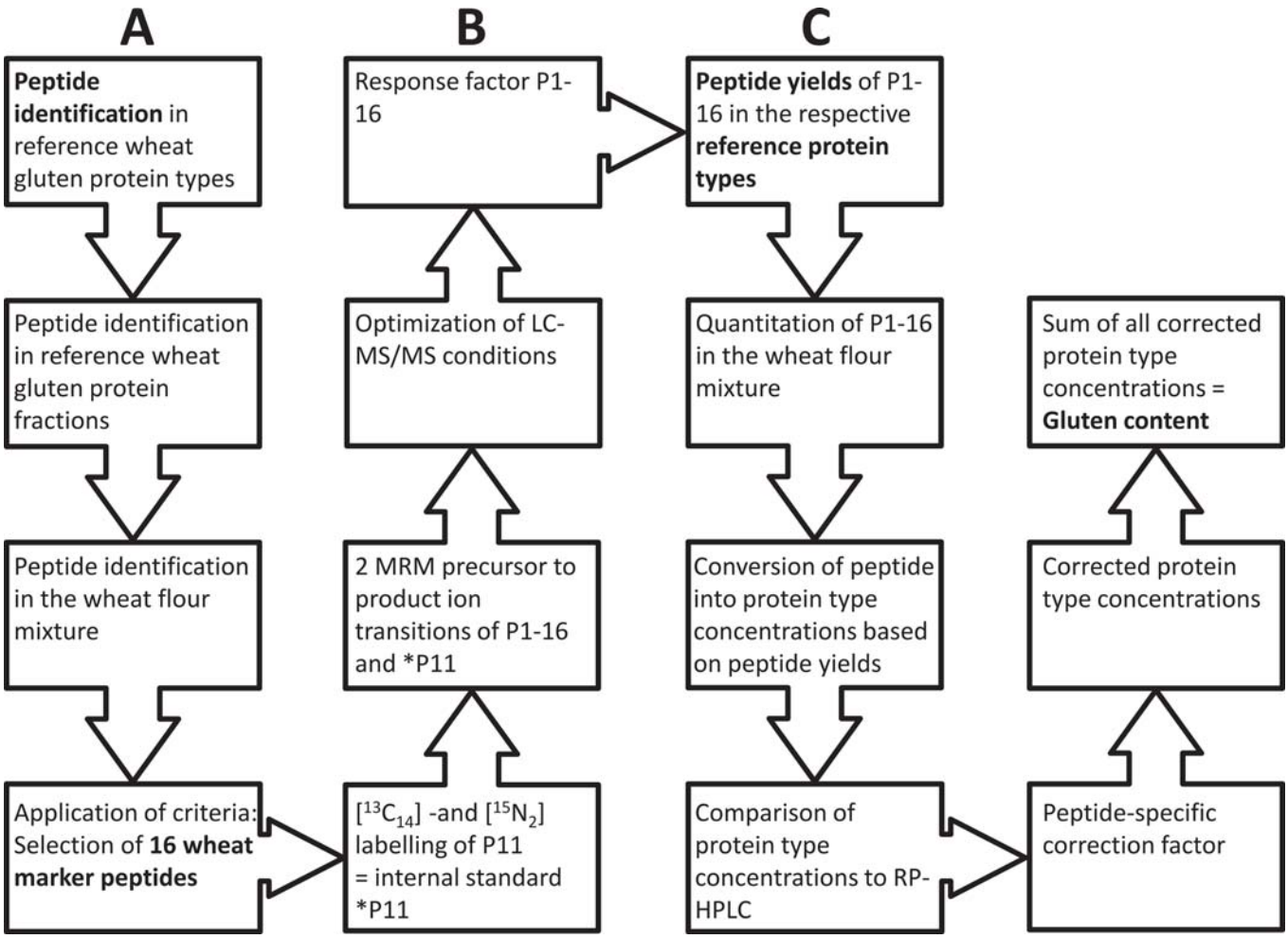

Fig 1. Schematic diagram showing the development of a method for the quantitation of gluten contents based on peptide yields. (A) Peptide identification and selection of 16 wheat marker peptides, (B) development of the liquid chromatography tandem mass spectrometry (LC-MS/MS) method with an isotopically labelled peptide as internal standard and optimization of the LC-MS/MS conditions, (C) quantitation of peptide yields in reference gluten protein types and conversion of peptide into protein type and gluten concentrations.

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modified caused by deletion or exchange [30] precluding its detection by targeted LC-MS/MS. To avoid this problem, more than one marker peptide was defined to be able to detect at least one peptide for each protein type. For ω5-gliadin, only 1 marker peptide was defined, because of the low concentration in flour [21]. In total, 16 wheat marker peptides (P1-16) were defined to quantitate the amount of gluten. Table 1 shows the amino acid sequences of P1-16 with the detected peptide ion scores in flour and their specificity for each protein type. P13 was already selected for quantitation by Sealey-Voyksner et al. (2010) [12] and P11 and P13 by van den Broeck et al. (2015) [15], both of whom specifically looked for immunogenic gliadin peptides. P8, P9, P11 and P13 were also identified as candidate wheat marker peptides by Fiedler et al. (2014) [13], who also focused on the gliadin fraction. Thus, the selection of P1-16 corresponds to earlier findings in 4 out of 6 cases for α- and γ-gliadins, with the advantage that additional peptides for the other gluten protein types were added. Of those, P2, P3, P4, P7 and P13 were already identified in one sample of gluten-free wheat starch and thus, appear to be

representative of gluten in different samples [11]. Further work will set about checking the validity of these wheat marker peptides across different wheat cultivars, also considering environmental variability.

Quantitation of wheat marker peptides

A targeted LC-MS/MS method was developed to quantitate the 16 wheat marker peptides (Fig 1B). For this purpose, P11 (LQLQPFPQPQLPYPQPQPF, monoisotopic mass 2263.2) was isotopically labelled and used as internal standard (*P11, LQLQPFPQPQLPYPQPQP*F* with F*: L-[$^{13}C_9$][^{15}N]-phenylalanine and P*: L-[$^{13}C_5$][^{15}N]-proline, monoisotopic mass 2279.2). P11 was chosen as internal standard, because the amino acid sequence contains the overlapping major immunogenic epitopes PFPQPQLPY (DQ2.5-glia-α1a) and PQPQLPYPQ (DQ2.5-glia-α2) [31]. P11 was isotopically labelled at the C-terminal end, because the y2-fragment (-PF) was detected as the most abundant product ion in the MS/MS spectrum and the label remained in the detected product ion in this way. All peptides except P11 were detected in the 2+ charge state as most abundant precursor ion. Only P11 and *P11 showed the highest intensity in the 3+ charge state of the precursor ion (P11, *m/z* 755.2, 3+; *P11, *m/z* 760.5, 3+). To define the most abundant transitions for MRM, the most abundant precursor ion of each P1-16 and *P11 was totally fragmented and a full MS/MS spectrum of each peptide was analysed. The most abundant MRM transition of each peptide was chosen for quantitation (quantifier) and the MRM transition following in intensity was used for qualification (qualifier) (Table 2). Fig 2 demonstrates the MRM transitions of P1-16 and *P11 which were used as quantifiers. The optimal fragmentation of each MRM transition was determined using different collision energies to induce the highest signal intensity [32] (Table 2). To confirm the identity of each marker peptide, the ratios of both monitored MRM transitions (i.e. precursor ion → quantifier to precursor ion → qualifier) were calculated in the response samples of each peptide. The stability of the determined ratios was monitored in each run and confirmed the identity of all peptides. The ratios were determined as follows: P1, 0.9; P2, 0.6; P3, 0.6; P4, 1.4; P5, 1.3; P6, 1.3; P7, 1.0; P8, 1.0; P9, 0.9; P10, 1.2; P11, 3.0, P12, 0.3, P12, 0.3; P13, 4.5; P14, 0.2; P15, 0.4, P16, 0.6, *P11, 3.0. The given ratios were constant in all analysed samples in this study.

Calibration and quantitation

The response factor (*RF*) of each peptide was determined using the peak area ratio A (*P11)/A (P1-16) at different values of n (*P11)/n (P1-16) between 0.05 and 12.0, that lay within the linear range. The concentration of P11 was determined by stable isotope dilution assay, because analyte and internal standard had the same amino acid sequence with the only difference that *P11 was [$^{13}C_{14}$]- and [$^{15}N_2$]-labelled. Therefore, P11 and *P11 had the same chemical properties, retention time and ionisation behaviour and as a consequence the response factor (*RF* = 1.277) determined from the slope of the regression line was close to 1.0. P1-10 and P12-16 were also quantitated using *P11 as standard, but because they had amino acid sequences different from *P11, the response factors ranged from 0.294 to 3.582.

Limit of detection (LOD) and limit of quantitation (LOQ)

The LOD and LOQ of the MS method to quantitate the 16 defined wheat marker peptides were determined by spiking P1-16 in seven different concentrations between 0.01 and 100 μg/g potato flour as matrix [22]. The absence of the marker peptides in hydrolysed gluten-free potato flour had been confirmed by LC-MS/MS. The LOD and LOQ for each marker peptide are shown in Table 3. The majority of peptides were detected with high sensitivity resulting in an LOD in a range between 0.2 and 3.4 μg/g and an LOQ between 0.9 and 10.5 μg/g. Only one

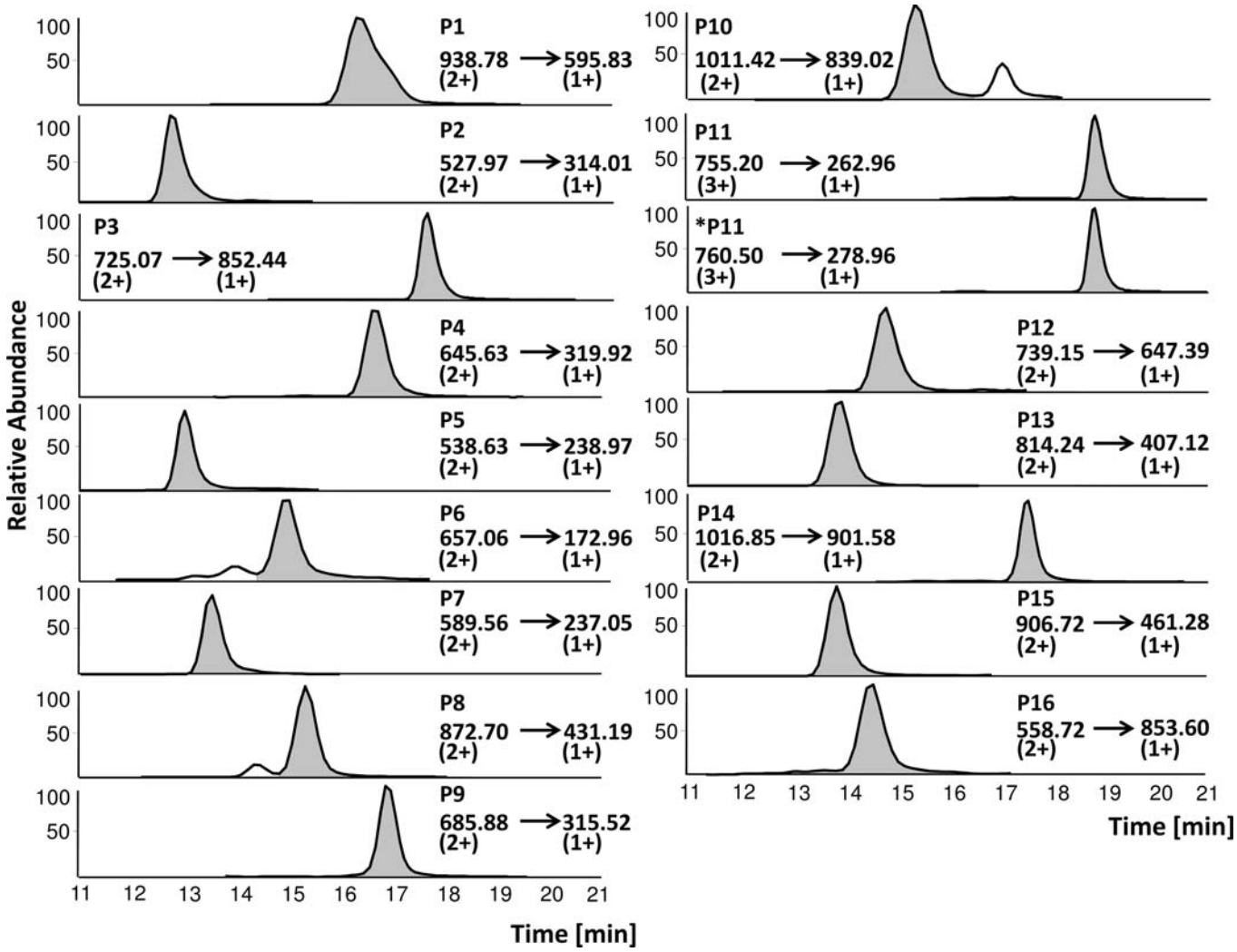

Fig 2. Precursor to product ion transition (*m/z*) of each marker peptide (P1-16) and the isotopically labelled standard (*P11). Marker peptides were quantitated in the respective protein type of wheat (multiple reaction monitoring mode, MRM). Two MRM transitions were monitored for each peptide and the most abundant MRM transition shown here was used for quantitation. HMW-GS, high-molecular-weight glutenin subunits; LMW-GS, low-molecular-weight glutenin subunits.

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marker peptide (P9) showed a relatively high LOD of 14.5 μg/g and three peptides (P6, 7, 9) showed a higher LOQ (16.8, 20.4 and 22.2 μg/g) as the other peptides.

Conversion of peptide into protein type concentrations

Each specific marker peptide was quantitated in the respective wheat protein type and the obtained peptide concentrations are shown in Table 4. Out of 16 peptides, 7 contained missed cleavages that are known to occur in gluten protein sequences [12,13,15], which is why the reproducibility of the chymotryptic digest of wheat protein types was confirmed first. The obtained peptide concentrations from ω5-, ω1,2-gliadins and HMW-GS (n = 3) as well as α-, γ-gliadins and LMW-GS (n = 6) showed a coefficient of variation (CV) ranging between 0.1% and 8.5% and 13 out of 16 marker peptides showed a CV of less than 5%. It appears that

Table 3. Limits of detection (LOD) and quantitation (LOQ) for the marker peptides P1-16 in potato flour [μg/g]. Correlation coefficients (r) were determined between peptide concentrations and gluten concentrations in the potato flour spiked to different gluten contents with the wheat flour mixture.

Peptide	Correlation coefficient (r)[1]	LOD [μg/g]	LOQ [μg/g]
P1	0.976	1.7	4.9
P2	0.912	0.2	0.9
P3	0.986	1.2	3.8
P4	0.994	0.5	5.7
P5	n.d	1.1	6.3
P6	0.943	7.5	22.2
P7	0.994	3.4	16.8
P8	0.997	0.8	3.0
P9	0.987	14.5	20.4
P10	0.985	0.8	3.0
P11	0.991	0.7	2.6
P12	0.847	3.1	10.5
P13	0.995	0.8	2.3
P14	0.970	1.9	5.6
P15	0.973	1.3	2.7
P16	n.d.	2.6	5.3

[1] Linear Pearson product correlation. Correlation coefficients (r): $0.0 < r \leq 0.54$, no correlation; $0.54 < r \leq 0.67$, weak correlation; $0.67 < r \leq 0.78$, medium correlation; and $0.78 < r \leq 1.0$, strong correlation [20].
n.d., not determined (only detected in two spiked samples)

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chymotrypsin digestion was suitable, but a profound comparison to trypsin digestion as reported by Colgrave et al. (2017) [33] would have to be done in further studies.

The peptide concentrations in the respective reference protein types formed the basis for the conversion of peptide into protein concentrations. To achieve this, the peptide yields of the chymotryptic digest obtained from a given amount of reference protein type were determined. Then, the peptide concentrations determined in the wheat flour mixture were converted into concentrations of protein type based on the respective peptide yields per protein type. In this way, a link between the obtained peptide concentrations and the respective protein types was established for all wheat marker peptides P1-16 and the efficiency of the chymotryptic digest and recovery were included in this method of calculation. In this approach, the peptide concentrations of P1-16 in the respective protein types (ω5-, ω1,2-, α-, and γ-gliadins, HMW-GS and LMW-GS) were used as reference values for the conversion of the amount of peptides determined by targeted LC-MS/MS into concentrations of wheat protein types (Fig 1C).

As an example, the calculation of the α-gliadin content using the peptide yield of P11 in the reference protein type (calculation in three steps) is explained. After the chymotryptic digest, 5879.6 μg of peptide P11 was formed from one gram of isolated α-gliadin (Table 4) (step 1). In step 2, P11 was quantitated in the wheat flour mixture and a concentration of 137.2 μg P11/g wheat flour mixture was determined. Based on a yield of 5879.6 μg P11/g α-gliadin, the wheat flour mixture contained 21.8 mg α-gliadin/g using the concentration of 137.2 μg P11/g wheat flour mixture (step 3). Then, the amount of α-gliadin in the wheat flour mixture determined by LC-MS/MS (21.8 mg/1 g) was compared to the amount of α-gliadin, which was quantitated by RP-HPLC-UV (29.1 mg/g). The amount of protein type determined by RP-HPLC-UV was taken as 100% and, thus, the recovery of LC-MS/MS was 75.3% based on peptide P11. The amount of each peptide P1-16 was converted into the concentration of the respective protein

Table 4. Concentrations of the marker peptides (P1-16) in the respective protein type [μg/g] and the wheat flour mixture [μg/g]. The concentrations of protein types in flour by LC-MS/MS [%] were calculated based on peptide concentrations in the specific protein types and compared to the contents [%] quantitated by RP-HPLC. The contents determined by RP-HPLC were taken as 100% to evaluate the recovery of LC-MS/MS. Protein type concentrations had to be multiplied by the individual correction factor to adjust to recoveries of 100%.

Peptide	Protein type	Content of protein type in flour by RP-HPLC	Peptide concentration in the specific protein type	Peptide concentration in the wheat flour mixture	Content of protein type in flour by LC-MS/MS	Recovery of LC-MS/MS compared to RP-HPLC[4]	Correction factor
		[%][1]	[μg/g][2]	[μg/g][1]	[%][3]	[%]	
P1	LMW-GS	1.99 ± 0.02	10823.2 ± 162.9	29.4 ± 0.2	0.27 ± 0.03	12.0	8.29
P2	LMW-GS		11909.8 ± 310.5	24.1 ± 0.4	0.20 ± 0.01	9.6	10.47
P3	LMW-GS		4903.4 ± 38.4	21.3 ± 0.7	0.43 ± 0.02	20.5	4.85
P4	LMW-GS		8893.1 ± 411.5	224.6 ± 16.7	2.53 ± 0.18	119.2	0.84
P5	HMW-GS	0.83 ± 0.02	5251.5 ± 366.0	90.6 ± 1.2	1.73 ± 0.08	195.2	0.51
P6	HMW-GS		3286.1 ± 111.6	n.d.	n.d.	-	-
P7	HMW-GS		7542.4 ± 250.0	86.3 ± 7.9	1.14 ± 0.04	129.5	0.77
P8	γ-gliadins	1.85 ± 0.15	18703.3 ± 304.0	639.4 ± 26.11	3.42 ± 0.09	172.3	0.58
P9	γ-gliadins		16830.2 ± 716.2	477.3 ± 33.6	2.84 ± 0.39	143.9	0.69
P10	γ-gliadins		1993.4 ± 187.2	16.1 ± 1.7	0.81 ± 0.08	41.1	2.43
P11	α-gliadins	2.91 ± 0.30	5879.6 ± 57.2	137.2 ± 13.7	2.33 ± 0.22	75.3	1.33
P12	α-gliadins		3890.9 ± 104.9	18.5 ± 0.7	0.48 ± 0.03	15.3	6.47
P13	α-gliadins		9501.9 ± 219.5	8.7 ± 0.2	0.09 ± 0.01	3.0	32.33
P14	ω5-gliadins	0.51 ± 0.02	11317.8 ± 49.4	25.6 ± 2.4	0.23 ± 0.02	39.9	2.55
P15	ω1,2-gliadins	0.67 ± 0.09	5391.7 ± 467.8	86.2 ± 2.9	1.60 ± 0.12	224.1	0.45
P16	ω1,2-gliadins		793.7 ± 17.4	n.d.	n.d.	-	-

[1] mean value ± standard deviation (n = 3)

[2] mean value ± standard deviation (HMW-GS, ω5-, ω1,2-gliadins n = 3; LMW-GS, α-, γ-gliadins n = 6) based on the concentration of protein type

[3] mean value ± standard deviation (n = 3) based on peptide concentrations (P1-16) in the respective protein type

[4] The amount of protein type, which was determined by RP-HPLC, was taken as 100% to evaluate the recovery of LC-MS/MS

LMW-GS, low-molecular-weight glutenin subunits; HMW-GS, high-molecular-weight glutenin subunits; n.d., not detected due to co-elution of other similar gluten components

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type following the same procedure, including the corresponding recoveries (Table 4). As a consequence, to calculate the amount of protein type in a real sample by LC-MS/MS, the obtained concentration had to be multiplied by the peptide-specific correction factor. The marker peptides from γ-gliadins, LMW- and HMW-GS were derived from several identical protein isoforms and therefore individual peptide correction factors were calculated. The marker peptides from α- and ω1,2-gliadins were derived from different protein isoforms and also summed up before comparison to amounts determined by RP-HPLC (S1 Table). The sum of P11, 12 and 13 yielded an α-gliadin content of 2.73%, which resulted in a recovery of 94% and a correction factor of 1.06. In case of detection of only one marker peptide from α-gliadin, the correction factor of 1.06 would overestimate the amount of α-gliadin and that is why individual peptide correction factors for P11, 12 and P13 were calculated (Table 4). The marker peptides P6 and P16 were only detected by untargeted LC-MS/MS, but the analysis of these two peptides by targeted LC-MS/MS showed interfering peaks at the same retention time (R_t; P6, R_t = 15.1 min; P16, R_t = 14.9 min) which made their quantitation impossible in the wheat flour mixture. Therefore, the ω1,2- gliadin content was only calculated based on P15. According to van den Broeck et al. [15], the amounts of protein types were calculated based on the average molecular weight (MW) of the respective protein type as described previously [22] (S2 Table). For example, the amount of peptide P11 [mmol] was converted into the corresponding

amount of α-gliadin using the average MW of α-gliadins (32286), which resulted in 0.2% α-gliadin in flour. The presented method, which considers peptide-specific yields from reference protein types and the efficiency of enzymatic digest, resulted in 1.9% α-gliadin in flour, which corresponded more accurately with the amount determined by RP-HPLC.

Matrix calibration

Each marker peptide (P1-16) was determined in the wheat flour mixture with known gluten content (89200 μg gluten/g) as well as in the wheat flour mixture spiked into gluten-free potato flour to obtain different gluten contents (44600, 22300, 8920, 4460, 2230 and 446 μg gluten/g). The gluten content of the wheat flour mixture was determined by RP-HPLC as sum of gliadins and glutenins. Gluten contents of the spiked samples were calculated based on the gluten content of the wheat flour mixture and the dilution factor. A strong correlation between peptide and gluten concentrations was observed for each marker peptide with correlation coefficients (r) > 0.847 (Table 3). The marker peptides P1, 2, 4, 11, and 14 were quantitated down to a content of 2230 μg gluten/g. In the spiked sample containing 446 μg/g, these five marker peptides were below the respective LODs (Table 3). The marker peptides P3, 10, 12, and 13 were only quantitated down to 4460 μg gluten/g in the spiked sample because the peptide contents were lower than the respective LODs (Table 3) in the samples with 2230 μg gluten/g and below. The marker peptides P6, 7, 8, 9, and 15 were quantitated down to a content of 446 μg gluten/g. The lowest quantitated peptide concentration of each marker peptide lay in between the determined LOQ and LOD of each peptide, but these concentrations still lay within the linear range. Fig 3 demonstrates the correlation between the concentrations of one peptide of each wheat protein type (P4, LMW-GS; P7, HMW-GS; P8, γ-gliadins; P11, α-gliadins; P14, ω5-gliadins; P15, ω1,2-gliadins) and the gluten contents of the spiked samples which showed the highest correlation within the same protein type.

This experiment confirmed that the marker peptides were sensitively detected at low levels of μg peptide/g flour. In the wheat flour mixture, the highest peptide yield was 639.4 μg/g of P8 and all other peptides had much lower concentrations than P8 (Table 4). Due to the comparatively low peptide concentrations compared to the high gluten content (89200 μg/g) of the wheat flour mixture, it was not feasible to quantitate the marker peptides at low levels of gluten concentrations using this approach. Further work will focus on improving sample preparation and clean-up and possibly selecting other precursor to product ion transitions less prone to interference to make the method more sensitive.

Quantitation of marker peptides in wheat starch, conversion into gluten contents and comparison to R5 ELISA and GP-HPLC-FLD

Seven wheat starches with different gluten contents were analysed by LC-MS/MS and the results compared to those obtained by sandwich R5 ELISA and GP-HPLC-FLD in a previous study [11]. Each of the methods had their own procedure to calculate the gluten content of the sample. By LC-MS/MS, the marker peptides were quantitated and selected marker peptides were used for the calculation of protein type concentrations. Afterwards, the obtained protein type concentrations were multiplied by the individual correction factor and the sum of all determined protein type concentrations resulted in the gluten content. By sandwich R5 ELISA, the gliadin content was determined and multiplied by a factor of 2 to calculate the gluten content [3]. By GP-HPLC-FLD, the concentrations of gliadins and glutenins were determined and the sum of both fractions resulted in the gluten content [11].

Only some marker peptides were detected and quantitated in all seven wheat starches (Table 5). The peptides P4 (LMW-GS), 7 (HMW-GS), 8 (γ-gliadins), 11 (α-gliadins) and 15

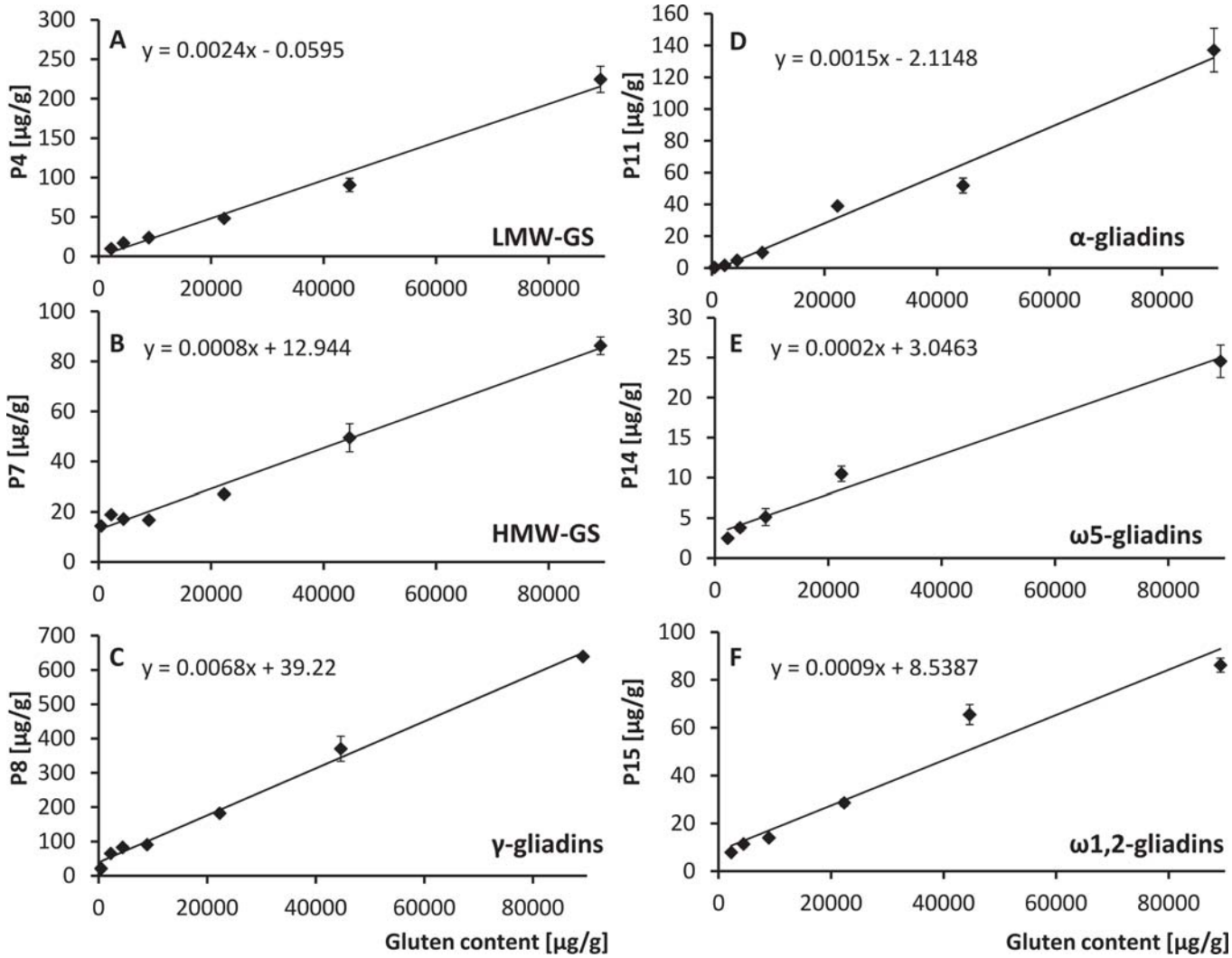

Fig 3. Linear Pearson correlations between gluten contents and concentrations of peptides from all wheat gluten protein types. (A) Peptide P4 from low-molecular-weight glutenin subunits (LMW-GS), (B) P7 from high-molecular-weight glutenin subunits (HMW-GS), (C) P8 from γ-gliadins, (D) P11 from α-gliadins, (E) P14 from ω5-gliadins, (F) P15 from ω1,2-gliadins. The presented peptides showed the highest correlation coefficients within the respective protein type (see Table 3).

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(ω1,2-gliadins) showed the highest correlation coefficients and the best recoveries compared to RP-HPLC within each protein type (Table 3), which is why these were selected for further calculations.

Peptide P4 (LMW-GS) was detected in all starches except W13 and P8 (γ-gliadins) in five out of seven starches. If the gluten content was calculated using P9, it showed significantly higher values (W13, W14) compared to the values obtained by R5 ELISA and GP-HPLC-FLD (Table 6). In W13, only peptide P9 was detected and the conversion resulted in a significantly higher gluten content compared to R5 ELISA and GP-HPLC-FLD. In contrast, the conversion of the peptide P8 concentrations into gluten contents (W4, W6, W8, W11, W15) resulted in values, which lay in the same range compared to R5 ELISA and GP-HPLC-FLD. Consequently, the concentration of peptide P9 seemed to be overestimated, which could be caused by co-elution of other similar gluten components. In wheat starch the MRM transitions of P9

Table 5. Concentrations of the marker peptides [μg/g] in seven wheat starches. The wheat starches used were W4, W6, W8, W11, W13, W14 and W15 as described in Scherf et al [11]. Those marker peptides not listed had concentrations below the respective limit of detection.

Wheat starch			
Peptide	**Protein type**	**Peptide concentration in wheat starch**	**Resulting protein type concentration**
		[μg/g][1]	**[μg/g][1]**
W4			
P4	LMW-GS	0.7 ± 0.0	63.3 ± 2.0
P8	γ-gliadins	0.7 ± 0.0	20.1 ± 1.5
P9	γ-gliadins	120.3 ± 10.8	4669.9 ± 420.1
W6			
P4	LMW-GS	0.9 ± 0.1	92.2 ± 20.1
P8	γ-gliadins	0.9 ± 0.1	25.3 ± 9.7
W8			
P2	LMW-GS	16.9 ± 0.1	13008.2 ± 1660.1
P3	LMW-GS	7.0 ± 1.8	6544.7 ± 169.7
P4	LMW-GS	27.9 ± 2.0	2538.7 ± 169.7
P7	HMW-GS	22.9 ± 2.3	1886.7 ± 580.8
P8	γ-gliadins	107.0 ± 1.9	2874.8 ± 418.4
P11	α-gliadins	5.9 ± 0.0	1291.0 ± 85.5
P15	ω1,2-gliadins	6.7 ± 1.0	523.2 ± 78.9
W11			
P4	LMW-GS	3.7 ± 0.2	330.9 ± 22.0
P8	γ-gliadins	3.5 ± 0.2	102.9 ± 7.1
P9	γ-gliadins	74.1 ± 7.5	2874.6 ± 289.2
W13			
P9	γ-gliadins	60.0 ± 2.7	2328.4 ± 105.3
W14			
P4	LMW-GS	0.5 ± 0.0	43.5 ± 2.2
P9	γ-gliadins	131.8 ± 0.6	5332.7 ± 377.0
W15			
P4	LMW-GS	8.5 ± 0.6	755.7 ± 56.6
P7	HMW-GS	7.7 ± 1.1	743.7 ± 107.8
P8	γ-gliadins	19.0 ± 2.5	554.2 ± 71.8
P11	α-gliadins	2.3 ± 0.2	479.4 ± 40.2
P15	ω1,2-gliadins	0.7 ± 0.1	132.7 ± 10.8

[1] mean value ± standard deviation (n = 3)

LMW-GS, low-molecular-weight glutenin subunits; HMW-GS, high-molecular-weight glutenin subunits

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showed interferences, which could explain the overestimation. Based on these results, P9 was eliminated for gluten calculation. Just two peptides (P4, 9) were detected in W14 and only P4 was used for the conversion into the gluten content, which yielded 43.7 μg gluten/g and showed a similar value compared to the other two methods. In W4 and W11, peptides P4 and P8 were used for the calculation of gluten contents, which showed similar results compared to R5 ELISA and GP-HPLC-FLD (Table 6). The gluten contents of W4, W11 and W14 were calculated based on all detected marker peptides except P9. In W8 and W15, one peptide of each protein type except ω5-gliadins was quantitated. In W15, all detected marker peptides were used for gluten calculation, because only marker peptides derived from different protein isoforms were detected. In W8, P2, P3 and P4 from LMW-GS were detected, which mainly

Table 6. Gluten contents [μg/g] of wheat starches W4, W6, W8, W11, W13, W14 and W15. Results from different methods, LC-MS/MS, GP-HPLC-FLD and R5 ELISA, were compared.

Sample	Method		
	LC-MS/MS[1]	GP-HPLC-FLD[2]	R5 ELISA[3]
	[μg/g]	[μg/g]	[μg/g]
W4	83.4 ± 0.7^{A}	158.6 ± 3.6^{B}	46.8 ± 2.1^{C}
W6	117.5 ± 2.8^{A}	103.6 ± 2.4^{B}	82.5 ± 0.5^{C}
W8	9114.4 ± 901.0^{A}	10371.8 ± 289.0^{AB}	11903.8 ± 1560.8^{B}
W11	433.8 ± 29.1^{A}	442.7 ± 13.7^{A}	424.4 ± 11.2^{A}
W13	2328.4 ± 105.3^{A}	196.0 ± 22.0^{B}	88.4± 1.5^{C}
W14	43.5 ± 2.2^{A}	87.2 ± 3.4^{B}	53.6 ± 2.1^{C}
W15	2665.7 ± 206.9^{A}	6543.3 ± 538.4^{B}	7022.0 ± 544.4^{B}

Values are given as mean ± standard deviation (n = 3)

Different capital letters designate significant differences ($p < 0.05$, one-way ANOVA, Tukey's Test) between the three methods within one wheat starch sample

[1] Gluten content expressed as sum of all determined protein type concentrations based on peptide concentrations

[2] Gluten content expressed as sum of gliadins and glutenins [11]

[3] Gluten content expressed as gliadins x 2 [3,11]

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derived from the same protein isoforms (S1 Table). The sum of all three peptides from the same protein isoforms would result in the overestimation of LMW-GS. Only P4 was used for the conversion into LMW-GS, because it gave the best recovery and correlation within this protein type and P4 was used for calculation in the other starches (W4, W6, W11, W14, W15) and therefore, provided better comparability between the different starches. The marker peptides P7, P8, P11 and P15 derived from different isoforms and were summed up. These two samples had the highest gluten contents compared to all others. The gluten content of W15 quantitated by LC-MS/MS was about 40% lower than those determined by R5 ELISA and GP-HPLC-FLD. The gluten content of W8 ranged between 9114 μg gluten/g (LC-MS/MS) and 11904 μg gluten/g (R5 ELISA) with an overall average of 10459 μg gluten/g. A significant difference was only observed between the gluten content of LC-MS/MS and R5 ELISA. This experiment showed that the lower the gluten content in wheat starch, the fewer marker peptides were quantitated, which may be caused by additional washing steps to decrease the gluten content of wheat starch [34,35]. As a result, several gluten proteins which contained the marker peptides were removed and not detected anymore in wheat starches with gluten contents below 100 μg/g. Looking at the gluten contents of all seven analysed wheat starches, the comparison of LC-MS/MS and GP-HPLC-FLD resulted in a strong correlation ($r = 0.909$, $p < 0.005$) as well as the comparison of LC-MS/MS and R5 ELISA ($r = 0.919$, $p < 0.005$). Overall, the results of the three different methods for gluten quantitation gave comparable results for W6, W8, W11 and W14. However, there was a rather large difference for W4, W13 and W15. The LC-MS/MS result for W4 lay in between those of GP-HPLC-FLD and R5 ELISA. Considering the gliadin/glutenin ratio of 0.76, it is likely that the gluten content was underestimated by R5 ELISA [11], because the gliadin content measured by ELISA is duplicated to obtain the gluten content assuming a ratio of 1. Further studies would be required to explain the difference between the two chromatographic methods, but the presence of γ-gliadins and LMW-GS as major residual gluten components in wheat starches as detected by LC-MS/MS is in line with earlier findings [11]. The very high gluten content in W13 detected by LC-MS/MS was due to the calculation based solely on P9, which was the only peptide above the LOQ, but the MRM trace showed interferences, as explained above. Therefore, the LC-MS/MS result for

gluten is likely too high compared to GP-HPLC-FLD and R5 ELISA. The gluten content of W15 was lower using LC-MS/MS compared to the other two methods, although peptides from all but one gluten protein types (except ω5-gliadins) were detected. It is, however, possible, that further gluten peptides/proteins were present that had no marker peptides in their amino acid sequences. At the moment, the ELISA R5 Mendez Method is considered as the "gold standard" in gluten analysis by Codex [3], but the current state of knowledge does not provide definite answers to the question which method provides the most accurate results. Even the use of different ELISA kits resulted in significantly different gluten contents for the same wheat starch sample [36] and this issue can only be addressed by further comparative analyses and enhancement of immunological and chromatographic gluten detection methods. When considering costs and time needed for one analysis, the three methods are all quite different. The extraction procedure takes about 2 h for R5 ELISA, about 3.5 h for GP-HPLC-FLD and about 39 h for LC-MS/MS, with an additional 2 h of measurement time per sample for R5 ELISA (up to 28 samples can be run in parallel in triplicates), 0.5 h for GP-HPLC-FLD and 0.75 h for LC-MS/MS. The costs are certainly highest for LC-MS/MS, because of the expensive instrumentation and skilled personnel required to perform the experiments, but it is difficult to put a number onto the cost of one analysis. ELISA is the cheapest method in comparison, with GP-HPLC-FLD in between, but certainly closer to ELISA than to LC-MS/MS. In total, ELISA seems to be preferable to the other two methods in terms of costs and time needed.

Conclusion

The present study is the first to establish a link between concentrations of 16 wheat marker peptides and gluten contents using a targeted, quantitative LC-MS/MS method. This was only possible using well-characterized reference proteins for all gluten types. With this novel approach, peptide yields after chymotryptic hydrolysis were determined and enabled the conversion of peptide into protein type concentrations and, finally, gluten contents. The conversion of the concentrations of peptides P4 (LMW-GS), 7 (HMW-GS), 8 (γ-gliadins), 11 (α-gliadins) and 15 (ω1,2-gliadins) into the respective concentrations of gluten protein types resulted in recoveries of 75 to 224% compared to RP-HPLC (100%). Gluten contents expressed as sum of all determined protein types did not significantly differ to those analysed by GP-HPLC-FLD and R5 ELISA in wheat starches with high gluten contents. In samples with low amounts of gluten (< 100 μg/g), the new method showed deficiencies regarding sensitivity, which could be improved using a different MS instrument. This study also highlighted that gluten quantitation by LC-MS/MS is still not applicable in routine analyses and requires a high level of expertise to obtain accurate results. It is, however, suitable for samples where a part of gluten has been removed by processing, as shown here for wheat starches. Further work will undertake a comparison to other previously published LC-MS/MS methods for gluten quantitation, but this would require a collaborative effort of many research groups, because no single laboratory has all the different LC-MS/MS instruments available to achieve this. For this study, marker peptides for the detection of wheat gluten were identified including CD-active peptides P10 (DQ2.5-glia-γ1), P11 (DQ2.5-glia-α1a and -α2) and P13 (DQ2.5-glia-α3) [29], but the selection was not limited by this criterion, inter alia, because wheat gluten proteins are also known allergens and the presence/absence of wheat needs to be determined also in this case. More CD-active peptides will be added to the LC-MS/MS method developed here and high-throughput techniques capable of monitoring the whole set of known CD-active peptides would be ideal to comprehensively monitor the gluten-free status of foods for CD patients.

Supporting information

S1 Table. Database search on the number of protein isoforms for each marker peptide. Number of isoforms for each marker peptide (P1-16) in *Triticum aestivum* and the number of similar isoforms for each marker peptide.
(PDF)

S2 Table. Concentrations of the marker peptides (P1-16) in the wheat flour mixture [μg/g and mmol]. Amounts of the respective protein types in the wheat flour mixture were calculated based on the molecular weight (MW) of the respective protein types.
(PDF)

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3.3 Quantitation of specific barley, rye and oat marker peptides by targeted liquid chromatography - mass spectrometry to determine gluten concentrations

According to chapter 3.2 chymotryptically digested gluten protein types and fractions from barley, oats and rye were analysed by untargeted LC-MS/MS to select suitable marker peptides for gluten quantitation. Based on specific selection criteria, 7 barley, 3 oats and 7 rye marker peptides were defined. Kathrin Schalk developed a targeted LC-MS/MS method in the MRM mode to enable the quantitation of all 33 selected marker peptides (including wheat peptides from chapter 3.2) and the identification of the source of gluten. For each type of grain, one marker peptide was chosen, isotopically labelled and used as grain-specific internal peptide standard. Kathrin Schalk quantitated marker peptides in the chymotryptically digested barley, oats and rye gluten reference proteins as described for wheat in chapter 3.2. This study resulted in peptide-specific yields, which enabled the conversion of peptide into protein concentrations and finally gluten contents, which showed a strong correlation between peptide and gluten contents.This new method was applied to quantitate the gluten content in samples from different stages of the beer brewing process. The obtained results were compared to those from competitve R5 ELISA and a strong correlation between both methods was observed. Furthermore, the method was applied for gluten quantitation in oat- and rye-based raw materials for sourdough fermentation as well as in dried sourdoughs and the results were compared to those obtained from competitive R5 ELISA and RP-HPLC. The comparison of gluten contents of all analysed raw materials resulted in a medium correlation of LC-MS/MS and RP-HPLC as wells as of LC-MS/MS and R5 ELISA, whereas no correlation was observed between RP-HPLC and R5 ELISA. In the sourdough samples, none of the marker peptides were detected, which was probably caused by microbial degradation of proteins during sourdough fermentation. These results were confirmed by RP-HPLC and SDS-PAGE. R5 ELISA gave higher gluten contents than LC-MS/MS and RP-HPLC, thus suggesting overestimation of gluten in fermented barley-based samples by ELISA.

Furthermore, Kathrin Schalk wrote the manuscript and revised it according to the comments of the reviewers.

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Quantitation of Specific Barley, Rye, and Oat Marker Peptides by Targeted Liquid Chromatography−Mass Spectrometry To Determine Gluten Concentrations

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Supporting Information

ABSTRACT: Celiac disease is triggered by the ingestion of gluten from wheat, barley, rye, and possibly oats. Gluten is quantitated by DNA-based methods or enzyme-linked immunosorbent assays (ELISAs). ELISAs mostly detect the prolamin fraction and potentially over- or underestimate gluten contents. Therefore, a new independent method is required to comprehensively detect gluten. A targeted liquid chromatography−tandem mass spectrometry method was developed to quantitate seven barley, seven rye, and three oat marker peptides derived from each gluten protein fraction (prolamin and glutelin) and type (barley, B-, C-, D-, and γ-hordeins; rye, γ-75k-, γ-40k-, ω-, and HMW-secalins). The quantitation of each marker peptide in the chymotryptic digest of a defined amount of the respective reference gluten protein type resulted in peptide-specific yields, which enabled the conversion of peptide into protein concentrations. This method was applied to quantitate gluten in samples from the brewing process, in raw materials for sourdough fermentation, and in dried sourdoughs.

KEYWORDS: *barley, celiac disease, gluten, liquid chromatography−mass spectrometry (LC−MS), marker peptide, oats, rye*

INTRODUCTION

About 1% of the Western population is affected by celiac disease (CD), an inflammatory disorder of the upper small intestine triggered by the ingestion of gluten in genetically predisposed individuals.[1] In the field of CD, gluten is defined as storage protein from wheat (gliadins and glutenins), barley (hordeins), rye (secalins), and oats (avenins). The CD toxicity of avenins is controversially discussed in the literature. Londono et al. showed that gluten epitopes from wheat, barley, and rye were not present in avenins.[2] However, two avenin peptides show an intestinal T-cell response that can cause mucosal inflammation.[3]

The only effective therapy known to date for CD patients is strict adherence to a gluten-free diet by consuming gluten-free food products[4] mostly based on rice, maize, or pseudocereals, such as buckwheat, quinoa, or amaranth.[5]

Currently, DNA-based methods (polymerase chain reaction assays)[6−8] or enzyme-linked immunosorbent assays (ELISAs) are most frequently used for gluten detection and quantitation in foods to ensure the safety of gluten-free products,[9,10] which must contain less than 20 mg of gluten/kg according to Codex Standard 118-1979.[11] Several ELISA kits based on various antibodies (e.g., R5,[12] Skerritt,[13] G12,[14] or α20[15]) are established on the market, whereas the ELISA based on the monoclonal R5 antibody is endorsed by legislation as the Codex Alimentarius type 1 method.[16] These antibodies mostly target the prolamin fraction, which is taken as 50% of gluten. Consequently, the prolamin content is multiplied by a factor of 2 to obtain gluten contents, which often resulted in over- or underestimation caused by various prolamin/glutelin ratios depending upon the type of grain.[17,18] Lexhaller et al. showed that the gluten contents of barley and rye were significantly overestimated by several ELISA kits.[19] Especially, the R5 antibody strongly reacted with rye prolamins, because it was raised against an ethanolic rye extract. This may result in an overestimation of gluten contents and unnecessarily ban actually gluten-free products from the diet of CD patients. Therefore, new independent methods are urgently needed to verify the results determined by ELISA and to identify the source of gluten.[20] Presently, mass spectrometry is the most promising non-immunochemical approach to gluten analysis to ensure the safety of gluten-free products.

Several approaches to the quantitation of gluten marker peptides by targeted liquid chromatography−tandem mass spectrometry (LC−MS/MS) were published in recent years, but many of these only focused on the quantitation of wheat marker peptides.[21−23]

Tanner et al. developed an untargeted LC−MS/MS method in the multiple reaction monitoring (MRM) mode, which enabled the relative quantitation of hordein peptides in 60 beers.[24] Knorr et al. quantitated two hordein-specific peptides derived from B- and γ-hordeins in gluten-containing and gluten-free beers relative to an internal peptide standard.[25] The conversion of peptide into gluten concentrations was not attempted as a result of the lack of a suitable reference material. Manfredi et al. were the first to focus on the quantitation of marker peptides derived from wheat, barley, rye, and oats.[26] The calibration was performed by spiking gliadin, ground oats, barley, and rye flour into rice flour. This new method allowed us to assess the presence of gluten-containing cereals in gluten-containing or gluten-free raw materials as well as processed

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Table 1. Amino Acid Sequences of the 17 Selected Barley, Oat, and Rye Marker Peptides (P17−P33), Their Specificity for the Respective Protein Type, and the Detected Peptide Scores in the Flour

peptide	amino acid sequence	specificity (protein type)	score[a]	NCBI accession[b]
P17	AIDTRVGV	γ/B-hordeins	35	P06471.1
P18	QQPQPQQGQQQQVPQSVF	γ/B-hordeins	23	P06471.1
P19	AQQQPSIEEQHQL	γ/B-hordeins	43	CAA51204.1
P20	GGGLTTEQPQGGKQPF	D-hordeins	36	BAA11642.1
P21	TQQKPGQGYNPGGTSPL	D-hordeins	56	BAA11642.1
P22	IIPQQPQQPFPLQPHQPY	C-hordeins	38	P17991.1
P23	RQLNPSSQEL	C-hordeins	35	P02864.1
P24	VQQQPPFVQQEQPF	avenins	57	CCC80640.1
P25	DPSEQYQPYPEQQEPF	avenins	15	Q09097.1
P26	LQPQLQQQL	avenins	38	CBL51494.1
P27	ASIETGIVGH	γ-75k-secalins	51	AEZ06411.1
P28	SQLEVVRSL	γ-75k-secalins	59	ADP95480.1
P29	QQFPQQPQQPFPQQPL	γ-75k-secalins	31	AEZ06411.1
P30	RQLNPSEQEL	ω-secalins	35	AAB37407.1
P31	AQQPEQLISQQPFPL	ω-secalins	63	ACQ83627.1
P32	LTSPQQPGQGQQGY	HMW-secalins	26	CAC40670.1
P33	STSPRQPGQGQQEY	HMW-secalins	24	CAC40670.1

[a]Individual peptide ion scores of >40 are considered to indicate identity ($p < 0.05$),[36] and scores of 15−40 were validated manually. [b]Accession number of the best match in the National Center for Biotechnology Information (NCBI) database.

food products. Recently, 16 wheat marker peptides were quantitated in well-characterized gluten reference proteins by targeted LC−MS/MS.[26] This study was the first to enable the conversion of wheat peptide into gluten protein concentrations. The limiting factor of LC−MS/MS is that peptide concentrations provide no further information on the gluten content of a sample. To declare food products as "gluten-free", the threshold of 20 mg of gluten/kg has to be met, and as a consequence, peptide concentrations must be converted into protein concentrations to comply with gluten legislation. Further points to consider are the choice of enzyme for gluten digestion (e.g., trypsin or chymotrypsin), cleanup of the protein digest, choice of the liquid chromatography−mass spectrometry (LC−MS) setup (i.e., untargeted or targeted analysis and acquisition mode), the data processing and evaluation procedure, and the right selection of marker peptides in the case of targeted analysis, because there will be no detection in the case of amino acid substitution, deletion, or insertion.

Therefore, the aim of the present study was the identification of suitable gluten marker peptides as well as the development of a targeted LC−MS/MS method for the quantitative determination of seven barley, three oat, and seven rye marker peptides, which were specific for each gluten protein type. The quantitation of marker peptides in the respective well-characterized barley, oat, and rye reference proteins allowed for the conversion of peptide into protein concentrations and, thus, to quantitate gluten by a non-immunochemical method independent from ELISA.

MATERIALS AND METHODS

Chemicals. The quality of all chemicals was of analytical grade or stated otherwise. Water for high-performance liquid chromatography (HPLC) was purified by an Arium 611VF water purification system (Sartorius, Goettingen, Germany). α-Chymotrypsin (from bovine pancreas, TLCK-treated, ≥40 units/mg of protein) was purchased from Sigma-Aldrich (Steinheim, Germany). The following peptides (Table 1) were purchased from GenScript (Hong Kong) with a purity of >90%: barley marker peptides (P17−P23) and the isotopically labeled peptide AQQQPSI*EEQHQL* (*P19), with I* being L-[$^{13}C_6$][^{15}N]-isoleucine and L* being L-[$^{13}C_6$][^{15}N]-leucine; oat marker peptides (P24−P26) and the isotopically labeled peptide VQQQPPFVQQEQPF* (*P24), with F* being L-[$^{13}C_9$][^{15}N]-phenylalanine; and rye marker peptides (P27−P33) and the isotopically labeled peptide ASIETGIV*G*H, with V* being L-[$^{13}C_5$][^{15}N]-valine and G* being L-[$^{13}C_2$][^{15}N]-glycine.

Grain Samples. Grains of four cultivars (cv.) each of barley (cv. Grace, cv. Marthe, Nordsaat Saatzucht, Langenstein, Germany; cv. Lomerit, KWS Lochow, Bergen, Germany; and cv. Sandra, I.G. Pflanzenzucht, Munich, Germany), oats (cv. Aragon, cv. Ivory, cv. Scorpion, Nordsaat Saatzucht; and cv. Flämingsgold, KWS Lochow), and rye (cv. Brasetto, cv. Conduct, cv. Palazzo, and cv. Visello, KWS Lochow), all harvested in 2013, were mixed in the ratio 1:1:1:1 (w/w/w/w). To achieve proper homogenization, each grain mixture was shaken overhead (Turbula, Willy A. Bachofen Maschinenfabrik, Muttenz, Switzerland) for 24 h. The barley and rye mixtures were milled on a Quadrumat Junior mill (Brabender, Duisburg, Germany) and sieved to a particle size of 0.2 mm (barley flour mixture and rye flour mixture). Oat grains were milled with a laboratory grinder (A10, IKA-Werke, Staufen, Germany) to obtain the oat flour mixture.

Methods. *Analytical Characterization of the Flour Mixtures.* According to the International Association for Cereal Science and Technology (ICC) Standard Method 167 (Dumas combustion method),[28] the crude protein content (nitrogen content × 5.7) of the barley, oat, and rye flour mixtures was determined. The moisture and ash contents were determined according to ICC Standards 110/1[29] and 104/1.[30] The quantitative determination of the Osborne fractions by analytical reversed-phase high-performance liquid chromatography (RP-HPLC) after sequential extraction of the flour mixtures was performed as described earlier.[17,31] The gluten content was calculated as the sum of prolamins and glutelins. The percentages of gluten protein types were calculated from the RP-HPLC absorbance area (210 nm) of each gluten protein type relative to the total absorbance area of the respective prolamin or glutelin fraction. All determinations and all extractions were performed in triplicates. The characterization of the raw materials for sourdough fermentation was performed accordingly.

Preparation of Gluten Reference Proteins. First, each flour mixture (100 g) was defatted with *n*-pentane/ethanol (95:5, v/v; 2 × 250 mL).[32] Second, reference gluten protein fractions and types were isolated on a preparative scale, and the obtained proteins were characterized as reported by Schalk et al.[33] To isolate reference gluten protein types (barley, C-, γ-, D-, and B-hordeins; rye, γ-75k-, γ-40k-, ω-, and HMW-secalins), reference gluten protein fractions (prolamins

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Table 2. MRM Parameters of the Seven Barley Marker Peptides (P17−P23), Three Oat Marker Peptides (P24−P26), and Seven Rye Marker Peptides (P27−P33) and the Respective Isotopically Labeled Peptide Standards (*P19, *P24, and *P27) and the Corresponding Response Factors[a]

peptide	precursor ion m/z (charge state)	product ions[b] m/z	collision energy (V)	retention time (min)	response factor (RF)
P17	415.93 (2+)	646.44 (y6)[c]	10	15.6	0.574
		531.38 (y5)[d]	14		
P18	1041.26 (2+)	577.43 (y5)[c]	16	15.2	3.119
		1505.34 (b13)[d]	16		
P19	768.71 (2+)	1081.20 (y9)[c]	16	13.7	1.498
		199.84 (b2)[d]	24		
*P19	775.46 (2+)	1094.87 (y9)[c]	16	13.7	
		200.08 (b2)[d]	24		
P20	801.71 (2+)	262.96 (b2)[c]	18	15.2	0.571
		858.51 (y8)[d]	18		
P21	865.60 (2+)	228.90 (y2)[c]	24	16.1	0.715
		627.90 (y7)[d]	20		
P22	719.62 (2+)	979.71 (y8)[c]	12	13.5	1.836
		641.22 (y5)[d]	10		
P23	586.56 (2+)	512.24 (b4)[c]	16	15.6	0.543
		911.68 (b8)[d]	14		
P24	850.86 (2+)	262.96 (y2)[c]	18	16.3	0.918
		1217.04 (y10)[d]	14		
*P24	855.66 (2+)	272.96 (y2)[c]	18	16.3	
		1227.12 (y10)[d]	14		
P25	991.70 (2+)	262.96 (y2)[c]	18	16.0	2.172
		874.61 (y7)[d]	14		
P26	548.68 (2+)	242.00 (b2)[c]	16	15.1	2.093
		466.31 (b4)[d]	16		
P27	492.53 (2+)	156.00 (b1)[c]	24	14.2	1.0902
		312.18 (y2)[d]	20		
*P27	496.90 (2+)	156.00 (b1)[c]	24	14.2	
		321.14 (y2)[d]	20		
P28	516.07 (2+)	216.00 (b2)[c]	12	15.7	0.3233
		815.66 (y3)[d]	12		
P29	968.80 (2+)	757.36 (b6)[c]	14	15.7	4.1747
		1180.56 (y10)[d]	14		
P30	607.45 (2+)	512.33 (b4)[c]	14	13.9	0.6491
		607.54 (b7)[d]	14		
P31	862.48 (2+)	228.80 (y2)[c]	34	18.0	0.4005
		908.80 (b8)[d]	12		
P32	745.08 (2+)	834.34 (y8)[c]	12	13.3	0.5217
		655.30 (b6)[d]	10		
P33	782.20 (2+)	181.84 (y1)[c]	34	11.7	9.0824
		529.16 (b5)[d]	26		

[a]Barley marker peptides were referred to *P19; oat marker peptides were referred to *P24; and rye marker peptides were referred to *P27. [b]Charge state of 1+. [c]Precursor to product ion transition was used as quantifier. [d]Precursor to product ion transition was used as qualifier.

and glutelins) were isolated by modified Osborne fractionation, followed by preparative RP-HPLC. Avenins were not further fractionated, because this fraction contained only six major peaks. Oat glutelins were not extracted, because they are mainly composed of polymeric 12S globulins and not considered to be CD-active.[34]

Digestion of Gluten Reference Proteins. Each gluten protein type obtained from the respective flour mixture (C-, γ-, D-, and B-hordeins, γ-75k-, γ-40k-, ω-, and HMW-secalins, and avenins; 5 mg), each gluten fraction (prolamins and glutelins; 5 mg) and the respective flour mixture (50 mg) were suspended in a tris(hydroxymethyl)-aminomethane (TRIS)−HCl buffer (2.0 mL, 0.1 mol/L TRIS−HCl at pH 7.8 and 120 mg/mL urea) and hydrolyzed with α-chymotrypsin (enzyme/protein ratio of 1:200, w/w) for 24 h at 37 °C. Trifluoroacetic acid (TFA; 5 μL) was added to stop the hydrolysis.[35] The obtained peptide mixtures were purified by solid-phase extraction (SPE), and the dried eluates were analyzed by untargeted LC−MS/MS, as described in detail by Schalk et al.[27] Preliminary experiments using RP-HPLC−ultraviolet (UV) to monitor the amount of peptides generated after 8, 16, 24, 36, and 48 h of chymotryptic hydrolysis had shown that the maximum amount of peptides was reached after 24 h and no further changes occurred after longer incubation, so that the digestion procedure was verified to be exhaustive.

Peptide Identification. Peptides were identified using the Mascot software (Matrix Science, London, U.K.) based on the National Center for Biotechnology Information (NCBI) database (National Library of Medicine, Bethesda, MD, U.S.A.) using peptide ion scores as criteria for the identification. Scores of >40 were considered to indicate identity ($p < 0.05$). As reported by Chen et al.,[36] all peptide identifications with peptide ion scores between 15 and 40 were manually validated.

Identification of Marker Peptides. The following criteria for acceptable gluten marker peptides were defined:[26] sequence specificity for each protein type, number of amino acids (8−20), and no cysteine present in the amino acid sequence. Peptides that fulfilled all criteria

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and had the highest peptide scores within one protein type were considered as ideal candidates. For each protein type, two to three marker peptides were defined.

Quantitation of Marker Peptides in Each Reference Protein Type. The quantitation was performed using three different internal standards (*P19 for hordein types, *P24 for avenins, and *P27 for secalin types). All reference gluten protein types of the flour mixtures (C-, γ-, D-, and B-hordeins, γ-75k-, γ-40k-, ω-, and HMW-secalins, and avenins; 5 mg) were suspended in TRIS−HCl buffer; the respective labeled standard *P19, *P24, or *P27 was added (75 μL; 100 μg/mL); and the protein−peptide mixture was hydrolyzed with α-chymotrypsin (enzyme/protein ratio of 1:200, w/w) for 24 h at 37 °C. To stop the digestion, TFA (5 μL) was added and the obtained peptide mixtures were dried using a vacuum centrifuge (40 °C, 6 h, 800 Pa), redissolved in formic acid (FA; 0.1%, v/v, 1 mL), filtered (0.45 μm), diluted with FA (0.1%, v/v, 1 + 14), and analyzed by targeted LC−MS/MS.

Targeted LC−MS/MS. The quantitation of the marker peptides P17−P23 (barley), P24−P26 (oats), and P27−P33 (rye) was performed on a triple-stage quadrupole mass spectrometer (TSQ Vantage, Thermo Fisher Scientific, Dreieich, Germany). The LC conditions and source parameters were set as described by Schalk et al.[27] MS was operated in the timed MRM mode (retention time of ±3 min). For each marker peptide, two MRM transitions were monitored and used as a quantifier (most abundant MRM transition) and qualifier. Table 2 shows the transitions from the precursor ions of P17−P33, *P17, *P24, and *P27 to the respective product ions (b and y fragments) and the experimentally optimized collision energies. This targeted LC−MS/MS method enabled the detection of 33 marker peptides from wheat (Table S1 of the Supporting Information), barley, oats, and rye and the identification of the source of gluten. The MRM transitions of 16 wheat marker peptides were described by Schalk et al.[27] All peptides were dissolved in FA (0.1%, v/v, 10 μg/mL). Peptide stock solutions were mixed in molar ratios *n* (*P17)/*n* (P17−P23) for barley, *n* (*P24)/*n* (P24−P26) for oats, and *n* (*P27)/*n* (P27−P33) for rye (1 + 9, 1 + 4, 1 + 1, 4 + 1, and 9 + 1) for calibration.

Matrix Calibration. Each of the defatted flour mixtures (barley, oats, or rye) was mixed with commercially available potato flour (RUF Lebensmittelwerk KG, Quakenbrück, Germany) in different ratios (barley and oats, 1 + 1, 1 + 3, 1 + 9, 1 + 19, 1 + 39, and 1 + 200; rye, 1 + 1, 1 + 3, 1 + 4, 1 + 5, 1 + 9, and 1 + 14) to obtain different gluten contents ranging from 423 to 21 150 μg/g for barley, from 129 to 6450 μg/g for oats, and from 2053 to 15 400 μg/g for rye. As a result of the different initial gluten contents of the flour mixtures, the resulting target concentrations were different. Albumins and globulins (ALGL) of the respective defatted flour mixture (500 mg) and all spiked samples (500 mg) were extracted with a buffered salt solution (2 × 2.0 mL of 0.067 mol/L K_2HPO_4/KH_2PO_4 buffer and 0.4 mol/L NaCl at pH 7.6) at 22 °C and discarded. The residue was extracted using gluten extraction solvent [3 × 2 mL; 50% (v/v) 1-propanol, 0.1 mol/L TRIS−HCl at pH 7.5, and 0.06 mol/L (w/v) dithiothreitol (DTT)] at 60 °C under nitrogen. Each flour suspension was vortexed for 2 min and stirred for 10 min (ALGL) or 30 min (gluten), after the addition of the respective solvent. The samples were centrifuged (20 min, 3550*g*, 22 °C), dried in a vacuum centrifuge, and resuspended in TRIS−HCl buffer. After the addition of the respective standard (*P19, *P24, or *P27; 100 μL; 100 μg/mL), the samples were hydrolyzed with α-chymotrypsin, as described above, and analyzed by targeted LC−MS/MS.

Limit of Detection (LOD) and Limit of Quantitation (LOQ) of the MS Method. To determine the LOD and LOQ of the quantitation method for the marker peptides P17−P33, potato flour was used (RUF Lebensmittelwerk KG) as a blank. First, potato flour was extracted with buffered salt solution and gluten extraction solvent, as reported above. The gluten-free extract was spiked at seven different concentrations (0.01−100 mg/kg) with each marker peptide, followed by hydrolysis with α-chymotrypsin and targeted LC−MS/MS analysis. A signal-to-noise (S/N) ratio of 3 was used to calculate the LOD, and a S/N ratio of 10 was used to calculate the LOQ, similar to Schalk et al.[35] An interfering peak next to the analyte was considered as noise, because it could have an influence on the detection of the marker peptide.

Quantitation of Barley Marker Peptides in Samples of Different Stages of the Brewing Process. The brewing process was described in detail by Knorr et al.[25] The wort, green beer, unfiltered beer, and kieselguhr-filtered beer were filtered (0.45 μm), and the respective supernatants were dried using a vacuum centrifuge. The residues were resuspended in TRIS−HCl buffer, and the barley standard *P19 was added (100 μL; 100 μg/mL), followed by incubation with α-chymotrypsin and analysis by targeted LC−MS/MS, as described above. The malt sample (500 mg) was extracted with gluten extraction solvent (3 × 2 mL) at 60 °C under nitrogen and analyzed like the other beer samples.

Quantitation of Oat and Rye Marker Peptides in Raw Materials for Sourdough Fermentation and in Oat- and Rye-Based Sourdoughs. Oat and rye marker peptides were quantitated in 12 different raw materials for sourdough fermentation (oat flour, OF; rye whole grain flour 1, 2, and 3, RWF; and rye semolina bran 1−8, RSB) as well as in dried oat- and rye-based sourdoughs (Boecker Sauerteige, Minden, Germany; oat sourdough, OS; whole grain sourdough, WGS; rye sourdough extracts 1 and 2, RSE; and rye- and wheat-based sourdough, RWS). The sourdough samples (500 mg) were extracted with gluten extraction solvent (3 × 2 mL) at 60 °C under nitrogen, as mentioned above, and dried using a vacuum centrifuge. The supernatants were resuspended in TRIS−HCl buffer; the oat (*P24) or rye standard (*P27) was added (100 μL; 100 μg/mL); and the mixture was incubated with α-chymotrypsin and analyzed by targeted LC−MS/MS, as described above. Additionally, the wheat standard *P11[26] was added to RWS. The raw materials were extracted similar to the flour mixtures. Because the final LC−MS/MS method also included the 16 wheat marker peptides in the same run, these were also monitored in all samples described here, but none was detected.

Competitive R5 ELISA. Gluten contents of samples from different stages of the brewing process and raw materials for sourdough fermentation were determined by competitive R5 ELISA (RIDASCREEN Gliadin competitive; R-Biopharm, Darmstadt, Germany). The extraction procedure of the samples from the brewing process followed that by Knorr et al.[25] The extraction of sourdough raw materials as well as dried sourdough samples was performed according to the instructions of the manufacturer.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS−PAGE). The sourdough samples (25 mg) were characterized by SDS−PAGE using a homogeneous NuPAGE 10% polyacrylamide−Bis-TRIS gel, a 3-morpholinopropane-1-sulfonic acid (MOPS)−TRIS running buffer (pH 7.7) containing DTT (5 mmol/L), and a marker mixture of 13 proteins (M_r of 15 000−200 000).[33]

Statistics. Pearson's product moment correlations were calculated between contents of each peptide (P17−P33) and the gluten content of the respective flour mixture and the spiked samples. Correlation coefficients (*r*) were defined ($r > 0.78$, strong correlation; $r = 0.67$−0.78, medium correlation; $r = 0.54$−0.66, weak correlation; and $r < 0.54$, no correlation).[37] Statistically significant differences between the gluten contents analyzed by LC−MS/MS, R5 ELISA, and RP-HPLC were determined by one-way analysis of variance (ANOVA) with Tukey's test as all pairwise multiple comparison procedure at a significance level of $p < 0.05$ using SigmaPlot 12.0 (Systat Software, San Jose, CA, U.S.A.). Furthermore, Pearson's product moment correlations were determined between the gluten contents obtained by LC−MS/MS, R5 ELISA, and RP-HPLC.

RESULTS AND DISCUSSION

Analytical Characterization of the Barley, Oat, and Rye Flour Mixture and Preparation of Reference Proteins. The selection of marker peptides from barley, oat, and rye was based on the respective flour mixture of four cultivars widely grown in Germany, each to involve genetic variability between different cultivars. Because all flour samples were from the same harvest year (2013), environmental variability was not accounted for as a result of practical

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Table 3. Analytical Characterization of the Barley, Oat, and Rye Flour Mixtures (Contents of Crude Protein, Moisture, Ash, Prolamins, Glutelins, and Gluten)[33] [a]

flour mixture	crude protein (g/100 g of flour)	moisture (g/100 g of flour)	ash (g/100 g of flour)	prolamins (g/100 g of flour)	glutelins (g/100 g of flour)	gluten (g/100 g of flour)
barley	7.7 ± 0.1	12.9 ± 0.1	0.87 ± 0.00	3.1 ± 0.1	1.1 ± 0.0	4.2 ± 0.1
oats	8.1 ± 0.0	11.8 ± 0.2	1.03 ± 0.00	1.3 ± 0.0		1.3 ± 0.0
rye	7.1 ± 0.1	11.3 ± 0.1	1.14 ± 0.01	2.5 ± 0.0	0.6 ± 0.0	3.1 ± 0.0

[a]Values are given as the mean ± standard deviation (n = 3).

considerations related to the rather time-consuming procedure of purifying sufficient amounts of gluten reference proteins. The cultivars were chosen considering their production yields relative to the total production of rye (three hybrid and one population rye cultivars), two-row and six-row winter and two-row summer barley, and oats in the year 2012 in Germany.[33,38] The crude protein contents, the ash contents, and the gluten contents (sum of prolamins and glutelins) of the flour mixtures are shown in Table 3, and the results were in agreement with earlier studies.[19,33] To obtain well-defined reference proteins, gluten protein fractions and types from the respective flour mixture were isolated and characterized according to Schalk et al.[33] Oat gluten includes only oat prolamins (avenins), because oat glutelins mostly contain 12S globulins.[34] Avenins were not further fractionated.

Identification of Barley, Oat, and Rye Marker Peptides. The strategy to define wheat marker peptides was explained in detail by Schalk et al.[27] In all isolated barley protein types, 45 peptides were identified by untargeted LC−MS/MS (D-hordeins, 9; C-hordeins, 11; and γ/B-hordeins, 25). γ/B-hordeins could not be separated by RP-HPLC;[33] therefore, they were expressed as the sum of both types. Only two peptides from C-hordeins did not fulfill one criterion, because these peptides were longer than 20 amino acids. This resulted in 43 potential barley marker peptides. Of these 43 peptides, 26 were identified in the protein type, the fraction, and the flour (three stages; C-hordeins, 6; D-hordeins, 6; and γ/B-hordeins, 14). In avenins, 37 specific peptides were identified, of which 14 were potential marker peptides. In rye gluten protein types, 78 characteristic peptides were identified (HMW-secalins, 34; γ-75k-secalins, 11; ω-secalins, 18; and γ-40k-secalins, 16). In total, 13 potential marker peptides were identified in all three stages (HMW-secalins, 2; γ-75k-secalins, 8; and ω-secalins, 3). For γ-40k-secalins, no potential marker peptide was verified, because no reliable reference sequence was available in the NCBI or the UniProt Knowledgebase (UniProtKB) database.[33] Peptides, which were detected with the highest peptide score, were defined as marker peptides for the quantitative determination of gluten. In total, seven peptides from barley (P17−P23), three from oats (P24−P26), and seven from rye (P27−P33) were chosen as marker peptides (Table 1). The wheat marker peptides P1−P16[26] are shown in Table S1 of the Supporting Information.

Quantitation of Barley, Oat, and Rye Marker Peptides. For the quantitative determination of marker peptides from barley, oat, and rye by targeted LC−MS/MS, three peptides P19 (AQQQPSIEEQHQL), P24 (VQQQPPFVQQEQPF), and P27 (ASIETGIVGH), one for each type of grain, were isotopically labeled and used as internal standards (*P19, *P24, and *P27). These peptides were chosen for labeling, because they were detected with the highest peptide score in flour. The most abundant precursor ion of each P17−P33 and *P19, *P24, and *P27 was totally fragmented to define the most abundant transitions for MRM. The labels were placed in such a way that they remained in the most abundantly detected product ions in the MS/MS spectra. The most abundant MRM transition of each peptide was selected for quantitation (quantifier), and the MRM transition following in intensity was used for qualification (qualifier) (Table 2). The optimal fragmentation of each MRM transition was determined by different collision energies to induce the highest intensity.[39] Furthermore, the ratios of both monitored MRM transitions (i.e., precursor ion → quantifier to precursor ion → qualifier) were calculated in the response samples of each peptide to ensure the identity of the peptides. The following ratios were monitored in each run and were constant in this study: P17, 2.5; P18, 8.6; P19, 1.4; P20, 6.5; P21, 2.3; P22, 2.1; P23, 3.5; P24, 2.2; P25, 2.2; P26, 1.7; P27, 2.5; P28, 0.9; P29, 2.0; P30, 2.1; P31, 3.4; P32, 1.4; and P33, 1.4. This targeted LC−MS/MS method was developed to detect all selected 33 marker peptides from wheat, barley, oats, and rye (P1−P33) and enabled the identification of the source of gluten, which is not possible by ELISA.

Calibration and Quantitation. The peak area ratio A (*P19)/A (P17−P23) at different values of n (*P19)/n (P17−P23) between 0.05 and 12.0 was used to calculate the response factor (RF) of each barley peptide that lay within the linear range. The same procedure was applied to each oat (*P24/P24−P26) and each rye marker peptide (*P27/P27−P33). The concentrations of P19, P24, and P27 were determined by the stable isotope dilution assay (SIDA), because analytes and the grain specific standards differed only in the number of placed labels (*P19, $[^{13}C_{12}][^{15}N_2]$-labeled; *P24, $[^{13}C_9][^{15}N]$-labeled; and *P27, $[^{13}C_7][^{15}N_2]$-labeled). The RF values were determined from the slope of the regression line and were close to 1.0 in the case of SIDA (P19, 1.488; P24, 0.918; and P27, 1.090). P17−P18 and P20−P23 were quantitated using *P19 as the internal barley-specific standard; P25 and P26 were quantitated using *P24 as the internal oat-specific standard; and P28−P33 were quantitated using *P27 as the internal rye-specific standard. The RF values ranged from 0.323 to 9.082 (Table 2) as a result of various ionization behaviors of analytes and standards caused by differences in the amino acid sequences. At best, each marker peptide should have an isotopically labeled standard, which would guarantee the same ionization behavior of analyte and standard during the MS measurement. As a result of high costs of isotopically labeled peptides, only one marker peptide of each grain type was labeled in this study.

LOD and LOQ. LOD and LOQ of the MS method to quantitate 17 barley, oat, and rye marker peptides were determined. Hydrolyzed gluten-free potato flour was used as the matrix, and the absence of the marker peptides had been confirmed by LC−MS/MS. The peptides P17−P33 were spiked in seven different concentrations between 0.01 and 100 μg of peptide/g of potato flour. The LOD and LOQ for each

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marker peptide are shown in Table 4. The majority of peptides were detected with high sensitivity, resulting in a LOD in a range between 0.05 and 1.9 μg/g and a LOQ in a range between 0.1 and 4.5 μg/g.

Table 4. LOD and LOQ for the Marker Peptides P17−P33 in Gluten-Free Potato Flour (μg/g)[a]

peptide	correlation coefficient (r)[b]	LOD (μg/g)	LOQ (μg/g)
P17	0.999	0.4	0.9
P18	0.997	0.7	2.4
P19	0.999	0.3	0.8
P20	nd[c]	0.5	1.3
P21	0.998	0.5	1.4
P22	0.999	0.9	2.9
P23	0.987	0.5	1.4
P24	0.999	0.07	0.5
P25	0.995	0.2	1.1
P26	0.988	1.9	2.7
P27	1.000	1.4	4.5
P28	0.998	0.05	0.1
P29	nd[c]	0.7	1.9
P30	nd[c]	0.1	0.6
P31	nd[c]	0.1	0.6
P32	nd[c]	1.1	3.8
P33	nd[c]	0.5	1.5

[a]Correlation coefficients (r) were determined between peptide concentrations and gluten concentrations in potato flour spiked to different gluten contents with the respective flour mixture (barley, oat, or rye). [b]Linear Pearson product correlation. Correlation coefficients (r): $0.0 < r \leq 0.54$, no correlation; $0.54 < r \leq 0.67$, weak correlation; $0.67 < r \leq 0.78$, medium correlation; and $0.78 < r \leq 1.0$, strong correlation. [c]nd = not detected.

Conversion of Peptide into Protein Type Concentrations. The strategy and explanation to convert peptide into protein concentrations was described in detail by Schalk et al.[27] In this approach, the peptide concentrations of P17−P33 in the respective protein types (γ/B-, C-, and D-hordeins, avenins, and γ-75k-, ω-, and HMW-secalins) were used as reference values for the conversion of the amount of peptides determined by targeted LC−MS/MS into concentrations of the respective protein type. In this way, the efficiency of the chymotryptic digest was included in the method of calculation based on these experimentally determined peptide yields. Table 5 shows the determined marker peptide concentrations in the respective reference protein type as well as in the respective flour mixture. Because 6 out of 16 marker peptides contained missed cleavages, the reproducibility of the chymotryptic digest was confirmed as reported by Colgrave et al.[40] The coefficient of variation (CV) of the determined peptide concentrations (n = 3) ranged from 0.8 to 18%, and the CV of 11 out of 16 peptides was less than 5%.

The marker peptide concentrations were converted into protein type concentrations, and the results were compared to protein type concentrations obtained from RP-HPLC, which were taken as 100%. Furthermore, the corresponding recoveries of LC−MS/MS for each peptide P17−P33 were evaluated in comparison to the amount of protein type determined by RP-HPLC. To calculate the amount of protein type in a real sample by LC−MS/MS, the obtained concentration had to be multiplied by the peptide-specific correction factor, which considered enzymatic cleavage efficiency (Table 5). The marker peptide P32 was only detected by untargeted LC−MS/MS, but the analysis of this peptide by targeted LC−MS/MS showed interfering peaks at the same retention time (R_t; 13.3 min), which made its quantitation impossible in the rye flour mixture. The rye marker peptides P29−P31 and P33 and the barley marker peptide P20 as well as the oat marker peptides P25 and P26 showed low concentrations in the respective flour mixture and resulted in low recoveries compared to RP-HPLC. The marker peptides P19, P24, and P27 were quantitated with the corresponding standard peptide and showed good recoveries close to 100%, which allowed for the conversion of peptide into corresponding protein type concentrations. For each marker peptide, individual peptide correction factors were calculated because the peptides of the respective protein type were derived from several identical protein isoforms (Table S2 of the Supporting Information). This is also the reason why the peptide concentrations of the same protein type were not summarized before comparison to amounts determined by RP-HPLC.

As reported in the literature, the amounts of protein types were also calculated on the basis of the average molecular weight (MW) of the respective protein type for comparison to the explained calculation, which included the efficiency of the enzymatic digest (Table S3 of the Supporting Information).[23] The recoveries showed that the calculation based on peptide-specific yields from reference protein types corresponded more closely to the amounts determined by RP-HPLC than the calculation based on average MW.

Matrix Calibration. The marker peptides (P17−P33) were quantitated in the respective flour mixture with known gluten content (barley, 42 300 μg/g; oats, 12 900 μg/g; and rye, 30 800 μg/g) determined by RP-HPLC. Furthermore, the respective flour mixture was spiked into gluten-free potato flour to obtain different gluten contents (barley, 21 150, 10 575, 4230, 2115, 1076, and 423 μg of gluten/g; oats, 6450, 3225, 1290, 645, 323, and 129 μg of gluten/g; and rye, 15 400, 10 267, 7700, 5133, 3080, and 2053 μg of gluten/g). The gluten content of the flour mixtures was determined by RP-HPLC as a sum of prolamins and glutelins. Gluten contents of the spiked samples were calculated on the basis of the gluten content of the respective flour mixture and the dilution factor. With correlation coefficients (r) of >0.987, a strong correlation between peptide and gluten concentrations was observed for each marker peptide (Table 4), as expected. The marker peptides P20 and P29−P33 were only quantitated in the respective flour mixture with amounts near the LOQ, and therefore, a correlation was not feasible. P23 was quantitated down to 2115 μg of gluten/g and was below the respective LOQ (Table 4) in the spiked samples containing 1076 and 423 μg of gluten/g. The oat marker peptides P25 and P26 were only quantitated down to 645 μg of gluten/g. The study showed that the sensitive detection of marker peptides from barley, oats, and rye at low level concentrations was possible. However, it was not feasible to quantitate marker peptides at very low levels of gluten concentrations, because a factor of more than 100 lay in between the marker peptide concentrations and the gluten concentration of the respective flour mixture. Further work will focus on clarifying the reasons for this discrepancy and improving sensitivity.

Quantitation of Marker Peptides in Beer, Conversion into Gluten Contents, and Comparison to R5 ELISA. The barley marker peptides were quantitated at different stages of the beer brewing process (malt, wort, green beer, unfiltered

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Table 5. Concentration of the Marker Peptides (P17−P33) in the Respective Protein Type (μg/g) and Flour Mixture (μg/g)[a]

peptide	protein type	content of protein type in flour by RP-HPLC (%)[b]	peptide concentration in the specific protein type (μg/g)[c]	peptide concentration in the flour mixture (μg/g)[b]	content of protein type in flour by LC−MS/MS (%)[d]	recovery of LC−MS/MS compared to RP-HPLC[e] (%)	correction factor
P17	γ/B-hordeins	2.95 ± 0.04	39047.2 ± 2996.5	1794.0 ± 1.6	4.59 ± 0.00	155.7	0.64
P18	γ/B-hordeins		35178.9 ± 4589.3	909.7 ± 53.8	2.59 ± 0.15	87.7	1.14
P19	γ/B-hordeins		3173.1 ± 138.4	59.8 ± 2.4	1.88 ± 0.07	63.9	1.57
P20	D-hordeins	0.32 ± 0.01	5925.0 ± 253.4	0.5 ± 0.0	0.04 ± 0.00	11.1	7.75
P21	D-hordeins		48815.7 ± 6320.7	29.0 ± 1.8	0.06 ± 0.00	18.6	5.33
P22	C-hordeins	0.96 ± 0.01	32827.8 ± 1209.1	113.9 ± 3.1	0.35 ± 0.00	36.2	2.74
P23	C-hordeins		26346.1 ± 430.2	23.7 ± 1.1	0.09 ± 0.00	9.4	10.67
P24	avenins	1.29 ± 0.03	13216.4 ± 792.3	141.9 ± 21.4	1.07 ± 0.16	83.2	1.21
P25	avenins		6396.6 ± 424.7	4.0 ± 0.7	0.06 ± 0.00	4.9	21.50
P26	avenins		13351.2 ± 1442.2	17.0 ± 0.2	0.13 ± 0.00	9.9	9.92
P27	γ-75k-secalins	1.41 ± 0.04	3337.1 ± 64.8	50.5 ± 1.2	1.51 ± 0.02	106.0	0.93
P28	γ-75k-secalins		950.7 ± 31.8	15.4 ± 1.9	1.62 ± 0.20	117.7	0.87
P29	γ-75k-secalins		856.9 ± 19.9	0.7 ± 0.0	0.09 ± 0.01	6.7	15.67
P30	ω-secalins	0.58 ± 0.02	482.6 ± 7.9	0.2 ± 0.0	0.04 ± 0.01	6.1	14.50
P31	ω-secalins		2081.2 ± 58.7	0.1 ± 0.0	0.02 ± 0.00	3.8	29.00
P32	HMW-secalins	0.17 ± 0.01	598.8 ± 111.9	<LOD[f]	<LOD		
P33	HMW-secalins		9227.4 ± 207.5	0.5 ± 0.0	0.01 ± 0.00	1.5	17.00

[a]The concentrations of protein types in flour by LC−MS/MS (%) was calculated on the basis of peptide concentrations in the specific protein types and compared to the contents (%) quantitated by RP-HPLC. The contents determined by RP-HPLC were taken as 100% to evaluate the recovery of LC−MS/MS. Protein type concentrations had to be multiplied by the individual correction factor to adjust for recoveries of 100%. [b]Mean value ± standard deviation (n = 3). [c]Mean value ± standard deviation (n = 3) based on the concentration of protein type. [d]Mean value ± standard deviation (n = 3) based on peptide concentrations (P1−P16) in the respective protein type. [e]The amount of protein type, which was determined by RP-HPLC, was taken as 100% to evaluate the recovery of LC−MS/MS. [f]LOD = limit of detection.

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Table 6. Marker Peptide Concentrations (P19 and P22) and Gluten Contents (μg/g) of Samples from Different Stages of the Brewing Process with Different Gluten Contents Quantitated by LC−MS/MS, and R5 ELISA[a]

	method			
sample	LC−MS/MS marker peptide		LC−MS/MS[b] gluten (μg/g)	R5 ELISA[c] gluten (μg/g)
malt	P19	32.2 ± 0.3[d]	19647.1 ± 153.4 A	97077.2 ± 6401.9 B
	P22	44.8 ± 0.1[d]		
wort	P19	0.5 ± 0.0[e]	228.9 ± 0.8 A	90.6 ± 7.8 B
green beer	P19	0.4 ± 0.0[e]	199.1 ± 11.1 A	197.9 ± 15.3 A
unfiltered beer	P19	0.6 ± 0.0[e]	299.8 ± 5.2 A	236.2 ± 7.9 B
kieselguhr-filtered beer	P19	0.8 ± 0.0[e]	377.8 ± 24.5 A	89.0 ± 6.7 B

[a]Values are given as the mean ± standard deviation (n = 3). Different capital letters indicate significant differences (p < 0.05; one-way ANOVA; Tukey's test) between the two methods within one sample. [b]Gluten content expressed as the sum of all determined protein type concentrations based on peptide concentrations. [c]Gluten content expressed as gliadins × 2.[11] [d]In units of μg/g. [e]In units of μg/mL.

Table 7. Marker Peptide Concentrations (P27 and P24) and Gluten Contents (μg/g) of Untreated Rye- and Oat-Based Raw Materials for Sourdough Fermentation with Different Gluten Contents Quantitated by LC−MS/MS, RP-HPLC, and R5 ELISA[a]

	method				
sample	LC−MS/MS marker peptide (μg/g)		LC−MS/MS[b] gluten (mg/g)	RP-HPLC[c] gluten (mg/g)	R5 ELISA[d] gluten (mg/g)
OF	P24	60.7 ± 4.6	5.5 ± 0.4 A	3.6 ± 0.0 B	n/a[e]
RSB 1	P27	31.9 ± 1.3	8.9 ± 0.4 A	23.3 ± 0.9 B	191.1 ± 17.3 C
RSB 2	P27	36.5 ± 4.3	10.2 ± 1.1 A	21.0 ± 0.4 B	134.3 ± 21.2 C
RSB 3	P27	43.6 ± 2.5	12.2 ± 0.7 A	24.5 ± 0.5 B	230.6 ± 46.0 C
RSB 4	P27	47.8 ± 1.4	13.4 ± 0.4 A	32.3 ± 0.7 B	193.9 ± 8.7 C
RSB 5	P27	53.0 ± 0.8	14.8 ± 0.2 A	31.7 ± 5.1 B	243.5 ± 10.5 C
RSB 6	P27	35.8 ± 1.8	10.0 ± 0.5 A	26.8 ± 2.0 B	105.6 ± 4.1 C
RSB 7	P27	34.0 ± 2.7	9.5 ± 0.6 A	27.2 ± 0.6 B	167.4 ± 11.3 C
RSB 8	P27	37.5 ± 1.5	10.5 ± 0.4 A	41.5 ± 0.2 B	132.9 ± 65.3 C
RWF 1	P27	22.2 ± 0.4	6.2 ± 0.1 A	12.5 ± 0.3 B	155.8 ± 21.0 C
RWF 2	P27	42.9 ± 3.5	12.0 ± 1.0 A	25.1 ± 0.4 B	314.6 ± 12.6 C
RWF3	P27	38.2 ± 2.1	10.7 ± 0.6 A	25.0 ± 0.5 B	178.1 ± 27.1 C

[a]OF, oat flour; RSB, rye semolina bran; and RWF, rye whole grain flour. Values are given as the mean ± standard deviation (n = 3). Different capital letters indicate significant differences (p < 0.05; one-way ANOVA; Tukey's test) between the two methods within one sample. [b]Gluten content expressed as the sum of all determined protein type concentrations based on peptide concentrations. [c]Gluten content expressed as the sum of prolamins and glutelins; the gluten content of OF is only expressed as prolamins because glutelins are mainly composed of polymeric 12S globulins.[34] [d]Gluten content expressed as gliadins × 2.[11] [e]n/a = not applicable, because the R5 antibody does not cross-react with oats.

beer, and kieselguhr-filtered beer). The obtained peptide concentrations were converted into hordein concentrations and multiplied by the individual correction factor, and the sum of all determined protein type concentrations resulted in the gluten content, which was compared to that analyzed by competitive R5 ELISA. The marker peptides P19, P21, and P22 showed the highest correlation coefficient and recovery within one protein type. P19 and P22 were used for gluten quantitation in beer, and the other marker peptides P17, P18, P20, P21, and P23 were not detected in any of the five samples. P19 was detected in all stages of the brewing process, but P22 was only detected in malt. By LC−MS/MS, the gluten content of wort, green beer, unfiltered beer, and kieselguhr-filtered beer was based on P19 concentrations (γ/B-hordeins), whereas the content of malt was based on the sum of γ/B-hordeins and C-hordeins calculated from P19 and P22. The gluten contents of wort, unfiltered, and kieselguhr-filtered beer were significantly different between LC−MS/MS and ELISA, whereas the gluten content of green beer showed no significant difference (Table 6). The gluten content of wort seems to be underestimated by R5 ELISA compared to LC−MS/MS, because it appeared unlikely that the gluten content of wort is lower than in green beer (R5 ELISA) and no further source of gluten was added at this stage. In general, during the malting process, peptidases are activated and gluten is degraded. In comparison to the barley flour mixture with a gluten content of about 42 mg/g (by RP-HPLC), the gluten content of malt (97.1 ± 6.4 mg/g) was likely to be overestimated by ELISA. A content of 19.6 ± 0.2 mg/g was determined by LC−MS/MS. The determination of the gluten content of malt by Knorr et al. resulted in 113 mg/g by ELISA and 22.7 mg/g by RP-HPLC and showed similar results compared to this study.[25] The decrease of the gluten content from wort to green beer by LC−MS/MS occurred as a result of microbial degradation of gluten during fermentation. However, at the same time, the content of gluten peptides increases, some of which might still be immunogenic but not detectable by either ELISA or targeted LC−MS/MS. A recent study on beers revealed that large and possibly CD-active protein fragments were present in gluten-reduced barley-based beers, even after treatment with a prolylendopeptidase.[41] A final risk assessment for CD patients could not be made as a result of the lack of absolute quantitation and will have to be the subject of further in-depth studies. It is unclear why an increase of the gluten content was observed by R5 ELISA, because no gluten source was added at this stage. An increase of the gluten content determined by both methods was observed between green beer and unfiltered beer, because wort was added to green beer before secondary fermentation. The decrease of the

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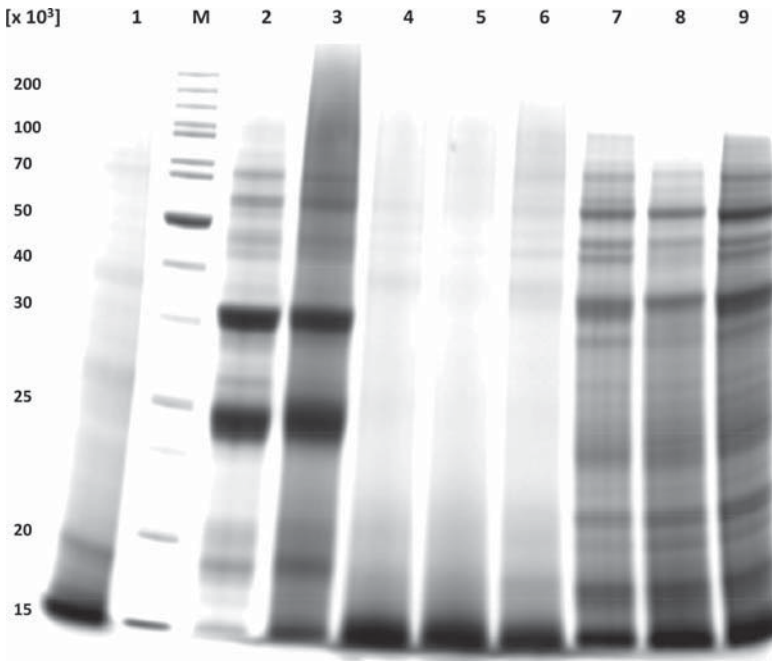

Figure 1. SDS−PAGE (reducing conditions) of dried oat-, rye-, and wheat-based sourdough samples and the raw materials for sourdough fermentation: M, marker (M_r of 15 000−200 000); 1, rye- and wheat-based sourdough; 2, oat flour; 3, oat sourdough; 4, rye sourdough extract 1; 5, rye sourdough extract 2; 6, whole grain sourdough; 7, rye whole grain flour; 8, rye semolina bran 1; and 9, rye semolina bran 2.

gluten content between unfiltered and kieselguhr-filtered beer was only observed by R5 ELISA, which resulted from the filtration process. The comparison of gluten contents of all analyzed samples from the brewing process resulted in a strong correlation (r = 0.999; p < 0.005) of LC−MS/MS and R5 ELISA. In general, R5 ELISA is known to overestimate barley gluten when calibrated to a wheat standard,[19] but this was only observed in the case of malt, because the gluten content was higher than that of the initial barley flour mixture. In all other samples from wort to filtered beer, the determination by LC−MS/MS resulted in higher gluten contents compared to ELISA. This is in line with earlier findings,[42] and thus, LC−MS/MS appears to be more suitable for foods containing (extensively) hydrolyzed gluten.

Quantitation of Marker Peptides in Untreated Raw Materials for Sourdough Fermentation and Oat- and Rye-Based Sourdoughs, Conversion into Gluten Contents, and Comparison to R5 ELISA and RP-HPLC. To quantitate marker peptides, in the first step, gluten proteins from raw materials were extracted similar to that by Fallahbaghery et al., who showed that the extraction was highly efficient and reproducible using propanol and DTT.[43] Second, gluten proteins were hydrolyzed with chymotrypsin. In the rye-based raw materials (RWF 1−3 and RSB 1−8), the quantitation of gluten contents was based on the peptide concentration of P27, because it was the only peptide that was detected. In OS, only P24 was detected and used for conversion of peptide into protein concentrations. The quantitated amounts of P24 and P27, which were converted into gluten contents, as well as the determined gluten contents by RP-HPLC and R5 ELISA of different raw materials for sourdough fermentation are shown in Table 7. There were significant differences between gluten contents determined by LC−MS/MS, RP-HPLC, and ELISA. The gluten contents determined by LC−MS/MS ranged from 6.2 to 14.8 mg of gluten/g in rye-based raw materials. The quantitated amounts by RP-HPLC were higher by a factor of about 2 compared to LC−MS/MS. The amounts determined by R5 ELISA were in a range from 132.7.0 to 314.6 mg of gluten/g and seemed to be very high. R5 ELISA is also known to overestimate rye gluten when calibrated to a wheat standard by a factor of up to 8,[19] which corresponds quite well to the average discrepancy observed between RP-HPLC and ELISA (Table 7). The comparison of gluten contents of all analyzed raw materials resulted in a medium correlation (r = 0.743; p < 0.005) of LC−MS/MS and RP-HPLC and a medium correlation (r = 0.705; p < 0.01) of LC−MS/MS and R5 ELISA, although the absolute values were quite different, as discussed above. No correlation (r = 0.453) was observed between RP-HPLC and R5 ELISA.

In the oat- and rye-based sourdoughs, none of the oat or rye marker peptides were detected by LC−MS/MS, which appeared to be caused by microbial degradation of the marker peptides during sourdough fermentation. The degradation of gluten proteins, as reported before,[44,45] was confirmed by SDS−PAGE and RP-HPLC (Figure 1 and Figure S1 of the Supporting Information). The samples WGS, RSE 1 and 2, and RWS showed no characteristic bands in the range of M_r from 15 000 to 200 000. Only OS showed two bands with M_r of 23 000 and 35 000. RP-HPLC showed no characteristic peaks in the prolamin and glutelin extracts of the sourdough samples, which made the exact determination of the gluten contents impossible. In contrast, R5 ELISA showed high gluten contents, which ranged from 7.5 mg of gluten/g (RSE 2) to 73.5 mg of gluten/g (RWS). Although rye gluten is known to be overestimated by R5 ELISA, it may well be that targeted LC−MS/MS missed peptides that were modified during sourdough fermentation. Further work is needed to precisely elucidate which immunogenic peptides remain in sourdough samples and address this limitation of targeted LC−MS/MS.

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Taken together, the present study is the first to establish a link between concentrations of seven barley, three oat, and seven rye peptides and protein contents by targeted LC−MS/MS. The determination of peptide yields obtained from chymotryptic hydrolysis of well-characterized reference gluten protein types from prolamins as well as glutelins enabled the conversion of peptide into protein type concentrations and, finally, gluten contents. This new LC−MS/MS method including a total of 33 marker peptides for wheat, rye, barley, and oats was applied to gluten quantitation in samples from different stages of the brewing process of barley-based beer, in untreated wheat-, rye-, and oat-based raw materials for sourdough fermentation, and in dried sourdoughs. The comparison of gluten contents analyzed by LC−MS/MS compared to R5 ELISA and RP-HPLC resulted in medium to strong correlations, although the absolute values did show differences. Despite careful experimental design, the implementation of the strategy illustrated here for gluten quantitation turned out to be fairly complicated, requiring a high level of expertise, because the conversion of marker peptide contents to gluten content was not as straightforward as originally thought, despite the use of well-characterized reference proteins. One major drawback of targeted LC−MS/MS approaches for gluten detection is the fact that only a limited number of pre-selected marker peptides are included and all other peptides with amino acid substitution, deletion, or insertion are missed, although this is known to occur very frequently within gluten and may or may not have an influence on CD immunogenicity. Another outcome of this study was that every peptide needs its corresponding stable isotope-labeled standard to account for potential losses during sample cleanup. Care is therefore advised regarding the general applicability of the LC−MS/MS method, especially in the case of foods containing hydrolyzed gluten, where ELISA and RP-HPLC cannot be regarded as fully reliable methods either. The multifaceted nature of gluten and, specifically, hydrolyzed gluten presents a variety of challenges to any analytical method and warrants further fundamental research to better understand the peptide fingerprint and its relation to CD immunogenicity. The LC−MS/MS method showed deficiencies regarding sensitivity to detect gluten trace levels as a result of low peptide yields and co-eluting substances. Furthermore, the method has to be improved to increase peptide recoveries and the abundance of marker peptides in processed food products to enable quantitation of trace levels of gluten (<20 mg/kg). One promising possibility to enhance selectivity, dynamic range, and sensitivity is the application of a more advanced MS instrument, which is also suitable for untargeted analyses, to gain a better understanding of the complexity of gluten hydrolyzates.[46]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.7b05286.

RP-HPLC chromatograms of the prolamin and glutelin fractions from oat-, rye-, and wheat-based sourdoughs: (A) rye whole grain sourdough, prolamins, (B) rye whole grain sourdough, glutelins, (C) oat sourdough, prolamins, (D) rye sourdough extract, prolamins, (E) rye sourdough extract, glutelins, (F) rye- and wheat-based sourdough, prolamins, and (G) rye- and wheat-based sourdough, glutelins (Figure S1), RP-HPLC chromatograms of the prolamin and glutelin fractions from raw materials for sourdough fermentation: (H) oat flour prolamins, (I) rye whole grain flour prolamins, (J) rye whole grain flour glutelins, (K) rye semolina bran prolamins, and (L) rye semolina bran glutelins (Figure S2), amino acid sequences of the 16 selected wheat marker peptides (P1−P16), their specificity for wheat protein types, and the detected peptide scores in the flour (Table S1), number of isoforms of each marker peptide in *Hordeum vulgare* (P17−P23), *Avena sativa* (P24−P26), and *Secale cereale* (P27−P33) and the number of similar isoforms of each marker peptide (Table S2), and concentrations of the marker peptides (P17−P33) in the respective flour mixture (barley, oats, and rye) (μg/g and mmol/g), with amounts of the respective protein types in the flour mixture calculated on the basis of the MW of the respective protein types (Table S3) (PDF)

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Notes

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■ ABBREVIATIONS USED

ALGL, albumins/globulins; CD, celiac disease; cv, cultivar; ELISA, enzyme-linked immunosorbent assay; FA, formic acid; HMW-GS, high-molecular-weight glutenin subunits; ICC, International Association for Cereal Science and Technology; LC−MS/MS, liquid chromatography−tandem mass spectrometry; LMW-GS, low-molecular-weight glutenin subunits; LOD, limit of detection; LOQ, limit of quantitation; MOPS, 3-morpholinopropane-1-sulfonic acid; MRM, multiple reaction monitoring; OF, oat flour; OS, oat sourdough; RP-HPLC, reversed-phase high-performance liquid chromatography; RSB, rye semolina bran; RSE, rye sourdough extract; RWF, rye whole grain flour; RWS, rye- and wheat-based sourdough; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; S/N, signal-to-noise; SPE, solid-phase extraction; TFA, trifluouroacetic acid; TRIS, tris(hydroxymethyl)-aminomethane; WGS, whole grain sourdough

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3.4 Quantitation of the immunodominant 33-mer peptide from α-gliadin in wheat flours by liquid chromatography tandem mass spectrometry

The highly immunodominat 33-mer peptide from α-gliadin (LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF) plays a central role in coeliac disease, because of its resistance towards proteolytic breakdown and due to the fact that it triggers a strong immune response. Because data about the presence of the 33-mer in common wheat flours were not available, Kathrin Schalk developed a SIDA combined with targeted LC-MS/MS for the quantitative determination of the 33-mer peptide in different wheat species and cultivars.

As isotopically labelled internal standard, a [$^{13}C_{28}$]- and [$^{15}N_4$]-labelled 33-mer peptide with L-[$^{13}C_9$][^{15}N]-phenylalanine (*F) and L-[$^{13}C_5$][^{15}N]-proline (*P) was used (LQLQP*FPQPQLPYPQPQLPYPQPQLPYPQ*PQ*P*F).

The 33-mer content of 23 hexaploid modern and 15 old common wheat cultivars and two spelt cultivars were determined by Kathrin Schalk and her co-author. All flours contained the 33-mer peptide at levels ranging from 91–603 µg/g flour. Principal component analysis of the data demonstrated that the contents of 33-mer were not suitable to distinguish old and modern common wheat cultivars, because cultivars with high 33-mer contents were found within both flour sets.

Furthermore, the amount of 33-mer of two tetraploid durum wheat, emmer and diploid einkorn cultivars was determined, respectively. In each of these wheat species, the 33-mer was not detected (< LOD), which could be explained by the absence of D-genome, that encodes α2-gliadins.

Due to the presence of the 33-mer in all common wheat and spelt flours analysed here, the special focus in the literature on this highly immunodominant peptide appears to be justified.

Kathrin Schalk partly designed and performed the experiments, collected and evaluated the data, wrote the manuscript and revised it according to the comments of the reviewers.

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Quantitation of the immunodominant 33-mer peptide from α-gliadin in wheat flours by liquid chromatography tandem mass spectrometry

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Coeliac disease (CD) is triggered by the ingestion of gluten proteins from wheat, rye, and barley. The 33-mer peptide from α2-gliadin has frequently been described as the most important CD-immunogenic sequence within gluten. However, from more than 890 published amino acid sequences of α-gliadins, only 19 sequences contain the 33-mer. In order to make a precise assessment of the importance of the 33-mer, it is necessary to elucidate which wheat species and cultivars contain the peptide and at which concentrations. This paper presents the development of a stable isotope dilution assay followed by liquid chromatography tandem mass spectrometry to quantitate the 33-mer in flours of 23 hexaploid modern and 15 old common (bread) wheat as well as two spelt cultivars. All flours contained the 33-mer peptide at levels ranging from 91–603 μg/g flour. In contrast, the 33-mer was absent (<limit of detection) from tetra- and diploid species (durum wheat, emmer, einkorn), most likely because of the absence of the D-genome, which encodes α2-gliadins. Due to the presence of the 33-mer in all common wheat and spelt flours analysed here, the special focus in the literature on this most immunodominant peptide seems to be justified.

With a prevalence of about 1% in the Western population[1], coeliac disease (CD) is one of the most common food hypersensitivities. This inflammatory disorder of the upper small intestine results in villous atrophy and consequently malabsorption of nutrients as well as extra- and intraintestinal symptoms. In genetically predisposed individuals, the precipitating factor of CD is the intake of storage proteins from wheat (gliadins, glutenins), barley (hordeins), rye (secalins), and possibly oats (avenins), which are called gluten in the field of CD[2]. CD patients need to follow a strict lifelong gluten-free diet to ensure mucosal healing and prevent complications.

All gluten protein fractions, namely the alcohol-soluble prolamins and the insoluble glutelins, contain CD-active epitopes[3]. The prolamin fraction is particularly rich in proline and glutamine and the numerous proline residues lead to a high resistance to complete proteolytic digestion by human gastric, pancreatic, and brushborder enzymes. Studies by Shan *et al.* (2002) showed that a large 33-mer peptide (LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF) from α2-gliadin (position in the amino acid sequence of α2-gliadin: 56–88) is resistant to cleavage by intestinal peptidases[4,5]. The 33-mer is widely called the most immunodominant gluten peptide[4,6,7], because it contains three overlapping T-cell epitopes, namely PFPQPQLPY (DQ2.5-glia-α1a, one copy), PYPQPQLPY (DQ2.5-glia-α1b, two copies) and PQPQLPYPQ (DQ2.5-glia-α2, three copies)[3], which result in the initiation of a strong immune response. As such, it plays an important role in the field of CD, e.g., as a model peptide to study CD mechanisms[7–9] or the efficiency of gluten-degrading enzymes[10–12]. It consists of three heptamers (PQPQLPY) framed by a hexamer (PQPQPF) at the C-terminal end and a hexamer (LQLQPF) at the N-terminal end, which (positions 56–73) is also known to be CD-toxic from *in vivo* studies[9]. Arentz-Hansen *et al.* (2000) were the first to identify the 33-mer in α2-gliadin from the Norwegian common (bread) wheat (*Triticum aestivum*) cultivar (cv.) Mjølner (MJO). DNA-sequencing revealed the entire

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amino acid sequences of eleven α-gliadins (α1–α11) of this cultivar, but only α2-gliadin contained the 33-mer[13]. T-cell proliferation assays demonstrated that treatment of the 33-mer with tissue transglutaminase (TG2) resulted in a higher T-cell immune response after specific deamidation of the glutamine residues in positions 65 and 72[14,15], followed by strong binding to HLA-DQ2[15–17].

The high relevance of the 33-mer is reflected by the production of two monoclonal antibodies (A1 und G12) against the 33-mer peptide[18]. These are used in commercially available enzyme-linked immunosorbent assays (e.g., GlutenTox ELISA, Biomedal, Sevilla, Spain and AgraQuant® ELISA Gluten G12, Romer Labs, Tulln, Austria) for the immunochemical quantitation of gluten in supposedly gluten-free foods[19].

Due to its unique CD-epitope-rich structure, the 33-mer peptide plays an important role in the literature with 636 results for a search in the database ScienceDirect with "33 mer" and "celiac disease" as keywords (as of February 07, 2017). Although about 20 papers per year were published since 2000 and 53 for the year 2016, information about the quantities of 33-mer in different wheat species and cultivars is still missing. According to a BLAST search within 897 entries for α-gliadins from *Triticinae* in the UniProtKB database, the amino acid sequence of the 33-mer was found in only 16 protein sequences from *T. aestivum* and in three from *T. spelta* with an identity of 100% (as of February 07, 2017). Of these 19 sequences, only three have evidence at transcript level (Q9M4L6, Q1WA39 and A5JSA6) inferred from the three Chinese wheat cv. Gaocheng 8901, Zhongyou 9507, and Chinese Spring[20,21], but only one (P18573) has evidence at protein level based on data of the Norwegian wheat cv. MJO. Taken together, the available data are insufficient to judge whether or not the 33-mer occurs frequently in different wheat species and cultivars.

Therefore, the aim of the present study was to develop a stable isotope dilution assay (SIDA) combined with targeted liquid chromatography tandem mass spectrometry (LC-MS/MS) for the quantitative determination of the 33-mer. The amount of 33-mer was determined in 57 samples of different wheat species from around the world (Table 1), including hexaploid common wheat (*T. aestivum*) and spelt (*T. aestivum* ssp. *spelta*), tetraploid durum wheat (*T. turgidum durum*) and emmer (*T. turgidum dicoccum*), and diploid einkorn (*T. monococcum*) to make a precise assessment of the importance of this peptide associated with CD.

Results

Development of a SIDA. To develop a SIDA, a [$^{13}C_{28}$]- and [$^{15}N_4$]-labelled *33-mer peptide (LQLQP*FPQPQLPYPQPQLPYPQPQLPYPQ*PQ*P*F, with *F: L-[$^{13}C_9$][^{15}N]-phenylalanine and *P: L-[$^{13}C_5$][^{15}N]-proline, monoisotopic mass 3943.0) was used as isotopically labelled internal standard which differed by 32 mass units compared to the unlabelled analyte (33-mer, monoisotopic mass 3911.0). Based on the fragmentation pattern of the 33-mer (Fig. 1), the [^{13}C]/[^{15}N]-labelled amino acids were positioned in such a way that the label remained in the detected product ions. To define the most abundant transitions for multiple reaction monitoring (MRM), the 3+ and 4+ charge states of the 33-mer with *m/z* 1305.2 (3+) and *m/z* 979.0 (4+) and *33-mer standard with *m/z* 1316.0 (3+) and *m/z* 987.0 (4+) were totally fragmented. The 4+ charge state of the 33-mer was more abundant than the 3+ charge state state (ratio charge state (4+)/(3+) = 2/1) and, therefore, the most abundant MRM transition of the 4+ charged 33-mer analyte and isotopically labelled *33-mer standard was chosen for quantitation (quantifier for 33-mer *m/z* 979.0 → 263.3 and *33-mer *m/z* 987.0 → 279.2). The three MRM transitions (Table 2) following in intensity were used for qualification (qualifiers) as well as the MRM transitions of the 3+ charge state. The collision energy was optimised for each MRM transition to achieve the highest possible product ion intensity[22].

The Norwegian wheat cv. MJO was used as a positive control to develop a SIDA, because it is known to contain the 33-mer[13]. Two different approaches were taken: quantitation of the 33-mer directly in chymotryptically hydrolysed wheat flour and quantitation in hydrolysed gliadins which had been extracted from the flour. The results showed that it was not possible to quantitate the 33-mer directly in hydrolysed flour, because the peptide signal was overlaid by signals originating from the flour matrix. The 33-mer showed signals with high intensity in hydrolysed gliadins and interfering matrix effects were reduced, because only one protein fraction was taken for analysis instead of the entire wheat protein present in flour. To balance out the loss of analyte during sample preparation, the isotopically labelled standard was added prior to chymotryptic digestion of the gliadins.

Resistance of the 33-mer to enzymatic hydrolysis. Preliminary experiments using a combination of pepsin and trypsin/chymotrypsin (PTC) for enzymatic hydrolysis were performed with the 33-mer. This PTC hydrolysate of the 33-mer was analysed by untargeted LC-MS/MS followed by data evaluation using the MS/MS ions search module of the Mascot software based on the NCBI database (National Library of Medicine, Bethesda, MD, USA). In addition to the original 33-mer, the truncated forms after N-terminal removal of leucine resulting in a 32-mer (*m/z* 950.0, 4+ and *m/z* 1266.3, 3+) and pyro-32-mer (*m/z* 945.7, 4+ and *m/z* 1260.6, 3+) were identified, but no 30-mer (*m/z* 889.7, 4+ and *m/z* 1185.9, 3+) after potential removal of N-terminal LQL. The ratio 33-mer/32-mer/pyro-32-mer was about 12/76/12. To see whether cleavage of the N-terminal leucine could be minimised, only chymotrypsin was used for further experiments. Therefore, the 33-mer peptide was incubated with chymotrypsin using exactly the same conditions as described for the samples to check its resistance towards cleavage, because it contains two potential chymotryptic cleavage sites at positions L1 and L3. In this case, untargeted LC-MS/MS revealed no truncated forms. To ascertain this, full MS/MS scans looking only for the above *m/z* values were acquired and these showed no detectable amounts of 32-mer, pyro-32-mer or 30-mer, only the intact 33-mer. Corresponding experiments, specifically looking for the potential isotopically labelled *32-mer (*m/z* 954.0, 4+ and *m/z* 1271.3, 3+), pyro-*32-mer (*m/z* 949.7, 4+ and *m/z* 1265.6, 3+) and *30-mer (*m/z* 893.4, 4+ and *m/z* 1190.9, 3+), confirmed that the *33-mer standard was also stable under the conditions applied. Full MS/MS scans were also done for the chymotryptic hydrolysates of the albumin/globulin and gliadin fractions obtained from wheat cv. MJO. Again, only the intact 33-mer was detected in the gliadin hydrolysate, but no detectable traces of 33-mer in the albumin/globulin hydrolysate. These experiments confirmed that no truncated

Species / Cultivar	Abbreviation	Cultivation region	Harvest year	Source
Hexaploid common wheat				
Chara	CHA	Australia (Victoria)	2014	A
Frame	FRA	Australia (Victoria)	2014	A
Westonia	WES	Australia (Victoria)	2014	A
Wyalkatchem	WYA	Australia (Victoria)	2014	A
	WYW	Australia (West Australia)	2014	A
Yitpi	YIT	Australia (Victoria)	2014	A
Capo	CAP	Austria	2014	A
Mulan	MUL	Austria	2014	A
Carberry	CAY	Canada	2015	A
Cardale	CAR	Canada	2015	A
CDC Go Pen West Seeds	GPS	Canada	2014	A
CDC Go Sara Weigum	GSW	Canada	2014	A
CDC Go Wes Froese	GEF	Canada	2014	A
Glenlea	GLE	Canada	2012	A
Yumai-34	Y11	China	2011	A
	Y12		2012	
	Y14		2014	
Akteur	A13	Germany	2013	A
	A14		2014	
Dekan	D05	Germany	2005	A
	D13		2011	
Tommi	TOM	Germany	2013	A
Winnetou	WIN	Germany	2014	A
Ackermanns Brauner Dickkopf[b]	ABD	Germany	2015	C
Breustedts Extra Dickkopf[b]	BED	Germany	2015	C
Cimbals Großherzog von Sachsen[a]	CGS	Germany	2015	C
Dippes Strum Weizen[c]	DSW	Germany	2015	C
Erbachshofer Braun[c]	EBR	Germany	2015	C
Firlbeck I[c]	FIR	Germany	2015	C
Janetzkis Früher Kreuzung[b]	JFK	Germany	2015	C
Kraffts Siggerländer[b]	KSI	Germany	2015	C
Rimpaus Dickkopf[a]	RPD	Germany	2015	C
Rimpaus Früher Bastard[a]	RFB	Germany	2015	C
Ruppiner Brauner Landweizen[b]	RBL	Germany	2015	C
Steigers Leutewitzer Dickkopf[a]	SLD	Germany	2015	C
Strengs Marschall[c]	STM	Germany	2015	C
Strubes Dickkopf[a]	STD	Germany	2015	C
Walz Oberrheinperle[c]	WOP	Germany	2015	C
Hexaploid spelt				
Franckenkorn	FRK	Germany	2014	D
Oberkulmer	OBE	Germany	2014	D
Tetraploid durum wheat				
Auradur	AUR	Germany	2014	D
Wintergold	WIG	Germany	2014	D
Tetraploid emmer				
Osiris	OSI	Germany	2014	D
Ramses	RAM	Germany	2014	D
Diploid einkorn				
Tifi	TIF	Germany	2014	D
Terzino	TER	Germany	2014	D
Hexaploid common wheat				
Mv Magvas	M11	Hungary	2011	A
	M12		2012	
	M14		2014	

Continued

Species / Cultivar	Abbreviation	Cultivation region	Harvest year	Source
Mv Mazurka	Z11	Hungary	2011	A
	Z12		2012	
	Z14		2014	
Mv Verbunkos	V11	Hungary	2011	A
	V12		2012	
	V14		2014	
Mjølner	MJO	Norway	2012	B
Bezostaja-1	BEZ	Russia	2012	A

Table 1. Overview of all 57 samples of modern and old common wheat, spelt, durum wheat, emmer, and einkorn cultivars, their abbreviations, cultivation regions, harvest years, and sources. [a]Year of first registration: 1891–1900; [b]year of first registration: 1901–1910; [c]year of first registration: 1941–1950; A: MoniQA Association (Monitoring and Quality Assurance in the Total Food Supply Chain, Neutal, Austria); B: kindly provided by Anette Moldestad (Nofima, Ås, Norway); C: kindly provided by Andreas Börner (Leibniz Institute of Plant Genetics and Crop Plant Research, Resources Genetics and Reproduction, Gatersleben, Germany); D: kindly provided by Friedrich Longin (University of Hohenheim, LSA-Research Group Wheat, Stuttgart, Germany).

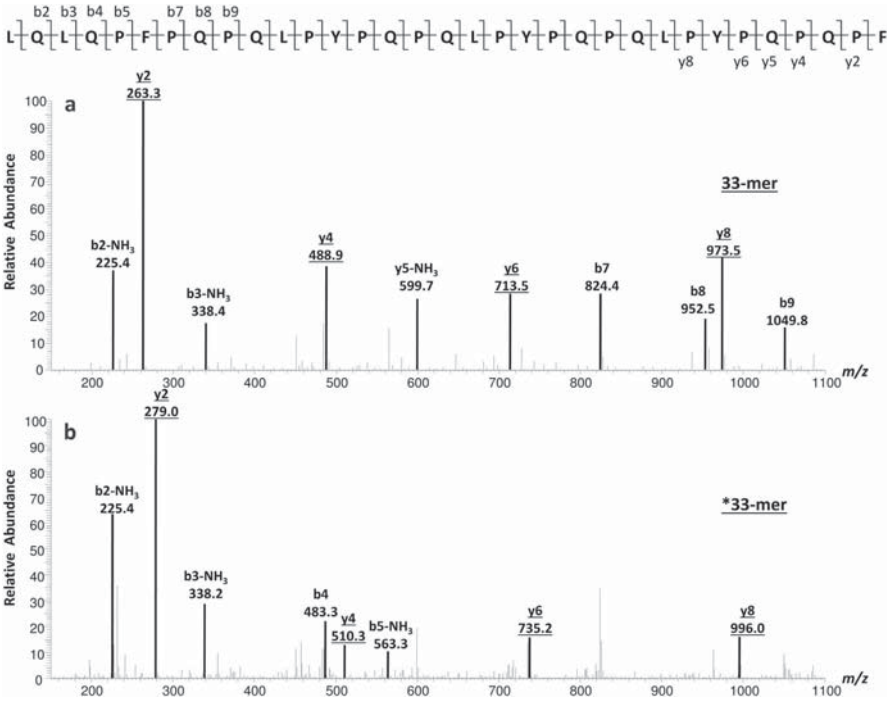

Figure 1. MS/MS product ion mass spectra of the 33-mer peptide (**a**) and the isotopically labelled *33-mer (**b**). The four most abundant product ions (underlined) were used for identification. The most abundant product ion (y2) was used for quantitation.

forms of the 33-mer were generated during chymotryptic hydrolysis using the applied conditions. This is consistent with the original report by Shan *et al.*[4] who only observed pepsin-catalysed cleavage of the N-terminal leucine. Additionally, no analyte loss is expected to take place during sample preparation, which involves the removal of albumins/globulins.

Calibration and quantitation. The response factor of the 33-mer peptide was determined using the peak area ratio A (*33-mer)/A (33-mer) at different values of n (*33-mer)/n (33-mer) between 0.02 and 9.2, that lay within the linear range. Quantitative ^{1}H nuclear magnetic resonance spectroscopy (^{1}H qNMR) was used to determine the exact concentrations of the methanolic solutions of the 33-mer (1.90 μmol/mL) and the *33-mer standard (1.81 μmol/mL). The characteristic signals located in the aromatic field (δ/ppm: 6.5–8) of the two phenylalanine residues (5 protons each) and the three tyrosine residues (4 protons each) were integrated (22 protons in total) and compared to a reference solution containing L-tyrosine[23]. The area ratio A (*33-mer)/A (33-mer)

Peptide	Precursor ions *m/z* (charge state)	Product ions[1] *m/z*	Collision energy (V)	Retention time (min)
33-mer	979.0 (4+)[2] 1305.2 (3+)[3]	263.3 (y2)[2]	14	19.0
		488.9 (y4)[3]	26	
		713.5 (y6)[3]	14	
		973.5 (y8)[3]	12	
*33-mer	987.0 (4+)[2] 1316.0 (3+)[3]	279.0 (y2)[2]	14	19.0
		510.3 (y4)[3]	26	
		735.2 (y6)[3]	14	
		996.0 (y8)[3]	12	

Table 2. Multiple reaction monitoring (MRM) parameters of the 33-mer and the isotopically labelled *33-mer peptides. [1]Charge state: 1+. [2]Precursor to product ion transitions were used as quantifier. [3]Precursor to product ion transitions were used as qualifier.

was obtained from the MS analysis of the MRM transitions *m/z* 987.0 → 279.2 (*33-mer) and *m/z* 979.0 → 263.3 (33-mer). The response factor determined from the slope of the regression line was 0.999. As expected from SIDA, it was very close to 1.0, because analyte and isotopically labelled standard demonstrated the same chemical properties and ionisation behaviour[24]. Quantitation of the 33-mer in all flours was based on this response factor.

Optimisation of sample preparation. Studies by Fiedler *et al.*[25] demonstrated that protein digestion was improved with reduction/alkylation in the first step followed by digestion in the second step, because this approach resulted in a higher number of identified peptides. Therefore, reduction of disulphide bonds with tris-(2-carboxyethyl)phosphine (TCEP) followed by iodoacetamide (IDAM) alkylation of liberated free cysteine residues of the gliadin fraction from wheat cv. Akteur (A13) were performed to see if the quantitated amount of 33-mer was influenced compared to native hydrolysed gliadins. After reduction and alkylation, the amount of 33-mer was 6.3 ± 0.4 mg/g gliadin compared to 6.2 ± 0.4 mg/g of native gliadin. The values showed no significant difference ($p = 0.705$). α-Gliadins typically contain six cysteine residues and form three intrachain disulphide bonds located in the C-terminal domain consisting of sections III, IV, and V[26]. The 33-mer is located within section I and represents a part of the N-terminal domain[27]. After cleavage of disulphide bonds following reduction, the N-terminal domain containing the 33-mer was apparently not affected regarding accessibility to enzymatic attack, which resulted in no significant change of 33-mer contents. To simplify sample preparation, the reduction/alkylation step was omitted.

After chymotryptic hydrolysis of the gliadins extracted from wheat cv. A13, the obtained peptide mixture was purified by solid phase extraction (SPE) using C_{18}-cartridges in order to reduce matrix effects. The 33-mer content was 6.3 ± 0.3 mg/g gliadin after purification in comparison to 6.2 ± 0.4 mg/g gliadin without purification of the hydrolysate. The quantitative values were not significantly different ($p = 0.506$). The only impact was a higher signal intensity (by a factor of 2) of both 33-mer and *33-mer after SPE purification, which did not influence the quantitated amount of 33-mer, because the ratio of analyte to standard did not change. Having ascertained that purification did not influence the content of the 33-mer, the gliadin hydrolysates were analysed by targeted LC-MS/MS without SPE to speed up sample preparation.

Limit of detection (LOD) and limit of quantitation (LOQ). The LOD and LOQ of the MS method to quantitate the 33-mer were determined according to Vogelgesang and Haedrich[28]. The analyte was spiked in seven different concentrations between 0.1 and 200 μg/g to rye prolamins as matrix, which did not contain the 33-mer peptide. The absence of the 33-mer in rye flour (cv. Visello) had been confirmed by LC-MS/MS of the hydrolysed flour and prolamin fraction. The 33-mer was identified with high sensitivity resulting in an LOD of 13.1 μg/g rye flour and an LOQ of 47.0 μg/g rye flour.

Analysis of the 33-mer content in different common wheat and spelt cultivars. The quantitative determination of the 33-mer was performed in flours of 23 hexaploid modern and 15 old common wheat cultivars from different harvest years and two spelt cultivars harvested in 2014 (Table 1). In this context, old common wheat is defined as a cultivar from *T. aestivum* with its year of first registration prior to 1950. All flours were characterised including determination of crude protein contents according to ICC Standard No. 167[29] and quantitation of α-gliadins, gliadins and glutenins after modified Osborne fractionation combined with RP-HPLC as reported by Wieser *et al.*[30]. Total gluten contents were calculated as sum of gliadin and glutenin contents (see Supplementary Table S1).

The 33-mer was present in all common wheat and spelt flours in a range from 90.9 to 602.6 μg/g of flour (Fig. 2a). The modern wheat Y14 had the highest amount of 33-mer (602.6 μg/g flour), that was significantly different to all analysed cultivars with the exception of Z14 (see Supplementary Table S2). The old wheat ABD with the lowest 33-mer content of 90.9 μg/g flour differed significantly to all wheat and spelt cultivars. In contrast, the old wheat SLD (528.0 μg/g flour) contained one of the highest 33-mer amounts of the analysed flours and showed no significant difference to the modern wheats A13, A14, D05, Z14, V12, and MJO and spelt OBE. Special attention was directed to MJO, because the 33-mer was first identified in this cultivar[13]. The content of 33-mer in MJO (515.0 μg/g flour) showed no significant difference to A13, A14, CAP, D05, GLE, Z12, Z14, V12, and OBE. Most of the modern and old wheat flours contained the 33-mer in a range of 200–400 μg/g flour with an overall average

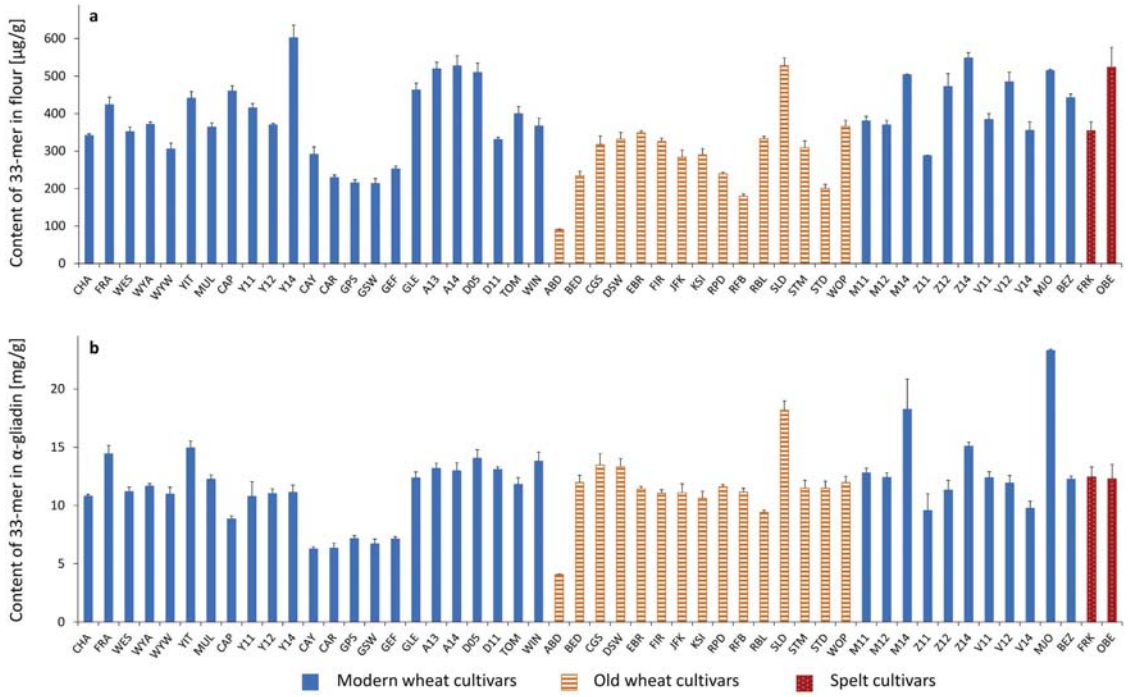

Figure 2. Contents of 33-mer based on flour [µg/g] (**a**) and based on α-gliadins [mg/g] (**b**). 23 modern and 15 old common wheat cultivars (49 samples in total due to multiple harvest years (see Table 1)) and two spelt cultivars were analysed. Wheat cultivars registered prior to 1950 were designated as old. For abbreviations of the cultivars, see Table 1.

of 368 ± 109 µg/g flour. As a result, only some differences in 33-mer contents between these wheat cultivars were significant. A certain trend, e.g., that modern wheat cultivars generally contain higher amounts of 33-mer than old cultivars could not be derived from the data. Considering the amounts of 33-mer in the two spelt cultivars, it was noticeable that OBE contained one of the highest amounts of the 33-mer peptide (523.4 µg/g flour). The content of 33-mer in FRK (353.9 µg/g flour) was in the range of 200–400 µg/g flour, and did not differ significantly from the common wheat cultivars.

The 33-mer contents of all analysed flours were also calculated based on the amount of α-gliadins (Fig. 2b) determined after modified Osborne fractionation by RP-HPLC[30]. MJO had the highest content of 33-mer in α-gliadin (23.2 mg/g α-gliadin). It was significantly different to all other cultivars (see Supplementary Table S3) and was caused by the high 33-mer content and the low amount of α-gliadins (2.2%) in flour. SLD and M14 had similar 33-mer contents (17–18 mg/g α-gliadin) and differed significantly to all other varieties. ABD had the lowest amount of 33-mer in α-gliadin (4.1 mg/g α-gliadin) and did not show significant differences to CAY, CAR, GPS, GSW, and GEF, but differed statistically to the other cultivars. The overall average content was 11.7 ± 3.1 mg/g α-gliadin.

Many studies in the literature have focused on peptide quantitation in enzymatically hydrolysed prolamin extracts[25,31], hydrolysed gluten extracts[32] or hydrolysed wheat flours[22], but the putative immunodominant 33-mer was not quantified. Because of the missing data for 33-mer contents, it was difficult to compare the peptide contents to existing data. Only studies by van den Broeck *et al.* reported the quantitation of the 33-mer using LC-MS with external calibration, but not SIDA. Peptide concentrations were converted into the corresponding contents of α-gliadin per microgram digested gluten protein extract using the average mass of 32,285.5 of α-gliadins. The 33-mer contents determined for two wheat cultivars corresponded to 10.3 and 5.8 mg/g of α-gliadin[33], which agreed well with the data in Fig. 2b.

Correlations and principal component analysis. The 33-mer contents of the 51 modern and old common wheat and spelt cultivars (based on flour) were correlated to the contents of α-gliadin, total gliadin and total gluten analysed by RP-HPLC after modified Osborne fractionation and to crude protein contents (see Supplementary Table S1). A weak correlation ($r = 0.568$, $p < 0.001$) was observed between 33-mer and α-gliadin contents, but there was no correlation to gliadin contents ($r = 0.469$, $p < 0.001$), gluten contents ($r = 0.526$, $p < 0.001$) or crude protein contents ($r = 0.481$, $p < 0.001$) (Fig. 3).

Principal component analysis (PCA) with 33-mer, α-gliadin, gliadin, gluten, and crude protein contents of the 49 common wheat and 2 spelt flours was performed to assess whether these variables could be used to differentiate between spelt, modern common wheat, and old common wheat cultivars (Fig. 4). Both principal components together accounted for 94.9% of data variability. Component 1 was positively correlated with 33-mer

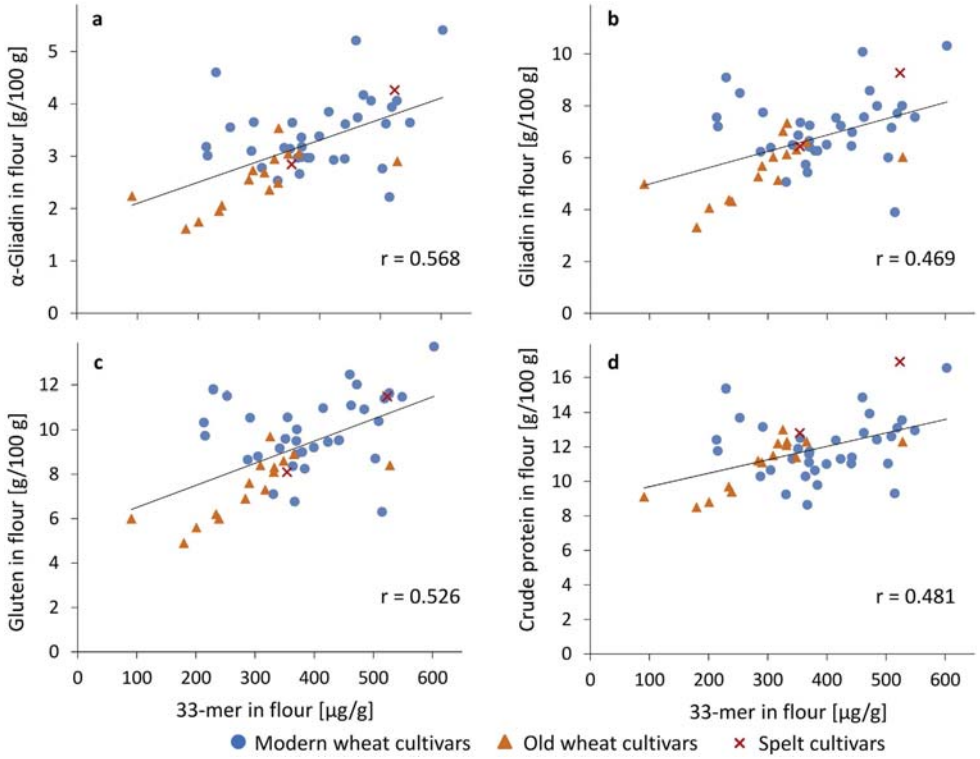

Figure 3. Linear Pearson correlations between contents of 33-mer and contents of α-gliadin (**a**), gliadin (**b**), gluten (**c**), and crude protein (**d**). 23 modern and 15 old common wheat (49 samples in total due to multiple harvest years (see Table 1)) and two spelt cultivars were analysed. Wheat cultivars registered prior to 1950 were designated as old.

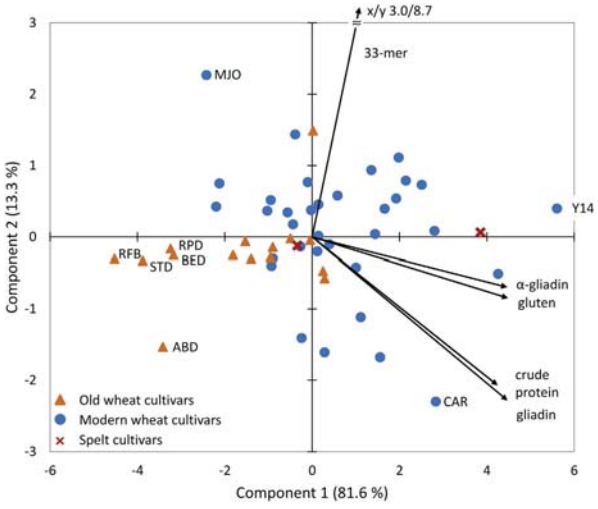

Figure 4. Principal component analysis biplot of data for 33-mer, α-gliadin, gliadin, gluten, and crude protein contents. 23 modern and 15 old common wheat (49 samples in total due to multiple harvest years (see Table 1)) and two spelt cultivars were analysed. Wheat cultivars registered prior to 1950 were designated as old.

contents ($r = 0.643$), but even more so with α-gliadin, gliadin, gluten, and crude protein contents ($r \geq 0.920$). In contrast, component 2 was only positively correlated with 33-mer contents ($r = 0.765$), but negatively associated

with α-gliadin, gliadin, gluten, and crude protein contents ($r \leq -0.061$). The vector indicating the contribution of the content of 33-mer was downsized for visibility reasons, but it pointed to 3.0/8.7 as x- and y-coordinates. The contents of α-gliadin, gliadin, gluten, and crude protein all had strong positive correlations in all possible pairwise combinations ($p \geq 0.850$). PCA essentially confirmed the results of the correlation analyses that had already shown the 33-mer contents to be mostly unrelated to α-gliadin, gliadin, gluten, and crude protein contents. Cv. MJO was placed in the top left corner, because of its high content of 33-mer, but comparatively low contents of gluten proteins, especially α-gliadins. In comparison, cv. Y14 appeared on the far right, because of high contents of all five variables whereas cv. CAR was located in the bottom right corner, because of high (gluten) protein contents, but a comparatively low 33-mer content (229.4 μg/g flour). In addition, PCA revealed that these five variables were unsuitable to differentiate between spelt, modern common wheat, and old common wheat cultivars. The five old common wheat cv. ABD, BED, RFB, RPD, and STD were placed on the far left, but the other ten old cultivars were located right in the middle at similar coordinates as the modern common wheat cultivars. The two spelt cv. FRK and OBE were situated next to the common wheat cultivars. Therefore, the hypothesis that spelt may be less CD-immunoreactive than modern common wheat cultivars could not be confirmed. This finding is in accordance with Ribeiro *et al.*[34] who compared modern common wheat to spelt cultivars and showed that spelt cultivars had a higher amount of toxic epitopes than common wheats.

Influence of harvest year and cultivar on the contents of 33-mer. To see whether harvest year or cultivar had a greater influence on 33-mer contents, four wheat cultivars (Mv Magvas, Mv Mazurka, Mv Verbunkos, and Yumai-34) grown at the same location in Hungary (Martonvásár) and harvested in three years (2011, 2012, and 2014)[35] were studied. The harvest year significantly influenced the 33-mer contents ($p < 0.001$), whereas the cultivars did not ($p = 0.391$). There were no significant differences in 33-mer contents between the four cultivars within the harvest year 2011, two out of six differences (V12 vs. M12 and V12 vs. Y12) were significant ($p < 0.05$) within the harvest year 2012, and three out of six (V14 vs. Z14, V14 vs. Y14, and V14 vs. M14) within the harvest year 2014. Apparently, the environmental factor had a greater influence on 33-mer contents than the genetic background of the four wheat cultivars, because the results for each combination of harvest years (2011 vs. 2012, 2011 vs. 2014 and 2012 vs. 2014) were significantly different ($p \leq 0.034$).

Analysis of durum wheat, emmer and einkorn. The 33-mer peptide was also analysed in two durum wheat and two emmer cultivars (genome AABB) as well as two diploid einkorn cultivars (genome AA) (Table 1). In each of these wheat species, the 33-mer was not detected (<LOD). In comparison to hexaploid common wheat, durum wheat, emmer, and einkorn do not contain the D-genome, which originated from hybridisation of *T. turgidum dicoccum* (genome AABB) with *Aegilops tauschii* (genome DD)[36]. The absence of the 33-mer peptide can be explained by the fact that this peptide is encoded by genes located in the Gli-2 locus on chromosome 6D, which is missing in durum wheat, emmer, and einkorn. Studies by Molberg *et al.* showed clear variations in intestinal T-cell responses between common wheat and tetra- or diploid species due to different degrees of T-cell immunoreactivity between the gluten proteins encoded on the A-, B-, and D-genome. Einkorn cultivars were only recognized by DQ2.5-glia-α1a-specific T-cell clones, but not by DQ2.5-glia-α1b- and DQ2.5-glia-α2-specific T-cell clones. Emmer and durum wheat cultivars were all recognized by DQ2.5-glia-α1a-specific T-cell clones, but only two out of four emmer cultivars and three out of ten durum wheat cultivars activated DQ2.5-glia-α1b- and DQ2.5-glia-α2-specific T-cell clones[37]. Consistent with our results, Prandi *et al.*[38] found that the 33-mer was not present in durum wheat. As a consequence, this peptide was used as a marker peptide to identify the presence of common wheat in durum wheat flours. One durum wheat cultivar was also analysed by van den Broeck *et al.*[33] and the 33-mer peptide was not detected either.

Discussion

The present study is the first to establish a SIDA combined with targeted LC-MS/MS for the quantitative determination of the immunodominant 33-mer peptide in wheat flours. Due to the use of a stable-isotope-labelled *33-mer standard, sample preparation could be simplified without reduction/alkylation and SPE purification.

Although the UniProtKB database had only 19 out of 897 entries for α-gliadin sequences from *Triticinae* containing the 33-mer with an identity of 100%, all 40 analysed modern and old common wheat and spelt cultivars contained the immunodominat 33-mer peptide (51 flour samples in total, because several flours were available from different harvest years). The focus on this peptide seems to be legitimated not only because of its unique structure containing six copies of three overlapping T-cell epitopes, but also because of its presence in all hexaploid wheat cultivars analysed in this study. PCA analysis of the data demonstrated that the contents of 33-mer were not suitable to differentiate old from modern common wheat cultivars, because cultivars with high 33-mer contents were found within both flour sets. The 33-mer was not detected in two cultivars each of tetraploid emmer and durum wheat as well as diploid einkorn, which do not contain the D-genome. This observation may be explained by the fact that the 33-mer is encoded on the Gli-2 locus on chromosome 6D, but a larger set of durum wheat, emmer and einkorn cultivars would have to be analysed to conclude whether these wheat species generally lack the 33-mer peptide. Further work will focus on correlating the content of 33-mer analysed by LC-MS/MS with the gluten content determined by ELISA using the G12 monoclonal antibody.

Materials and Methods

Chemicals. The quality of all chemicals was of analytical grade, unless stated otherwise. Disodium hydrogen phosphate dihydrate, ethanol, formic acid (FA; 98–100%), hydrochloric acid (32%, w/w), pentane, 1-propanol, potassium dihydrogen phosphate, sodium chloride, tris(hydroxymethyl)-aminomethane (TRIS), and urea were purchased from Merck (Darmstadt, Germany). IDAM was from Applichem (Darmstadt, Germany). α-Chymotrypsin (from bovine pancreas, TLCK-treated, ≥40 U/mg protein), pepsin (from porcine gastric

mucosa, 3200–4500 U/mg protein), TCEP, trifluoroacetic acid (TFA; 99%), and trypsin (from bovine pancreas, TPCK-treated, ≥10 000 BAEE U/mg protein) were obtained from Sigma-Aldrich (Steinheim, Germany), and deuterated methanol-d_4 containing tetramethylsilane (TMS) was from Euriso-Top (Gif sur Yvette Cedex, France). The peptide LQLQPFPQPQLPYPQPQLPYPQPQPLPYPQPQPF (33-mer) and the isotopically labelled peptide LQLQP*FPQPQLPYPQPQLPYPQPQLPYPQ*PQ*P*F (*33-mer) with *P: L-[$^{13}C_5$][^{15}N]-proline and *F: L-[$^{13}C_9$][^{15}N]-phenylalanine, were purchased from Genscript (Hongkong, PR China) with a purity of >90%. Water for HPLC was purified using an Arium 611VF water purification system (Sartorius, Goettingen, Germany).

Grain samples. Grains of 23 modern and 15 old (year of first registration before 1950) common wheat cultivars from different harvest years grown worldwide, and one rye cultivar (cv. Visello, harvested 2013, donated by KWS Lochow, Bergen, Germany) were either obtained as flours or milled on a Quadrumat Junior mill (Brabender, Duisburg, Germany) and sieved to a particle size of 0.2 mm. Two spelt, two durum wheat, two emmer, and two einkorn cultivars, grown in Germany (harvested 2014) were milled on a Laboratory 3100 cross beater mill (Perten Instruments, Hamburg, Germany) to wholemeal flours. In total, 57 samples were analysed, because some modern wheat cultivars were available from two to three different harvest years or two cultivation regions (Table 1).

Characterization of the flours. *Crude protein content.* The crude protein content (nitrogen content × 5.7) of the flours was determined by the Dumas combustion method according to ICC Standard Method 167[29] using a TruSpec Nitrogen Analyzer (Leco, Kirchheim, Germany).

Qualitative and quantitative composition of flour proteins: Osborne fractionation. Flours (100 mg) were extracted with a buffered salt solution (2 × 1.0 mL 0.067 mol/L K_2HPO_4/KH_2PO_4-buffer, 0.4 mol/L NaCl, pH = 7.6) at 22 °C (room temperature) to obtain albumins and globulins (ALGL). The residues were extracted with ethanol (60%, v/v; 3 × 0.5 mL) at 22 °C (gliadins, GLIA) followed by the glutenin extraction solvent (2 × 1 mL; 50% (v/v) 1-propanol, 0.1 mol/L TRIS-HCl, pH 7.5, 0.06 mol/l (w/v) dithiothreitol) at 60 °C under nitrogen (glutenins, GLUT). After addition of the respective solvent, each flour suspension was vortexed for 2 min and stirred for 10 min (ALGL, GLIA) or 30 min (GLUT). The suspensions were centrifuged for 20 min at 3550 *g* and 22 °C. The corresponding supernatants were combined, diluted to 2 mL with the respective extraction solvent and filtered (WhatmanTM, Spartan 13/0.45 RC, GE Healthcare)[30].

Reversed-phase high-performance liquid chromatography (RP-HPLC). An UltiMate 3000 HPLC system (Dionex, Idstein, Germany) was used to analyse the extracted ALGL, GLIA, and GLUT fractions. Protein separation was carried out using an Acclaim™ 300 C_{18} column (2.1 × 150 mm, 3 μm, 30 nm, Thermo Fisher Scientific, Braunschweig, Germany). The following conditions were set: solvent A, TFA in water (0.1%, v/v) solvent B, TFA in acetonitrile (0.1%, v/v); linear gradient for ALGL: 0 min 0% B, 0.5 min 20% B, 7 min 60% B, 7.1–11 min 90% B, 11.1–17 min 0% B; linear gradient for GLIA and GLUT: 0 min 0% B, 0.5 min 24% B, 20 min 56% B, 20.1–24.1 min 90% B, 24.2–30 min 0% B; flow rate 0.2 mL/min; temperature, 60 °C; injection volume, 10 μL (GLIA), 20 μL (ALGL, GLUT); detection, UV absorbance at 210 nm. The absorbance areas of PWG-gliadin[39] (11.6–46.5 μg) were used as calibration standard to calculate the ALGL, GLIA, and GLUT contents of the extracts. The amounts of α-gliadins were calculated from the absorbance area of α-gliadins (retention time 13.5–17.3 min) relative to the total absorbance area of the gliadin fraction. All determinations were done in triplicates.

^{1}H qNMR. ^{1}H qNMR was carried out at 25 °C using a Bruker AV III system (Bruker, Rheinstetten, Germany), equipped with a Z-gradient 5 mm multinuclear observe probe and operated at a frequency of 400.13 MHz. The two peptides (33-mer and *33-mer) were dissolved in methanol-d_4 and 600 μL of each peptide solution were analysed in 5 × 178 mm NMR tubes (USC tubes, Bruker, Faellanden, Switzerland). The concentrations of the peptide solutions were determined according to Frank *et al.*[23] using L-tyrosine as reference standard. The signals in the aromatic field were used for integration. ^{1}H NMR, δ/ppm (TMS): 6.5–8 (m, 22 H).

Sample preparation. First, flours (150–200 mg) were defatted with pentane/ethanol (95/5, v/v; 2 × 2.0 mL)[40]. The gliadin fractions from the different defatted flours were extracted as described above[30]. The gliadin fractions were dried by centrifuging under reduced pressure (40 °C, 6 h, 800 Pa) and re-suspended in a TRIS-HCl-buffer (2.0 mL, 0.1 mol/L TRIS-HCl, pH 7.8, urea 120 mg/mL). The labelled *33-mer peptide was added (300 μL; 10 μg/mL) and the protein-peptide-mixture was hydrolysed with α-chymotrypsin (enzyme-to-protein (E:P) ratio of 1:200) for 24 h at 37 °C. To stop the digestion, TFA (5 μL) was added[41]. The obtained peptide mixture was dried using a vacuum centrifuge (40 °C, 6 h, 800 Pa), re-dissolved in FA (0.1%, v/v, 500 μL), filtered (0.45 μm) and analysed by targeted LC-MS/MS. The same hydrolysis procedure was applied to the 33-mer peptide (10 μg/ml), the *33-mer standard (10 μg/ml) and the albumin/globulin and gliadin fractions of wheat cv. MJO, which were all analysed by untargeted LC-MS/MS. Preliminary experiments were also done with the 33-mer peptide using pepsin (in 0.01 mol/L HCl, pH 2.0, 60 min, 37 °C, E:P ratio of 1:25) followed by trypsin and α-chymotrypsin (in 0.05 mol/L phosphate buffer, pH 6.5, 120 min, 37 °C, E:P ratio of 1:200) according to Dorum *et al.*[8]

Optimisation of sample preparation. *Reduction of disulphide bonds and alkylation of cysteine residues.* Reduction and alkylation were performed according to Rombouts *et al.*[41]. The extracted gliadin fraction (cv. A13) was re-suspended in a TRIS-HCl-buffer (1600 μL, 0.5 mol/L, pH 8.5) diluted to 50% (v/v) 1-propanol and the reducing agent (TCEP, 0.05 mol/L, 40 μL) was added, followed by incubation for 30 min at 60 °C under nitrogen atmosphere. For alkylation, IDAM was added (0.5 mol/L, 60 μL) and the suspension was incubated for 30 min at 60 °C in the dark. The solution was dried by a vacuum centrifuge (40 °C, 6 h, 800 Pa), the *33-mer was

added and the reduced and alkylated gliadins were hydrolysed with α-chymotrypsin and analysed by targeted LC-MS/MS accordingly.

Purification of peptides. After hydrolysis, the peptide mixtures were purified by solid phase extraction (SPE) on Supelco DSC-C_{18} tubes (Supelco, Steinheim, Germany). The C_{18}-cartridges were conditioned with methanol (1 mL), and equilibrated with TFA (0.1%, v/v, 1 mL). After loading the peptide mixtures, the cartridges were washed with water containing TFA (0.1%, v/v, 5 × 1 mL), and the peptides were eluted with methanol (2 mL). The peptide solution was dried by a vacuum centrifuge (40 °C, 6 h, 800 Pa) and analysed by targeted LC-MS/MS.

Untargeted LC-MS/MS. To confirm the resistance of the 33-mer and *33-mer towards chymotryptic cleavage, untargeted LC-MS/MS using an HCTultra PTM ion trap MS (Bruker Daltonics, Bremen, Germany) with collision-induced dissociation (CID) was performed as described in detail by Scherf *et al.*[42]. The untargeted approach was done with standard enhanced scan and auto-MS(n) settings. Additionally, full MS/MS scans of the following precursors were acquired (*m/z* range: target mass ± 1): 32-mer (*m/z* 950.0, 4+, *m/z* 1266.3, 3+), pyro-32-mer (*m/z* 945.7, 4+, *m/z* 1260.6, 3+), 30-mer (*m/z* 889.7, 4+ and *m/z* 1185.9, 3+), *32-mer (*m/z* 954.0, 4+, *m/z* 1271.3, 3+), pyro-*32-mer (*m/z* 949.7, 4+, *m/z* 1265.6, 3+) and *30-mer (*m/z* 893.4, 4+, *m/z* 1190.9, 3+).

Targeted LC-MS/MS. A triple-stage quadrupole mass spectrometer (TSQ Vantage, Thermo Fisher Scientific, Dreieich, Germany) was used. The ion source was operated in the ESI positive mode and the following source parameters were set: spray voltage, 4500 V; vaporizer temperature, 50 °C; sheath gas pressure, 40 arbitrary units (au); aux gas pressure, 5 au; capillary temperature, 300 °C. The mass spectrometer was operated in the MRM mode. The most abundant MRM transition was used as quantifier, and the three MRM transitions following in abundance were used as qualifiers. A declustering voltage of −10 V was set for all transitions. The transitions from the precursor ions of the 33-mer and *33-mer to the respective product ions (y-fragments) and the optimised collision energies are shown in Table 2. The 33-mer and the isotopically labelled *33-mer peptides were dissolved in FA (0.1%, v/v, 10 μg/mL). These two stock solutions were mixed in molar ratios n (*33-mer)/n (33-mer) between 9.2 and 0.02 (1 + 9, 1 + 4, 1 + 3, 1 + 1, 3 + 1, 4 + 1, 9 + 1, 14 + 1, 19 + 1, 29 + 1, and 39 + 1) for calibration.

For HPLC separation, an UltiMate 3000 HPLC system (Dionex, Idstein, Germany) was coupled to the mass spectrometer. An XBridge Peptide 3.5 μm BEH-C_{18} column (1.0 × 150 mm, 13 nm; Waters, Eschborn, Germany) was used for peptide seperation. The LC conditions were set as follows: solvent A, FA (0.1%, v/v) in water, solvent B, FA (0.1%, v/v) in acetonitrile; gradient 0–5 min isocratic 5% B, 5–22 min linear 5–55% B, 25–30 min isocratic 90% B; 30–35 min linear 90–5% B, 35–45 min isocratic 5% B, flow rate, 0.1 mL/min; injection volume, 10 μL, column temperature, 22 °C.

LOD and LOQ of the MS method. The LOD and LOQ of the quantitation method for the 33-mer peptide were determined. Rye flour (cv. Visello, harvest year 2013) was used as blank, because it was very similar to wheat regarding the gluten protein fractions, but did not contain α-gliadins. The prolamin extraction procedure and chymotryptic hydrolysis were performed as described above. To determine the LOD and LOQ of the targeted LC-MS/MS method, the prolamin extract was spiked at 7 different concentrations (0.1–200 mg/kg) of 33-mer peptide and the samples were hydrolysed by α-chymotrypsin followed by targeted LC-MS/MS analysis. The LOD and LOQ were derived statistically from the data[28]. The LOD was calculated based on a signal-to noise-ratio (S/N) of 3, and the LOQ on an S/N of 10.

Statistics. Statistically significant differences between 33-mer contents of different modern and old wheat cultivars and two spelt cultivars were determined by one-way analysis of variance (ANOVA) with Tukey's test as all pairwise multiple comparison procedure at a significance level of $p < 0.05$ using SigmaPlot 12.0 (Systat Software, San José, CA, USA). The significance of differences between 33-mer contents of the cv. Mv Magvas, Mv Mazurka, Mv Verbunkos, and Yumai-34 harvested in 2011, 2012, and 2014 were analysed by two-way ANOVA accordingly with harvest year and cultivar as factors. Pearson's product moment correlations were calculated between contents of 33-mer and α-gliadins, gliadins, gluten or crude protein for all analysed wheat and spelt cultivars. Correlation coefficients (r) were defined according to Thanhaeuser *et al.*[43] ($r > 0.78$, strong correlation; 0.67–0.78, medium correlation; 0.54–0.66, weak correlation; $r < 0.54$, no correlation). PCA was carried out with XLStat 2016 (Addinsoft, New York, NY, USA) to determine if the contents of 33-mer, α-gliadin, gliadin, gluten, and crude protein could be used to differentiate between spelt, modern common wheat, and old common wheat cultivars.

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Author Contributions

K.S. and C.L. performed the experiments and analyzed the data. K.S. wrote the main manuscript text and prepared the tables and figures. K.A.S. assisted in data analysis and manuscript preparation. K.A.S., H.W., and P.K. contributed reagents, materials, equipment, knowledge and help in conceiving the experiments. All authors reviewed the final manuscript.

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4 General discussion

The aims of the study were divided into three parts, which were achieved one by one: 1) the isolation and in-depth characterization of gluten reference proteins from wheat, barley, rye and oats for use as reference materials for LC-MS/MS; 2) the quantitation of 16 wheat, 7 barley, 3 oat and 7 rye marker peptides in the well-characterized reference proteins to enable the quantitative determination of gluten contents by LC-MS/MS; 3) the quantitative determination of the immunodominant 33-mer peptide from α-gliadin in different wheat flours by SIDA based on targeted LC-MS/MS.

Isolation and characterization of gluten protein types from wheat, rye, barley and oats for use as reference materials for LC-MS/MS

In the first part of the study, a strategy to isolate gluten protein fractions and types was developed. Furthermore, an intenisve analytical characterization was carried out to obtain well-characterized reference proteins. Defatted flours of mixtures of four cultivars each were used to account for the genetic variability between different cultivars. Prolamins and glutelins were isolated from flours according to Wieser et al. [1998]. By means of preparative RP-HPLC with UV detection, the different protein types were isolated from the protein fractions according to their retention times. Wheat gliadins were separated into ω5-, ω1,2-, α- and γ-gliadins, barley prolamins into C- and γ/B-hordeins and rye prolamins into ω-, γ-75k- and γ-40k-secalins. Oat avenins were not further separated. Wheat glutenins were subdivided into HMW- and LMW-GS, barley glutelins into D- and γ/B-hordeins and rye glutelins into HMW-secalins.

The isolated gluten protein types were characterized by five different analytical methods using analytical RP-HPLC, SDS-PAGE, N-terminal sequencing, LC-ESI-QTOF-MS and untargeted LC-MS/MS of chymotryptic gluten protein type hydrolysates.

The crude protein contents of each gluten protein type ranged from 74.3 ± 3.7 (HMW-secalins) to 100.8 ± 1.1% (ω1,2-gliadins) and demonstrated that the RP-HPLC isolation procedure yielded gluten protein types with high protein contents. Re-chromatography by analytical RP-HPLC confirmed the identities and purities of each gluten protein type in comparison to the chromatograms of the respective prolamin

and glutelin fraction. Only B- and γ-hordeins could not be separated by RP-HPLC. The identities and purities of all types were confirmed by SDS-PAGE. The Mr of all gluten reference protein types determined by LC-ESI-QTOF-MS were in good agreement with reference sequences in the NCBI database. Only in case of γ-40k-secalins, no reliable reference sequence was available in the NCBI or the UniProtKB database and, therefore, no marker peptide was selected for γ-40k-secalins. N-terminal sequencing was additionally used to confirm identity and purity of all gluten reference protein types and the sequences were also in agreement with reference sequences in the NCBI database. By targeted LC-MS/MS several protein type-specific peptides were detected in chymotryptic hydrolysates.

One the one hand, these well-characterized gluten reference proteins were used as reference material for the development of a targeted LC-MS/MS method to quantitate gluten in foods in this approach and on the other hand they were used in an immunisation study for gluten immunogenicity profiling [Röckendorff et al., 2017], which is not part described in this thesis.

Identification and selection of marker peptides

The identification of marker peptides was mainly based on the following three criteria: sequence specificity for each protein type, number of amino acids (8-20), and the absence of cysteine residues in the amino acid sequence [Martínez-Esteso et al., 2016]. Peptides, which were detected in gluten protein types, fractions and in the respective flour mixture, fulfilled the criteria and showed the highest score within one protein type were selected as suitable marker peptides. In this way, 33 gluten marker peptides (16 from wheat, 7 from barley, 3 from oats, and 7 from rye) were chosen for gluten quantitation (Table 1, chapter 3.2; Table 1, chapter 3.3). For each protein type, two to three marker peptides were selected to be able to detect at least one marker peptide in case of amino acid modification caused by deletion or substitution [Wieser et al., 2014].

Only peptides, which showed the highest score within one protein type, were considered as marker peptides. In general, the peptide score indicates the probability of a match of the measured mass spectra compared to the database. Individual peptide ion scores > 40 were considered to indicate identity or extensive similarity ($p < 0.05$). Additionally, all peptide identifications with peptide ion scores between 15

and 40 were manually validated [Chen et al., 2005]. The peptide score was an important criterion for correct identification of gluten peptides, but it did not express the intensity of the measured signals of the peptide precursor and the respective product ions. The intensity of the peptide signals would have been a non-negligible criterion, which received only little attention in this approach. Several selected marker peptides, such as P6 and P16 (Table 1, chapter 3.2) from wheat as well as P28-33 (Table 1, chapter 3.3) from rye were quantitated with low amounts in the respective gluten protein type and were not detected in samples with lower gluten contents compared to the respective flour mixture. This may be avoided and the marker peptides may be detected in low levels of gluten, if the selected marker peptides would have been detected with high intensities.

Development of a targeted LC-MS/MS method for the quantitative determination of 33 gluten marker peptides

In the second part of the study, a targeted LC-MS/MS method in the MRM mode was developed to quantitate 33 gluten marker peptides from gluten-containing grains (wheat, barley, rye and oats). For each type of grain, one marker peptide was isotopically labelled and used as grain-specific internal standard. For each marker peptide and isotopically labelled standard, two MRM transitions were monitored, which resulted in 74 transitions in each single run and enabled the identification of the source of gluten and quantitation of gluten. Table 4.1 shows the selected grain-specific marker peptides and the corresponding isotopically labelled peptide standard.

Table 4.1: Amino acid sequence of the grain-specific marker peptides and the corresponding isotopically labelled peptide standards

Type of grain	Marker peptide	Isotopically labelled standard
	Amino acid sequence	
Wheat	P11	*P11
	LQLQPFPQPQLPYPQPQPF	LQLQPFPQPQLPYPQPQP*F*
Barley	P19	*P19
	AQQQPSIEEQHQL	AQQQPS*IEEQHQ*L
Oats	P24	*P24
	VQQQPPFVQQEQPF	VQQQPPFVQQEQP*F
Rye	P27	*P27
	ASIETGIVGH	ASIETGI*V*GH

*F, L-[$^{13}C_9$][^{15}N]-phenylalanine; *G, L-[$^{13}C_2$][^{15}N]-glycine; *I, L-[$^{13}C_6$][^{15}N]-isoleucine; *L, L-[$^{13}C_6$][^{15}N]-leucine; P*, L-[$^{13}C_5$][^{15}N]-proline; *V, L-[$^{13}C_5$][^{15}N]-valine

The marker peptides P11, 19, 24 and 27 were quantitated by SIDA, because analyte and the corresponding standard differed only in the number of placed labels. Therefore, analyte and standard had the same chemical properties, retention times and ionisation behaviours and resulted in response factors (*RF*) close to 1 (*RF* P11: 1.277, P19: 1.488, P24: 0.918, P27: 1.090). The other marker peptides were quantitated relative to the respective internal isototopically labelled standard and provided absolute concentrations. Thus, the marker peptides P1-9 and 12-16 were quantitated using the wheat standard *P11, P17-18 and 20-23 using the barley standard *P19, P25-26 using the oat standard *P24 and P28-33 using the rye standard *P27. Because of various ionisation behaviours of the marker peptides and the corresponding grain-specific standards caused by differences in the amino acid sequences, the *RF*s ranged from 0.294 to 9.082 [Schalk et al., 2018a; Schalk et al., 2018b]. To obtain more precise results, it would be better to have an isotopically labelled standard for each peptide. Due to high costs of isotopically labelled peptides, only one marker peptide of each grain type was labelled in this approach.

Quantitation of marker peptides in gluten reference protein types and the conversion of peptide into protein type concentrations

Each marker peptide was quantitated in the respective well-characterized chymotryptically digested gluten reference protein type. In this way, the peptide yields of the chymotryptic digest obtained from a given amount of reference protein type were determined. The peptide concentrations in the respective reference protein types were the basis for the conversion of peptide into protein concentrations. In the next step, each gluten marker peptide was quantitated in the respective flour mixture and the obtained concentrations were converted into concentrations of protein type based on the respective peptide yields per protein type. In this way, a link between the obtained peptide concentrations and the respective protein types was established for all marker peptides P1-33. It has to be emphasised that the quantitation of P1-33 in the respective protein type resulted in relatively low peptide yields (0.5 to 48.8 mg/g) in spite of high gluten contents of flours. Consequently, the quantitation of P1-33 in the respective flour mixture with high gluten contents also resulted in low peptide amounts (0.1 to 1794.0 µg/g).

Furthermore, the corresponding recoveries of LC-MS/MS for each peptide P1-33 were evaluated in comparison to the amount of protein type determined by RP-HPLC (taken as 100%). As a consequence, to calculate the amount of protein type in a real sample by LC-MS/MS, the obtained concentration had to be muliplied by the peptide-specific correction factor.

The recoveries of LC-MS/MS compared to RP-HPLC-UV ranged from 1.5 to 224.1%, which resulted in peptide-specific correction factors ranging from 0.45 to 29.0. However, the marker peptides P11, 19, 24 and 27, which were quantitated by SIDA with their own isotopically labelled standards, showed good recoveries near 100% (P11: 75.3%; P19: 63.9; P24: 83.2%; P27: 106.0%), which resulted in correction factors near 1.0 (P11: 1.33; P19: 1.57; P24: 1.21; P27: 0.93). These results also indicated that it would be desirable to quantitate each marker peptide with its own isotopically labelled standard to obtain more precise results.

Matrix calibration

Each marker peptide was quantitated in the respective flour mixture with known gluten content, which was determined by RP-HPLC-UV (wheat: 89200 µg/g; barley: 42300 µg/g; oats: 12900 µg/g; rye: 30800 µg/g). Furthermore, the concentration of each marker peptide was determined in the respective flour mixture, which was spiked into gluten-free potato flour to obtain different gluten contents (wheat: 44600 - 446 µg/g; barley: 21150 - 423 µg/g; oats: 6450 – 129 µg/g; rye: 15400 – 2053 µg/g). A strong correlation between peptide and gluten concentrations was observed for each marker peptide with correlation coefficients (r) > 0.847. P6 and 16 were not detected by targeted LC-MS/MS, due to interfering peaks at the same retention time. For P20 and 29 – 33 no correlation coefficient was determined, because these peptides were quantitated in the respective flour mixture with amounts near the LOQ and therefore a correlation was not feasible. The linear Pearson correlations between gluten contents and concentrations of the peptides P11, 19, 24 and 27 are shown in Figure 4.1.

The marker peptides P11, 19, 24 and 27 showed correlation coefficients close to 1.0 (P11: 0.991; P19: 0.999; P24, 0.999; P27: 1.000), which also demonstrated that the quantitation by SIDA yielded more precise results compared to the quantitation by the grain-specific internal standard of the other marker peptides.

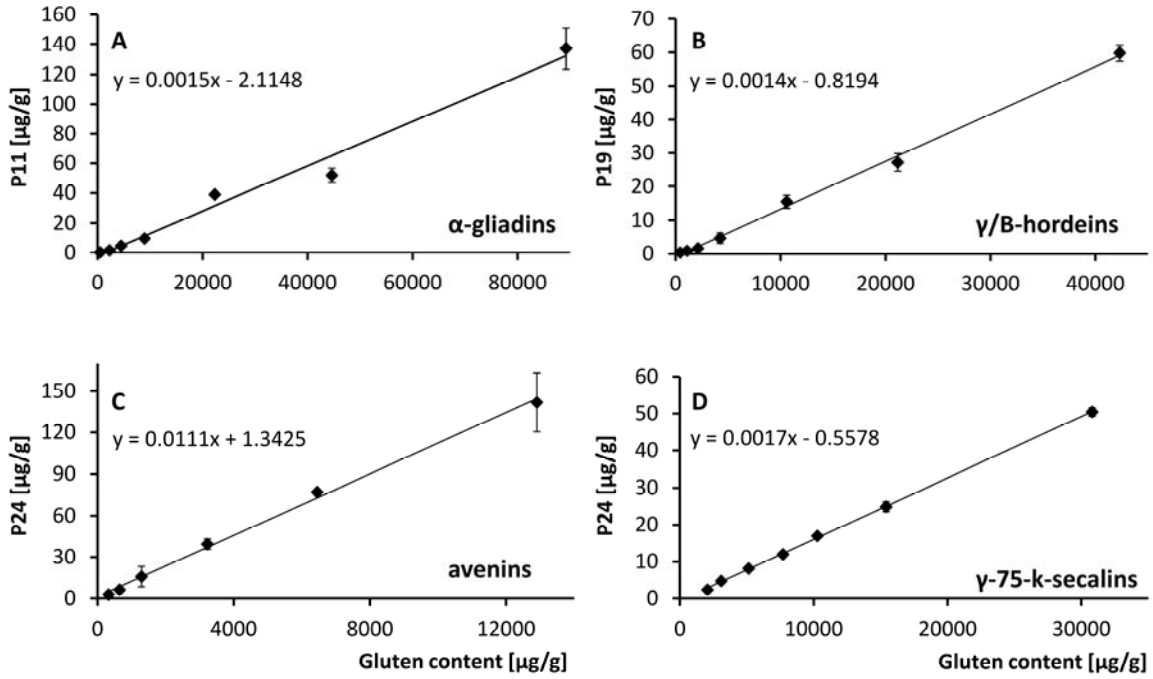

Figure 4.1: Linear Pearson correlations between gluten contents and concentrations of peptides from gluten reference protein types of each type of grain. Peptide P11 from α-gliadins (A), P19 from γ/B-hordeins (B), P24 from avenins (C), P27 from γ-75k-secalins (D).

This part of the study demonstrated that this new LC-MS/MS method enabled the sensitive detection of gluten marker peptides with LODs ranging from 0.05 (P28) to 14.5 µg/g (P9) [Schalk et al., 2018a; Schalk et al., 2018b]. However, the digestion of gluten reference protein types resulted in very low peptide yields compared to high gluten contents. A factor of more than 100 up to 1000 lay between peptide and protein concentrations and, therefore, only low peptide amounts were determined in the respective flour mixture with high gluten content. This spiking experiment showed that it was not possible to detect these marker peptides at low levels of gluten concentrations. None of the marker peptides were detected in the spiked wheat and barley samples with gluten contents less than 400 µg/g. P24 was detected down to 129 µg gluten/g. Rye marker peptides showed even lower peptide yields in the reference protein types than peptides from other grain types and, therefore, the marker peptides were not detected in spiked samples, which contained less than 2000 µg gluten/g. To detect these marker peptides in samples with gluten contents

near the threshold for gluten-free food (20 mg gluten/kg) and to implement a new non-immunochemical method to control ELISA results, the sensitivity has to be improved. The sensitivity might be enhanced using a different more sensitive mass spectrometer, e.g. a quadrupole-orbitrap MS instead of a triple quadrupole instrument [Gallien et al., 2012].

Application of the developed LC-MS/MS method for gluten quantitation in wheat-, barley-, oat- and rye-based food products

The new LC-MS/MS method was applied to quantitate gluten contents in wheat-, barley-, oat-, and rye-based food products and the obtained results were compared to those from R5 ELISA (sandwich or competitive) and HPLC (RP-HPLC-UV or GP-HPLC-FLD). Each method had its own procedure to calculate gluten contents. By LC-MS/MS, the marker peptides were quantitated and selected marker peptides were used for the calculation of protein type concentrations. Afterwards, the obtained protein type concentrations were multiplied by the individual correction factor and the sum of all determined protein type concentrations resulted in the gluten content. By R5 ELISA, the gliadin content was determined and multiplied by a factor of 2 to calculate the gluten content [Codex Stan 118, 2015]. By HPLC, the concentrations of gliadins and glutenins were determined and the sum of both fractions resulted in the gluten content [Scherf et al., 2016; Wieser et al., 1998].

The wheat marker peptides were quantitated in seven wheat starches (W4, W6, W8, W11, W13, W14 and W15) with different gluten contents and the results were compared to R5 ELISA (sandwich) and GP-HPLC-FLD. Only some marker peptides were detected and quantitated in all seven wheat starches. W8 had the highest gluten content of the analysed wheat starches and the highest number of marker peptides were detected (P2, 3, 4, 7, 8, 11, 15). In contrast, only 2 marker peptides were detected in W6 (P4, 8), which contained a low gluten content. The results showed that the lower the gluten content in wheat starch, the fewer marker peptides were quantitated, which may be due to extensive washing to decrease the gluten content of wheat starch [Scherf, 2016, van der Borght et al., 2005]. Consequently, several gluten proteins, which contained the marker peptides were removed and not detected anymore in wheat starches with gluten contents of less than 100 µg/g. The

peptides P4, 8, 11 and 15 were used for gluten calculation, due to high correlation coefficients and high recoveries compared to RP-HPLC. The peptide P9 seemed to be unsuitable for gluten calculation, which was maybe caused by co-elution of other similar gluten components, because the gluten contents based on P9 were significantly higher compared to ELISA and GP-HPLC-FLD. In six out of seven wheat starches, the gluten contents obtained by LC-MS/MS showed significant differences compared to ELISA. In five out of seven wheat starches, gluten contents by LC-MS/MS significantly differed to those analysed by GP-HPLC-FLD.
The comparision of all analysed wheat starches showed a strong correlation between LC-MS/MS and GP-HPLC-FLD ($r = 0.909$, $p < 0.005$) as well as between LC-MS/MS and ELISA ($r = 0.919$, $p < 0.005$). Thus, the LC-MS/MS method enabled the quantitation of gluten in wheat starch, in which a part of gluten had been removed by processing.

The barley marker peptides were quantitated in samples from different stages of the beer brewing process (malt, wort, green beer, unfiltered beer, kieselguhr-filtered beer) and the results were compared to R5 ELISA (competitive). By LC-MS/MS, the marker peptide P19 was detected in all stages of the brewing process and P22 was only detected in malt. P17, 18, 21 and 23 were not detected in any of the stages. The gluten contents of wort, unfiltered and kieselguhr-filtered beer were significantly different between LC-MS/MS and ELISA, whereas the gluten content of green beer showed no significant difference. The gluten content of malt seemed to be overestimated by ELISA compared to the gluten content of the barley flour mixture, whereas a 5-fold lower content was determined by LC-MS/MS. The comparison of gluten contents of all analysed samples from the beer brewing process, resulted in a strong correlation ($r = 0.999$, $p < 0.005$) of LC-MS/MS and R5 ELISA. The results showed that LC-MS/MS enabled gluten quantitation in samples from the beer brewing process and offered the opportunity to verify the results obtained from ELISA by an independent method.
Oat and rye marker peptides were quantitated in oat- and rye-based raw materials for sourdough fermentation and in dried sourdoughs and were compared to R5 ELISA (competitive) and RP-HPLC-UV. In oat- and rye-based raw materials, only P24 and P27 were detected and used for gluten calculation. Significant differences were observed between the gluten contents determined by LC-MS/MS, RP-HPLC and

ELISA. The gluten contents obtained by ELISA seemed to be overestimated compared to the gluten content of the rye flour mixture, which was also observed in studies of Lexhaller et al. [2016]. The comparison of gluten contents of all analysed raw materials resulted in a medium correlation ($r = 0.743$, $p < 0.005$) of LC-MS/MS and RP-HPLC and a medium correlation ($r = 0.705$, $p < 0.01$) of LC-MS/MS and R5 ELISA. No correlation ($r = 0.453$) was observed between RP-HPLC and R5 ELISA.
In dried oat- and rye-based sourdoughs, none of the marker peptides P24-33 were detected, which was maybe caused by microbial degradation of the marker peptides during sourdough fermentation. The degradation of gluten proteins was confirmed by SDS-PAGE and RP-HPLC, because no characteristic bands in the range of Mr 15 000 to 200 000 and no characteristic peaks in the prolamin and glutelin extract of the sourdough samples were observed. In contrast, R5 ELISA showed high gluten contents (3.2 - 73.5 mg gluten/g), which seemed to be overestimated in comparison to the other three methods or these three methods seemed to underestimate the gluten content compared to R5 ELISA.

Quantitation of the immunodominant 33-mer peptide from α-gliadin in different wheat flours by SIDA combined with targeted LC-MS/MS

The last part of the study included the development of a SIDA combined with targeted LC-MS/MS for the quantitative determination of the 33-mer. For this purpose, an isotopically labelled 33-mer was required. The first strategy to obtain an isotopically labelled standard was the synthesis of a 33-mer, in which L-proline residues were substituted by 3,4-dehydro-L-proline to introduce multiple deuterium labels by catalytic deuteration. Quantitative [^{1}H] nuclear magnetic resonance spectroscopy ([^{1}H] qNMR) was used to determine the absolute concentrations of the 33-mer and the deuterium labelled standard in methanolic solutions. The results showed that the deuterium labelled standard showed a quite low concentration (0.69 µmol/mL) compared to the 33-mer (1.9 µmol/mL). Furthermore, the deuterium labelled standard showed more NMR-signals than the 33-mer, which were not comparable and were maybe caused by polymerisation of the double bond of 3,4-dehydro-L-proline during the catalytic deuteration process. Due to these results, the deuterium labelled standard was deemed unsuitable for the development of a SIDA and a [$^{13}C_{28}$]- and [$^{15}N_{4}$]-labelled *33-mer peptide was purchased for this approach

(LQLQP*FPQPQLPYPQPQLPYPQPQLPYPQ*PQ*P*F with L-[$^{13}C_9$][^{15}N]-phenylalanine (*F) and L-[$^{13}C_5$][^{15}N]-proline (*P)).

The 33-mer was quantitated in flours of 23 hexaploid modern (currently cultivated) and 15 old common wheats (first registration prior to 1950) from different harvest years and two spelt cultivars harvested in 2014. The results showed that the 33-mer was present in all common wheat and spelt flours in a range from 90.9 to 602.6 µg/g of flour. Most of the modern and old wheat flours contained the 33-mer in a range of 200 - 400 µg/g flour with an overall average of 368 ± 109 µg/g flour. As a result, only some differences in 33-mer contents between these wheat cultivars were significant. A certain trend, e.g., that modern wheat cultivars generally contain higher amounts of 33-mer than old cultivars could not be derived from the data.
The 33-mer contents of the 40 modern and old common wheat and spelt cultivars (based on flour) were correlated to crude protein contents and to the contents of α-gliadin, total gliadin and total gluten analysed by RP-HPLC after modified Osborne fractionation. A weak correlation was observed between 33-mer and α-gliadin contents ($r = 0.568$, $p < 0.001$) as well as between 33-mer and gluten contents ($r = 0.563$, $p < 0.001$), but there was no correlation to gliadin contents ($r = 0.469$, $p < 0.001$), or crude protein contents ($r = 0.481$, $p < 0.001$).

Moreover, the 33-mer was analysed in two tetraploid durum wheat and two emmer cultivars (genome AABB) as well as two diploid einkorn cultivars (genome AA), in which the 33-mer was not detected (<LOD). The absence of the 33-mer peptide can be explained by the fact that this peptide is encoded by genes located in the Gli-2 locus on chromosome 6D, which is missing in durum wheat, emmer, and einkorn [Feuillet et al., 2008].

Although the 33-mer was found only in few entries of the UniProtKB database (20 out of 587 entries for α-gliadin sequences from *Triticum* sp. containing the 33-mer with an identity of 100 %), all 40 analysed modern and old common wheat and spelt cultivars contained the immunodominat 33-mer peptide. The special focus on this peptide seems to be legitimated not only because of its unique structure containing six copies of three overlapping T-cell epitopes, but also because of its presence in all hexaploid wheat cultivars analysed in this study.

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5 Future research

Future research on gluten analysis can be classified into several different topics. Further studies should focus on the production of a generally accepted gluten reference material including prolamins as well as glutelins to improve the reliability of analytical results. The use of such a reference material for method validation, proficiency testing and the development and calibration of novel approaches would improve the comparability of different methods.

Moreover, the development of a new ELISA based on a combination of two antibodies, e.g. the monoclonal R5 antibody and an antibody, which should be raised against protein types from the glutelins, would enable the quantitation of total gluten. In this way the multiplication of the prolamin content with the factor 2 would be avoided, which often resulted in an over- or underestimation of the gluten content according to previous studies.

The study reported here based on the quantitation of gluten marker peptides by targeted LC-MS/MS showed that it was possible to quantitate gluten contents based on peptide concentrations. However, further studies should concentrate on optimization of the enzymatic hydrolysis (choice of enzyme, efficiency of hydrolysis) to increase peptide yields and improve method sensitivity. Furthermore, the application of a different MS instrument (e.g. quadrupole-orbitrap MS instead of a triple quadrupole instrument) would be also one promising possibility to enhance selectivity, dynamic range and sensitivity. The selection of further marker peptides, which should be characteristic for processed food products, would enable the quantitation of gluten by LC-MS/MS in processed products, such as sourdough.

Further work with respect to the 33-mer should focus on the correlation of the 33-mer content determined by LC-MS/MS with the gluten content analysed by ELISA based on the G12 monoclonal antibody, which was raised against the 33-mer.

The results of this study have shown a great potential for future research, but there is still a lot of work to meet several challenges.

6 Summary

A strict gluten-free diet is the only effective therapy for coeliac disease (CD) patients. Currently, immunochemical methods (enzyme-linked immunosorbent assays, ELISAs) are most commonly used for gluten analysis to monitor the safety of gluten-free products, but these assays primarily target the alcohol-soluble prolamin fraction of gluten. The gluten content is then calculated by multiplying the prolamin content by a factor of 2. The problem is that different types of grains contain variable proportions of prolamins and alcohol-insoluble glutelins. As a result the calculated gluten content may be either over- or underestimated, which is a food safety issue for CD patients.
Therefore, the aim of the present study was the development of a new independent non-immunochemical method for the quantitation of prolamins and glutelins (=gluten) by targeted LC-MS/MS. To achieve this, gluten marker peptides should be quantitated by targeted LC-MS/MS. A strategy to calculate gluten concentrations based on marker peptide concentrations had to be developed.

For this purpose, well-characterized gluten proteins were required for use as reference materials. Gluten protein fractions (prolamins and glutelins) were isolated by modified Osborne fractionation from wheat, barley, oat and rye flour mixtures, respectively, to include genetic variability between different cultivars. By preparative RP-HPLC-UV, the isolated protein fractions were separated into the respective protein types (wheat: ω5-, ω1,2-, α-, γ-gliadins, HMW- and LMW-GS; barley: B/γ-, C-, D-hordeins, rye: γ-75k-, γ-40k-, ω-, HMW-secalins). Only oat avenins were not further separated. The purity and identity of all isolated gluten protein types were confirmed by five independent methods using analytical RP-HPLC, SDS-PAGE, N-terminal sequencing, LC-ESI-QTOF-MS and untargeted LC-MS/MS of hydrolysed protein types. The results showed that all protein types were reproducibly isolated in high purity from the flours and were suitable to be used as reference materials for targeted LC-MS/MS.

Chymotryptically hydrolysed well-characterized gluten reference protein types were analysed by untargeted LC-MS/MS to identify gluten-specific marker peptides. Peptides were defined as ideal candidates, if they fulfilled specified criteria (sequence specificity for each protein type, number of amino acids (8-20), and no cysteine

present in the amino acid sequence) and had the highest peptide scores within one protein type. In this way, two to three marker peptides for each protein type were chosen as marker peptides for gluten quantitation. In total, 33 marker peptides were selected (16 marker peptides for wheat, 7 for barley, 3 for oats and 7 for rye). A targeted LC-MS/MS method in the multiple reaction monitoring mode (MRM) was developed for gluten quantitation based on these 33 marker peptides, which enabled the detection of the source of gluten in contrast to ELISA. For each type of grain, one marker peptide was isotopically labelled (^{13}C and ^{15}N) and used as internal standard for quantitation. Thus, four peptides were quantitated by stable isotope dilution assay (SIDA) and the remaining peptides were quantitated relative to the respective grain specific isotopically labelled internal standard. For each marker peptide and isotopically labelled standard, two transitions were monitored, which resulted in 74 MRM-transitions for each single run.

The developed targeted LC-MS/MS method was used for the quantitation of 33 marker peptides in chymotryptic hydrolysates of the respective gluten reference protein types, which resulted in peptide-specific yields obtained from a given amount of reference protein type and formed the basis for the conversion of peptide into protein concentrations.

The quantitation of the 33 marker peptides in the respective flour mixture enabled the conversion into concentrations of protein type based on the respective peptide yields per protein type. The amount of each protein type determined by LC-MS/MS was compared to the respective amount obtained from RP-HPLC-UV, which was taken as 100% to evaluate the corresponding recoveries of LC-MS/MS. As a result, the obtained concentration had to be muliplied by the peptide-specific correction factor to calculate the amount of protein type in a real sample by LC-MS/MS. The recoveries ranged from 1.5 to 224.1%, which resulted in peptide-specific correction factors ranging from 0.45 to 29.0.

A matrix calibration by spiking the respective flour mixture into gluten-free potato flour followed by the quantitation of the respective marker peptide in each spiked sample resulted in a strong correlation between peptide and gluten concentrations with correlation coefficients (r) > 0.847.

The targeted LC-MS/MS method was applied to determine gluten contents in wheat-, barley-, oat-, and rye-based food products (wheat starches, samples from the

brewing process, oat- and rye-based raw materials for sourdough fermentation and sourdoughs) and the obtained results were compared to those from R5 ELISA (sandwich or competitive) and HPLC (RP-HPLC-UV or GP-HPLC-FLD).
In wheat starches, a strong correlation between LC-MS/MS and GP-HPLC-FLD ($r = 0.909$, $p < 0.005$) as well as between LC-MS/MS and R5 sandwich ELISA ($r = 0.919$, $p < 0.005$) was observed. The quantitation of gluten in samples from the brewing process also resulted in a strong correlation ($r = 0.999$, $p < 0.005$) of LC-MS/MS and R5 competitive ELISA. In oat- and rye-based raw materials for sourdough fermentation, a medium correlation ($r = 0.743$, $p < 0.005$) of LC-MS/MS and RP-HPLC as well as between LC-MS/MS and R5 competitve ELISA ($r = 0.705$, $p < 0.01$) was observed. Marker peptides were not detected in sourdough samples, caused by degradation of gluten proteins, which was confirmed by SDS-PAGE and RP-HPLC.
The results demonstrated that the quantitation of marker peptides by targeted LC-MS/MS enabled the quantitation of prolamins and glutelins (= gluten) and the identification of the source of gluten in different cereal-based food products.

Furthermore, a SIDA combined with targeted LC-MS/MS for the quantitative determination of the immunodominant 33-mer peptide from α-gliadin was developed. Due to its high resistance to proteolytic digestion by intestinal peptidases and its unique peptide structure characterized by six copies of three overlapping T-cell epitopes, the 33-mer plays an important role in previous studies. This part of the study should demonstrate whether the special focus on this peptide is justified or not and to allow a precise assessment of its importance associated with CD-research.
The 33-mer was analysed in flours of 23 hexaploid modern and 15 old common wheats from different harvest years and two spelt cultivars. Moreover, the 33-mer was determined in two tetraploid durum wheat and two emmer cultivars as well as two diploid einkorn cultivars.
The results showed that all common wheat and spelt flours contained the 33-mer at levels ranging from 91–603 µg/g flour. The comparison of the contents of α-gliadin, total gliadin and total gluten analysed by RP-HPLC after modified Osborne fractionation and to crude protein contents resulted in a weak correlation between 33-mer and α-gliadin contents ($r = 0.568$, $p < 0.001$) and between 33-mer and gluten contents ($r = 0.563$, $p < 0.001$). No correlation to gliadin contents ($r = 0.469$, $p < 0.001$) or crude protein contents ($r = 0.481$, $p < 0.001$) was observed. Thus,

quantitation of the 33-mer to determine the gluten content of food appears to be not possible.

In contrast, the 33-mer was absent (< limit of detection) from tetra- and diploid species (durum wheat, emmer, einkorn), most likely because of the absence of the D-genome, which encodes α2-gliadins.
In conclusion, the special focus in the literature on this most immunodominant peptide seems to be justified due to the presence of the 33-mer in all analysed common wheat and spelt flours.

7 Zusammenfassung

Die strikte Einhaltung einer glutenfreien Diät ist gegenwärtig die einzige Therapie für Zöliakiepatienten. Die Glutenanalytik erfolgt derzeit mittels einer immunchemischen Methode (ELISA), um die Sicherheit von glutenfreien Produkten zu gewährleisten. Diese Methode erfasst lediglich die alkohol-lösliche Prolaminfraktion des Glutens und Glutengehalte werden durch Multiplikation des Prolamingehalts mit einem Faktor von 2 berechnet. Problematisch ist hierbei jedoch, dass verschiedene Getreidearten unterschiedliche Verhältnisse von Prolaminen und alkohol-unlöslichen Glutelinen aufweisen, wodurch die Glutengehalte oftmals über- oder unterbestimmt werden. Besonders die Unterbestimmung des Glutengehalts beinhaltet ein Risiko für die Sicherheit von glutenfreien Lebensmitteln für Zöliakiepatienten.
Das Ziel dieser Arbeit war deshalb die Entwicklung einer nicht-immunchemischen Methode für die Quantifizierung von Prolaminen und Glutelinen (= Gluten) mittels zielgerichteter LC-MS/MS über Gluten-spezifische Leitpeptide. Dazu musste eine Strategie zur Berechnung des Glutengehalts basierend auf Gluten-Leitpeptid-Konzentrationen entwickelt werden.

Zu diesem Zweck wurden gut charakterisierte Glutenproteine als Referenzmaterialien benötigt. Mittels modifizierter Osborne-Fraktionierung wurden jeweils Glutenproteine (Prolamine, Gluteline) aus Weizen-, Gerste-, Hafer- und Roggen-Mehlmischungen isoliert. Die Mischungen wurden hergestellt, um die genetische Variabilität verschiedener Sorten mit einzubeziehen. Mittels präparativer RP-HPLC-UV wurden die isolierten Proteinfraktionen in die jeweiligen Proteintypen getrennt (Weizen: ω5-, ω1,2-, α-, γ-Gliadine, HMW- und LMW-Gluteninuntereinheiten; Gerste: B/γ-, C-, D-Hordeine; Roggen: γ-75k-, γ-40k-, ω-, HMW-Secaline). Lediglich Avenine aus Hafer wurden nicht weiter aufgetrennt. Die Reinheit und Identität aller isolierten Proteintypen wurden durch folgende fünf unabhängige Methoden bestätigt: analytische RP-HPLC, SDS-PAGE, N-terminale Sequenzierung, LC-ESI-QTOF-MS und nicht-zielgerichtete LC-MS/MS von hydrolysierten Proteintypen. Die Ergebnisse zeigten, dass alle Proteintypen in hoher Reinheit aus Mehlen reproduzierbar isoliert worden waren und diese als Referenzmaterialien für die zielgerichtete LC-MS/MS geeignet waren.

Um Gluten-spezifische Leitpeptide zu identifizieren, wurden chymotryptisch hydrolyiserte gut charakterisierte Gluten-Referenz-Proteintypen mittels nicht-zielgerichteter LC-MS/MS analysiert. Peptide wurden als ideale Kandidaten für Leitpeptide definiert, wenn sie festgelegte Kriterien erfüllten (Spezifität der Sequenz für den jeweiligen Proteintyp, Anzahl der Aminosäuren (8-20) und die Abwesenheit von Cystein-Resten in der Aminosäuresequenz) und den höchsten Peptidscore innerhalb eines Proteintyps aufwiesen. Auf diese Weise wurden zwei bis drei Leitpeptide für jeden Proteintyp zur Glutenquantifizierung ausgewählt. Insgesamt wurden 33 Leitpeptide ausgewählt (16 Leitpeptide für Weizen, 7 für Gerste, 3 für Hafer und 7 für Roggen). Eine zielgerichtete LC-MS/MS-Methode im MRM Modus basierend auf diesen 33 Leitpeptiden wurde entwickelt, welche im Gegensatz zu ELISA die Detektion der Art/Herkunft des Glutens ermöglichte. Für jede Getreideart wurde ein stabilisotopenmarkiertes Leitpeptid (^{13}C und ^{15}N) als interner Standard zur Quantifizierung eingesetzt. Somit wurden vier Leitpeptide mittels Stabilisotopenverdünnungsanalyse (SIVA) und die verbleibenden Leitpeptide relativ zum jeweiligen Getreide-spezifischen stabilisotopenmarkierten internen Standard quantifiziert. Für jedes Leitpeptid und jeden Getreide-spezifischen stabilisotopenmarkierten Standard wurden zwei Übergänge gemessen, somit 74 Übergänge für jeden einzelnen Lauf.

Die entwickelte zielgerichtetete LC-MS/MS-Methode wurde zur Quantifizierung der 33 Leitpeptide in chymotryptischen Hydrolysaten der jeweiligen Gluten-Referenz-Proteintypen eingesetzt. Dies führte zu Peptid-spezifischen Ausbeuten einer bestimmten Menge an Referenz-Proteintyp und bildete die Grundlage für die Umrechnung von Peptid- in Protein-Konzentrationen.
Die Quantifizierung der 33 Leitpeptide in den jeweiligen Referenz-Proteintypen ermöglichte die Umrechnung von Peptid- in Protein-Konzentrationen basierend auf den jeweiligen Peptidausbeuten pro Proteintyp. Die per LC-MS/MS ermittelte Menge an Proteintyp wurde mit dem jeweiligen per RP-HPLC-UV bestimmten Wert verglichen (angenommen als 100 %), um die entsprechende Wiederfindung der LC-MS/MS zu bestimmen. Infolge dessen mussten die erhaltenen Konzentrationen mit dem Peptid-spezifischen Korrekturfaktor multipliziert werden, um Proteintyp-Gehalte in einer realen Probe per LC-MS/MS zu berechnen. Die Wiederfindungen lagen im

Bereich von 1.5 bis 224.1 %, was zu Peptid-spezifischen Korrekturfaktoren von 0.45 bis 29.0 führte.
Durch Spiken von glutenfreiem Kartoffelmehl mit der jeweiligen Mehlmischung wurde eine Matrixkalibrierung erstellt. Anschließend wurden die jeweiligen Leitpeptide in jeder gespikten Probe quantifiziert. Es wurde eine starke Korrelation von Peptid- und Glutenkonzentrationen beobachtet. Die daraus resultierenden Korrelationskoeffizienten (r) waren größer als 0.847.

Die neue LC-MS/MS Methode wurde angewendet, um Glutengehalte in Weizen-, Gerste-, Hafer- und Roggen-basierten Lebensmitteln (Weizenstärke, Proben aus dem Brauprozess, Hafer- und Weizen-basierte Rohmaterialien für die Sauerteig-Fermentation und Sauerteige) zu bestimmen und die Ergebnisse wurden mit den durch R5 ELISA (sandwich oder kompetitiv) und HPLC (RP-HPLC-UV oder GP-HPLC-FLD) bestimmten Konzentrationen verglichen.
In Weizenstärke wurde eine starke Korrelation zwischen LC-MS/MS und GP-HPLC-FLD ($r = 0.909$, $p < 0.005$) und zwischen LC-MS/MS und R5 ELISA (sandwich) ($r = 0.919$, $p < 0.005$) beobachtet.
Die Glutenquantifizierung in Proben des Brauprozesses führte ebenfalls zu einer starken Korrelation ($r = 0.999$, $p < 0.005$) zwischen LC-MS/MS und R5 ELISA (kompetitiv).
In Hafer- und Roggen-basierten Rohmaterialien für die Sauerteig-Fermentation wurde eine mittlere Korrelation zwischen LC-MS/MS und RP-HPLC ($r = 0.743$, $p < 0.005$) und zwischen LC-MS/MS und R5 ELISA (kompetitiv) ($r = 0.705$, $p < 0.01$) beobachtet. Keines der Leitpeptide wurde in den untersuchten Sauerteig-Proben detektiert, aufgrund des Abbaus von Glutenproteinen, welcher mittels SDS-PAGE und RP-HPLC bestätigt wurde.
Die Ergebnisse veranschaulichten, dass die Quantifizierung von Prolaminen und Glutelinen (= Gluten) und die Identifizierung der Glutenart in Getreide-basierten Lebensmitteln durch die quantitative Bestimmung der Leitpeptide mittels zielgerichteter LC-MS/MS möglich war.

Des Weiteren erfolgte die Entwicklung einer SIVA mit anschließender LC-MS/MS-Detektion, um die Quantifizierung des immundominanten 33-mer Peptids aus α-Gliadin zu ermöglichen. Das 33-mer spielt in der Literatur eine wichtige Rolle,

aufgrund seiner hohen Resistenz gegenüber dem proteolytischen Abbau durch intestinale Peptidasen und der einzigartigen Peptidstruktur, welche durch sechs Kopien von 3 sich überlappenden T-Zell-Epitopen gekennzeichnet ist. Dieser Teil der Arbeit sollte demonstrieren, ob der besondere Fokus vieler Arbeiten auf dieses Peptid gerechtfertigt werden kann oder nicht und um den Stellenwert im Zusammenhang mit Zöliakie beurteilen zu können.

Der 33-mer Gehalt wurde in Mehlen von 23 modernen und 15 alten hexaploiden Weichweizensorten verschiedener Erntejahre und 2 Dinkelsorten analysiert. Des Weiteren wurde der 33-mer Gehalt in jeweils zwei tetraploiden Hartweizen- und Emmersorten und zwei diploiden Einkornsorten bestimmt.

Die Ergebnisse zeigten, dass alle Weichweizen- und Dinkelmehle das 33-mer beinhalteten. Die 33-mer Gehalte lagen im Bereich von 91–603 µg/g Mehl. Diese wurden anschließend mit α-Gliadin-, Gesamtgliadin-, Gesamtgluten- (ermittelt durch RP-HPLC nach modifizierter Obsborne-Fraktionierung) und Rohproteingehalten verglichen. Hieraus ergab sich eine schwache Korrelation zwischen den 33-mer- und α-Gliadin-Gehalten ($r = 0.568$, $p < 0.001$) und zwischen 33-mer- und Glutengehalten ($r = 0.563$, $p < 0.001$). Zwischen 33-mer und Gliadingehalten ($r = 0.469$, $p < 0.001$) oder Rohproteingehalten ($r = 0.481$, $p < 0.001$) wurde keine Korrelation beobachtet.

Im Gegensatz dazu erfolgte keine Detektion des 33-mer (< Nachweisgrenze) in tetra- und diploiden Sorten (Hartweizen, Emmer, Einkorn), aufgrund der Abwesenheit des D-Genoms, welches α2-Gliadine kodiert.

Aufgrund des Vorkommens des 33-mers in allen analysierten Weichweizen- und Dinkelsorten, scheint die besondere Bedeutung des immundominaten 33-mer Peptids in der Literatur gerechtfertigt zu sein.