SPECIAL GUEST EDITOR SECTION

Analytical Methods for Detection of Gluten in Food—Method Developments in Support of Food Labeling Legislation

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The current essential therapy of celiac disease is a strict adherence to a gluten-free diet. Besides food products that are naturally gluten-free, "very low gluten" and "gluten-free" bakery products have become available. The availability of immunochemical and other analytical methods to determine gluten markers in foods is of utmost importance to ensure the well being of gluten-sensitive individuals. The aim of this review was to evaluate if currently available methodologies are suitable to meet the requirements of food labeling standards for individual gluten source declaration, in order to achieve policy objectives. Codex Alimentarius and European Union (EU) legislation and gluten detection methodologies applicable at present have been summarized and compared. In 2009, the European Commission issued Regulation No. 41/2009 concerning the composition and labeling of foodstuffs suitable for people intolerant to gluten. This review constitutes a basis to investigate the possibility to develop a proteomic-based method for the specific detection of gluten-containing cereals in food products, especially at or around the limits specified in EU legislation.

Wheat allergy—including baker's asthma and wheat-dependent exercise-induced anaphylaxis (WDEIA)—is a food allergy that is immunoglobulin E (IgE) mediated and is limited to the seed storage proteins of wheat (1). Some reactions are restricted to wheat proteins, while others can be caused by many varieties of seeds and other plant tissues. Allergies to other cereals, such as maize, sorghum, and millet, are not common and are not related to reactions triggered by wheat, rye, barley, and oats. However, peptides with a high degree of similarity to these immunostimulatory sequences were identified in maize zein, oat avenin, some rice proteins, and some other noncereal foods (2). Gluten intolerance is a term for nonallergic food intolerance or hypersensitivity that is used widely for varied physiological responses associated with a particular food or compound found in a range of foods. Celiac disease (CD), or celiac sprue, is an immune mediated enteropathy triggered by gluten intake and mostly found in European individuals (3). CD and IgE-dependent wheat allergy are triggered by wheat gluten or the gluten-like proteins of other related cereals: rye, barley, kamut, spelt, and, in some cases, oat (4). Oat intolerance is rare but may be a reason for villous atrophy and inflammation in CD patients (5).

The celiotoxic proteins, referred to as toxic for the purpose of this work, are a result of the specific amino acid composition of gluten; its high prolin content makes gluten quite resistant to enzymatic degradation so that a high number of immunogenic gluten peptides reach the small intestine. In almost every case, the caused inflammation of the proximal small intestine becomes smooth, losing its ability to absorb nutrients. Symptoms include diarrhea, deficiencies in uptake of nutrients such as vitamins (6, 7), villous atrophy and crypt hyperplasia in the small intestine (8), extraintestinal manifestations like dermatitis herpetiformis (9), or a range of neurological dysfunctions (10). Gastrointestinal digestion of gluten releases proteolytically resistant immunotoxic peptide fragments that are deamidated at specific glutamine residues by tissue transglutaminase-2 (tTGase; 11, 12). Deamidated peptides bind to a certain class of human leukocyte antigens (HLA-DQ2; 13). The DQ2-gluten complexes on the surface of antigen presenting cells interact with gluten-specific $CD4^+$ T cells that mediate a T_H1 response comprising the secretion of proinflammatory cytokines (14).

CD occurs only in genetically predisposed individuals possessing HLA-DQ2 or HLA-DQ8 genes (15). The enzyme tTGase is the primary focus of the autoantibody response in celiac sprue (16). The tTGase catalyzed deamidation of toxic sequences dramatically increases their affinity for HLA-DQ2. Large variations were observed in the degree of deamidation between different peptides and between individual glutamine residues within each peptide (17). The rate of deamidation by tTGase appears to be a factor of importance for the T-cell response to gluten in celiac disease.

Celiac disease affects about 1% of the world's population

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(18, 19), and these people must follow a lifetime gluten-free (GF) diet (20). Therefore, there is a need to develop nondietary therapies (8). A few approaches to treat CD were published recently, and it should be noted that these methods are only in a trial stage and are not without risk (10, 21, 22).

Characterization of Cereal Proteins

Cereal proteins, more specifically wheat proteins, were initially characterized by their solubility (23, 24). These so-called Osborne fractions—water-soluble albumins, salt-soluble globulins, alcohol-soluble prolamins, and insoluble glutelins—are complex mixtures of different polypeptides, especially in the case of wheat (25). The nomenclature and classification systems for the most important cereals (e.g., wheat, barley, rye, maize, rice, and sorghum) are based on Osborne fractionation, while in the case of other cereals (e.g., pseudocereals—amaranth, quinoa, buckwheat, and millet) trivial names are in use for their protein fractions. (26–29). Soy proteins have been often classified according to their sedimentation coefficients, but they can be also characterized by their immunological properties (30).

Protein Characterization of Gluten-Containing Cereals

Wheat contains about 12–14% protein, barley 8–13%, and rye and oats 8–13%. Kamut is a variety of *Triticum turanicum* (or *T. turgidum*), with 12–18% protein content. Spelt is a hexaploid wheat variety (*T. spelta*) with 17% protein content (31). All gluten-containing cereals are rich in glutamine and proline and poor in basic amino acids.

Water-soluble albumins and salt-soluble globulins represent the small size proteins of gluten-containing cereals and are present at relatively low levels of total protein content. The bulk of these proteins are enzymes and related proteins, though some can have an immunotoxic effect. Prolamins are the alcohol-soluble protein fractions of cereal grains. The cereal prolamins, namely glutenins and gliadins in wheat, secalins in rye, and hordeins in barley, are the major storage proteins found in the endosperm of cereal grains. Gliadins and glutenins together form the gluten protein family with a size range of 30 to millions kDa (26). The ratio of gliadin to glutenin is approximately 65 to 35 and can vary depending on genetic and environmental influences (32–34). Glutenins and gliadins form a protein network during grain development that is stabilized by various physicochemical forces, such as hydrophobic interactions and hydrogen bonds but mainly disulfide covalent bonds (35–37). Storage proteins are localized within the wheat endosperm and provide the majority of the total proteins. Both gliadins and glutenins can be further characterized based on their electrophoretic mobility and structural features (25).

Wheat gliadins belong to the proline- and glutamine-rich prolamin family, and have been characterized by many authors (35, 38–41). Their acid polyacrylamide gel electrophoresis (A-PAGE) profile separates them into α-, β-, γ-, and ω-gliadin regions. The gliadins can be grouped also by their N-terminal sequence: S-rich α/β-, γ-, and S-poor ω-gliadins that contains no cysteine residue and are present in 60, 30, and 10% levels, respectively (25, 42). α-gliadins contain six conserved cysteine residues that form three intrachain crosslinks, and γ-gliadins contain eight conserved cysteine residues that form four intrachain crosslinks. ω-gliadins do not contain any cysteine, similar to barley C-hordeins and rye ω-secalins (43). Their solubility properties and size range (32–58 kDa) are overlapping with low molecular weight glutenin subunits (LMW-GS; 44, 45), and some of them may also become part of the glutenin fraction due to mutations affecting cystein number and distribution (46). Some gliadins are alcohol insoluble, and some glutenins are alcohol soluble (44, 47, 48).

Wheat gliadins are encoded on the short arm of chromosomes 6A, 6B, and 6D. α- and β-gliadins are encoded at the Gli-2 loci, and ω- and γ-gliadins at the Gli-1 loci. The gene family includes approximately 150 members (49), and most of them contain internal stop codons and are not expressed (50). Electrophoretic analyses have shown the existence in durum and bread wheat of genotypes lacking an entire cluster of gliadin components controlled by genes at a given complex locus (51).

α-gliadins contain a 20 amino acid signal peptide that is cleaved post-translationally, followed by an N-terminal region that contains repetitive sequences. The repetitive regions of some α-gliadins contain a number of T cell stimulatory epitopes (12, 52, 53). The C-terminal portion of the protein consists of two short polyglutamine regions (Poly Q Regions I and II) interspersed with two regions of unique sequences (Unique Regions I and II). An additional T cell stimulatory epitope and a toxic peptide have been reported in Unique Region II in some cases (54, 55).

Most α-gliadins are monomers containing six conserved cysteine residues that form three intramolecular disulfide bonds. Certain proteins with an extra cysteine residue can be incorporated into the glutenin polymer (56, 57). These proteins are structurally similar to gliadins but functionally similar to LMW-GS (56). α-gliadin fractions from bread and spelt wheat showed a close relationship (58). Based on N-terminal sequencing, α-gliadins of all wheat species contain amino acid sequences that are potentially toxic for CD patients (59). The prolin content of oat avenin is only half that in gluten, secalin, or hordein (60, 61), which partly explains its reduced toxicity in CD.

It is essential to be able to identify and distinguish individual α-gliadins because very minor differences in the sequences of these proteins can be important for human health. Genes encoding proteins containing the greatest number of CD epitopes are also the most highly expressed genes (62).

Wheat glutenins can be separated into high and low MW (HMW and LMW, respectively) glutenins based on their sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) mobility (63, 64). The size range of HMW-GS is 90–120 kDa and LMW-GS 30–70 kDa (65). LMW-GS are present in gluten three times more than HMW-GS. The characterization of LMW-GS at the gene and protein levels has been reviewed (43, 66). SDS-PAGE mobility divides LMW-GS into B type proteins (40–50 kDa) and C type (30–40 kDa). B type LMW-GS can be divided into three subgroups based on the length of their N-terminal sequences and referring to the first amino acid in the N-terminal sequence: s-, m-, and i-type according to serine (Ser), methionine (Met), or isoleucin (Ile), respectively (43, 67). Applying cleavage to the covalent disulfide bonds and interchain hydrogen bonds, glutenin proteins become alcohol soluble (47, 68).

The high MW glutelin fraction of barley (35–45%) is the family of D-hordeins. Their amino acid composition—rich in glutamic acid (Glu), proline (Pro), and glycine (Gly)—and molecular properties are similar to rye and wheat glutelins (67, 69, 70). HMW storage proteins of rye are the HMW secalins or rye glutelins, which are stabilized by disulfide bonds. Their size range is similar to those of wheat proteins. They contain glycin in large amount and are poor in proline. Based on N-terminal sequences, HMW secalins are homologous with D-hordeins and HMW-GS of wheat (71).

Protein Characterization of Gluten-Free Cereals

Gluten-free (GF) cereals contain proteins that are not able to form a gluten-type protein network (72). Nongluten proteins have a narrower molecular size range and contain less prolamine and glutamine, which is the major reason for the disability to form a gluten network. Their solubility properties, therefore, are much better than those of gluten-containing cereals. Despite the fact that some of these proteins contain cysteine residues allowing the formation of S-S bonds, none of these nongluten-containing cereals could replace the role of the gluten polymer. Due to the amino acid composition of nongluten proteins, they do not possess CD epitopes, which makes their use in a GF diet advantageous.

On an average dry matter basis, soybeans contain about 40% protein (73). Maize and rice contain 7–10% protein, and their classification is according to the Osborne fractionation scheme (67, 74, 75).

Sorghum's protein content is about 7–13% (76), but these proteins are enclosed in protein bodies, and their availability for extraction and enzymatic digestion is, therefore, limited. Sorghum storage proteins are the kafirin prolamins, which are 68–73% of the total protein content (76). Kafirins show hydrophobic properties that are very similar to zein (77). Maize seed proteins have alcohol-soluble prolamins (60%) and alkali-soluble glutelins (34%; 78). Maize prolamins (zeins) are rich in glutamine, proline, alanine, and leucine and almost completely lack lysine and tryptophan (79). Rice glutelin (80%) is a HMW protein, is soluble in dilute acid, and is composed of subunits bound by disulfide linkages (80).

When wheat flour is partially or totally replaced in producing GF or gluten-reduced food, a supplementation is needed that helps to improve the nutritional quality and texture of such foodstuffs. The incorporation of soy proteins can cause interaction with gluten proteins during dough mixing (81–83). There are also possible interactions between soy proteins and wheat puroindolines, which are bound to the surface of starch granules in wheat endosperm (84). Its importance is in the fact that gluten-reduced and GF foods often contain wheat starch. Gluten from different sources can also interact with each other (85). Sorghum proteins can form new structures with each other or with proteins from other sources during processing. Nevertheless, not only protein–protein interactions can occur among components in gluten containing foods. For example, phytate in cereals—especially rice—can interfere with proteins (86) as well as tannins in sorghum (87).

Toxicity Factors in Cereal Proteins

The main proteins responsible for CD and gluten intolerance were identified in 1959, but new toxic peptides and/or sequences are still being revealed (88, 89). From wheat albumins and globulins, the α-amylase inhibitor subunits (12–18 kDa) were identified to be toxic in baker's asthma (90–94). Another type of protein, an IgE-reactive 27 kDa wheat protein (92) and two IgE-reactive 14 and 13 kDa salt-soluble fractions, were also identified to be responsible for baker's asthma (93).

The toxicity of prolamins depends on the amino acid sequences present and the molecular properties. The celiac-harmful proteins are rich in glutamine and proline (95–97). The proline-rich repetitive region of wheat gliadins was shown to be responsible for carrying epitopes for a respective lymphocyte receptor and were connected to CD pathogenesis (98). The 33-mer from α-gliadin contains three overlapping glutamine (Gln)-Pro rich epitopes (12, 52, 95): PFPQPQLPY, PQPQLPYPQ (3 copies), and PYPQPQLPY (2 copies) were identified in many studies (53). Further DQ2 restricted α- and γ-gliadin epitopes were characterized, and their location was established to be in distinct proline-rich clusters of the gliadin protein. The toxicity of proteins is linked to the release of peptides upon digestion. Based on in vivo studies, these toxic peptides were shown to always contain one of the four motifs: PSQQ, QQQP, QQPY, or QPYP. However, many nontoxic proteins also contain these motifs, and therefore, these sequences are not etiological factors in CD and, thus, must be surrounded by other amino acids to cause toxicity (99). The optimal

length of T cell stimulating peptides is 10–15 amino acid residues (98, 100).

The α-gliadin 57–73 Q65E sequence was identified as a dominant toxic epitope in CD (95). Later, further CD-toxic cereal proteins were identified (53, 101–105). Two major CD causing peptides, one from α-gliadin, a 33-mer LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF (53), and one from γ-gliadin, a 26-mer FLQPQQPFPQQPQQPYPQQPQQPFPQ (106), were identified. *In silico* analysis of the gluten proteome identified about 60 putative peptides sharing characteristics of the 33- and 26-mer toxic peptides. Twenty-one celiac-specific gluten epitopes were reported up to 2005 (106).

Gliadins, secalins, and hordeins contain several of the PQPQLPY sequences or sequences rich in Pro-Gln residues. They are a high-affinity substrate for the tTGase that deamidates the second Q residue, forming the peptide sequence PQPELPY (107). The spacing between the targeted Gln and C-terminal Pro residues plays an essential role in the specificity of tTGase (100, 108, 109) and is often found in the cluster regions (52). Overall, toxicity of proteins and peptides can be summarized as follows: they are resistant to gastrointestinal proteases, can be efficiently proteolyzed by therapeutic glutenases, are able to penetrate across the intestinal epithelium, are recognized by the human TG2 or HLA-DQ2, and stimulate an inflammatory response from T cells.

Other than gliadins, a glutenin protein containing QQQPP motifs was described and identified as an allergen (110, 111). An ω-gliadin (Tri a 19) with 65 kDa mass is considered to be the major allergen for causing WDEIA (85, 112–115). Seven epitopes (QQIPQQQ, QQLPQQQ, QQFPQQQ, QQSPEQQ, QQSPQQQ, QQYPQQQ, and PYPP) were detected within the primary sequence of ω-gliadins and considered to cause WDEIA. Critical amino acid sequences for IgE binding were also determined in this research (116).

Oat proteins might not belong to the celiac-harmful protein family, because their prolamin composition and amino acid sequence differ from those found in wheat, rye, and barley (100). Initially, they were thought to be toxic, but later their effect was reinvestigated and it was concluded that peptides generated from oat proteins are less toxic for CD patients (4, 5, 9, 22, 117). As mentioned earlier, the reduced toxicity of oat avenin is due to its lower proline content compared to gliadin, secalin, or hordein (47, 61). Several avenin-derived peptides were synthesized and tested for T-cell recognition, and only one peptide (SEQYQPYPEQQEPFVQQQQ) was recognized by the regioselective T-cell lines (5). The recognition depends on tTG2 treatment proving the tTG2 specificity of deamidation (100, 108). By studying the possible cross-reactions between gluten and oat avenin (100), it was shown that at least two distinct peptides of oat exist that can elicit mucosal T cell responses in CD patients with oat intolerance (5).

Some authors have shown the potential toxicity of wheat glutenins as well (2, 48, 98, 118), especially the LMW-GS that are overlapping with some of the gliadin fractions in terms of size and solubility (44, 60). It is well known that storage protein composition of different cultivars within a species varies, and, therefore, the quality and ratio of toxic proteins/peptides may change among cultivars. The cultivar dependency of toxic gliadins was investigated in *T. aestivum* and *T. durum* species. It was concluded that the immunogenic gliadin epitopes are present in different amounts in various species (119, 120).

There are proteins less known than gliadins or glutenins in wheat that can cause allergy or intolerance. Sander et al. (121) identified more than 100 IgE binding proteins in wheat flour by means of immunoblotting with sera of 10 subjects with baker's asthma, leading to the conclusions that cereal allergy is a complex fact and its comprehensive understanding is far from complete (122). Wheatwins are pathogenesis-related (PR) proteins of the PR-4 family (123, 124). The PR protein family (especially chitinases) can cause allergic reactions in humans and animals (125). Thioredoxins from wheat and maize represent a novel family of cross-reactive allergens that might contribute to the symptoms of baker's asthma and, in addition, be related to grass pollen allergy (126). Interestingly, thioredoxins can also reduce the allergenicity by eliminating disulfide bonds, while they can cause allergy at the same time (127). The expansin superfamily of plant proteins is also one of the allergenic cereal protein repertoires (128).

Food Products Labeled GF and Very Low Gluten

As a consequence of adverse health reactions, food products that contain or may contain gluten, even at a trace level, shall be labeled to protect the well being of consumers intolerant to gluten. Depending on the geographical location and the type of gluten-containing cereals, the level of toxicity may differ to a certain extent due to their differences in protein composition and, thus, the varying qualitative and quantitative ratio of toxic peptides generated in the gut. The genetic specificity of the immune system of a certain human population also influences the extent of toxic effects due to cereal consumption (117). Population thresholds can help both the food industry and regulatory authorities to assess the public health risk and design appropriate food safety objectives to guide risk management (129).

Codex Alimentarius Standard 118 regulates how food for special dietary use for persons intolerant to gluten shall be described depending on the level of gluten proteins. According to this standard, GF foods are dietary foods not exceeding 20 mg/kg gluten in total, based on the food as sold or distributed to the consumer, while labeling standards for foods specially processed to reduce gluten content to a level from 20 to 100 mg/kg can be decided at the national level.

Compared to other countries, Australia apparently has the most stringent threshold regulations for GF labeling. There are regulations for food labeling in Canada, the United States, South Africa, Hong Kong/China, Europe, and Switzerland. Detailed analysis of legislation and labeling rules of these countries is in progress by working groups within the European Union (EU)-funded Network of Excellence "MoniQA" (www.moniqa.org).

In the EU, Commission Regulation No. 41/2009 defines GF foods as foods containing no more than 20 mg/kg gluten from wheat (i.e., all *Triticum* species, such as durum, wheat, spelt, and kamut), rye, and barley in the final food. The maximum tolerable daily intake of gluten was studied in some cases (130, 131) before the regulation was put in force (132). The Regulation excludes pure oats from these grains, given that current evidence indicates most people with CD are able to tolerate pure oats. Furthermore, the category "very low gluten" foods was introduced, in line with the stipulations made by Codex Standard 118-1979. These foods are not to contain gluten from wheat, barley, and rye at a level equal to or higher than 100 mg/kg.

Safety of Gluten-Free Food Products

Gluten is often a hidden contaminant of many foods. Scientifically, the definition of gluten is not exact as gluten represents a mixture of different proteins having different chemical properties and several origins like wheat, barley, and rye. The Codex Standard 118-1979 for example defines gluten as " *…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 M NaCl.*" Patients with gluten intolerance or CD have different clinical sensitivities for gluten and are facing safety issues when selecting GF products for consumption. GF products comprise mostly various types of biscuits, pastry, pizza, pasta, or bread, which are based on naturally GF flours from rice, maize, soya, amaranth, or wheat starch. These foods tend to be less tasty and have less nutritional value due to the lack of gluten (133). This indicates a strong need of quality GF foods (134). In addition, there are hidden sources of gluten originating from cross-contamination and certain ingredients (22) that are often not labeled. GF maize or rice flours are often contaminated with wheat, and detection of gluten at trace levels in a matrix that contains large amount of other prolamins is difficult, posing potential risks for the most sensitive celiac patients (135). Enzymatically altered foodstuffs (beer and malted barley) contain hydrolyzed gluten, and gliadin-like epitopes were detected in such products, which raises safety issues (9, 136). The tolerance threshold for oats also varies among CD patients (5, 137–141). Despite the fact that oat toxicity has not yet been completely resolved, there is a growing need to include oat into a GF diet due to its high nutritional value (22, 142). Uncertainties and doubts related to food products containing or not containing any kind of gluten can be properly addressed once a reliable gluten quantification methodology is made available that unambiguously reveals whether a toxic amount of gluten is present in certain foodstuffs and, thus, provides safety for human consumption.

Analytical Approach for the Extraction and Detection of Gluten in Food

A review on a variety of methods for the determination of gluten in food products has been published (143). In this section, we describe advantages and drawbacks of currently available methods for gluten detection and their applicability for enforcing regulatory limits. In practice, proteins or DNA are targeted for this purpose.

There are a few approaches so far that claim the successful detection of gluten toxic peptides or proteins present in various food matrixes. The figures of merit of these methods vary not only due to the limitations of methods per se, but many of the difficulties are due to the complexity of food matrixes and the physicochemical properties of gluten proteins. Their solubility may be incomplete due to molecular size, heterogeneous surface properties, inter- and intrachain covalent bonds, and sensitivity to temperature and chemicals.

Several analytical methods are used to characterize cereal proteins and their encoding genes: isoelectric focusing (IEF), A-PAGE, SDS-PAGE, reversed-phase (RP)-HPLC, size-exclusion HPLC (SE-HPLC), high-performance resolution capillary electrophoresis (HPCE), matrix-assisted laser desorption ionization time-of-flight MS (MALDI-TOF-MS), ELISA, immunoblotting, and PCR. A recent study has investigated 60 wheat germplasm accessions to characterise their HMW-GS composition by several of the above-mentioned methodologies and identified advantages and disadvantages of each technique; however, the authors did not come to a conclusion regarding the choice of the most appropriate technique (144). Selection of a method for gluten analysis is highly dependent on the application or the required specificity and sensitivity.

Method standardization for the determination of gluten content in foods, especially if it is labeled GF, has its limitation due to the fact that gluten refers to a complex mixture of proteins found in several different cereals with natural variability in their protein profile, which is determined genetically and influenced by the environment. Therefore, besides the selection of the most accurate and sensitive measurement technique, an internationally recognized gluten reference material should be produced to be able to carry out at least interlaboratory comparisons (145).

Detection of gluten is so far mainly based on immunological tests (mainly ELISA kits), the proteomic approach involving MS, and the genomic approach applying

PCR. However, none of them is considered universally acceptable for the reliable high sensitivity detection of gluten in food. All technologies have advantages and disadvantages that make them more suited to specific tasks. ELISAs, for example, are easy to use, and many analysts are familiar with this technique. Also, the equipment for ELISAs is relatively inexpensive and widely available. At the same time, MS has made considerable progress and can detect many allergenic proteins at low concentrations (mg/kg or ppm). While cleanup and extraction still present some problems, the potential benefits of multiscreening and quantification are many fold. A downside of MS is the price of the equipment. However, as with all developing technologies, the cost will inevitably decrease with market competition and increasing numbers of instruments (146).

Sample Preparation—Protein Extraction Methods

The three fundamental steps in sample preparation for subsequent protein analysis are cell disruption, inactivation or removal of interfering substances, and solubilization of the proteins (147–150). When selecting or developing a protein extraction method for cereal analysis, several important variables have to be controlled in order to achieve well-resolved and reproducible gels or chromatograms. First, all possible disturbing interactions between proteins and other components present in the sample should be taken into consideration during method development. The use of reducing agents to enable the extraction of large molecules is essential; blocking the reforming of disulfide bonds via a reducing agent is also necessary in sample preparation methods for further electrophoretic or chromatographic analyses. The blocking reaction is usually an alkylation, which can affect proteins' electrophoretic mobility, ionic strength, and, thus, the resolution of electrophoresis or chromatography (45, 151). Salt concentration also affects the separation of proteins and can cause baseline disturbances and lower resolution (152). Organic solvents, such as acetonitrile (ACN), provide a stacking agent and have an impact on the sample matrix (153–155). Solubility enhancers such as urea or SDS have been widely used in protein extraction methods for cereal analysis. The presence of these agents, however, disturbs the further analysis of proteins or peptides. The removal of SDS using acetone allows proteins to be further characterized by electrophoresis or chromatography (156). An alternative to resolubilize proteins can be a physical method such as sonication. The intensity and duration of such a treatment influence the integrity of protein polymers (157).

The stability of protein extracts is an important consideration as well. Factors such as aqueous extraction and short-term storage, handling chromatographic fractions, freeze-drying and reconstitution were investigated (158). Microbial contamination from water induced proteolytic breakdown products in the extracts, thus changing the polymeric protein distribution by reducing the number of polymers and increasing the amount of monomers.

Protein extraction based on solubility was most commonly used in early cereal protein analysis (23, 24). More specific extraction methods have been developed as the knowledge about cereal protein complexity increased (159). Depending on the analytical target and specificity of different techniques, protein extraction methodologies were developed including single or multiple steps (160–163).

Single step extraction methods in cereal analysis aim to produce the bulk of proteins that represents either the water-soluble or water-insoluble fraction of a given cereal. Single step protein extraction from GF cereals [brown rice, maize, and teff (Eragrostis tef)] was found to be specific in terms of use of buffers containing urea and dithiotreitol (DTT), but differing in the presence or absence of solubilizing agents (SDS and/or thiourea; 164). Although a one-step procedure for protein extraction would be highly desirable due to simplicity and reproducibility, there is no single method of sample preparation that can be universally applied to all kinds of samples analyzed (147).

A sequential extraction protocol allows the separation of protein fractions of a certain solubility or size that are suitable for further analysis. Although a large number of standard protocols have been published, these protocols have to be adapted and further optimized for the type of sample to be analyzed. Sample preparation should be as simple as possible to increase reproducibility, and protein modifications during sample preparation must be minimized. In particular, proteolytic enzymes in the sample must be inactivated. Samples containing urea must not be heated (147). Basic proteomic sample preparation protocols apply sequential extraction of water-soluble, hydrophobic, strongly hydrophobic, and membrane proteins. The extraction procedure of cereal proteins is quite different from this.

Sequential extractions with various organic solvents and the use of RP-HPLC are frequently applied to fractionate glutenin proteins to HMW and LMW glutenin subunits (165, 166). A selective precipitation of the LMW-GS fraction with 80% acetone following the prior extraction of gliadin was also introduced (167), and alcohol extraction of glutenins after reducing disulfide bonds was found to be useful (168). These methods were further optimized, and high purity and yield of individual protein fractions were achieved. Wheat flour was washed with Tris-HCl containing 4% Triton X114 before extracting the residual gluten with 70% ethanol at the preparative scale (169). The ethanol-washed and freeze-dried gluten fraction was used to extract glutenin from the pellet by suspension in 50% propanol, centrifugation, and dialysis against 0.04 M acetic acid. The supernatant contained the gliadins. Further extraction of the glutenin fraction with 50% propanol and 1% DTT was followed by an adjustment to 65% propanol and 1% DTT, and the separation of the pellet and the

supernatant led to the production of HMW- and LMW-GS, respectively (165, 169). This time-consuming process was further optimized, and a simultaneous extraction of both HMW- and LMW-GS was developed (170). Pre-extraction of gliadins with 50% propanol was followed by glutenin extraction with 1% DTT containing 50% propanol. A partial subsequent precipitation of C and B type LMW-GS was observed with increasing propanol concentration up to 85%. For sequential extraction of wheat flour proteins, extraction with 0.3 NaI in 7.5% 1-propanol followed by 2% SDS, 25 mM DTT in 25 mM Tris, proved to be successful (171).

A sample matrix containing gliadins together with other prolamins, such as hordeins, rice prolamins, and zeins, represents a challenge when gliadin detection is the aim. A large amount of other prolamins with a trace level of gliadins causes problems due to co-extraction of gliadins and other prolamins. The inclusion of an acetic acid solubilization step after a conventional alcoholic extraction allows the separation of gliadins from other prolamins and allows the further analysis of the gliadin fraction (172).

Most recently, a two-step extraction method was optimized for gluten proteins from wheat flour (173). This method does not use pre-extraction of albumins and globulins and claims an overall higher yield of gluten proteins. Extraction with 50% isopropanol (no DTT) removes ω-gliadins and LMW-GS encoded in the D genome, which was not possible in earlier methods that used reducing agent (DTT) at this step. This protocol also served to support the solution of another issue: wheat varieties showed large variation of epitope-specific T cell responses and antibody binding between varieties; therefore, the analysis of immune responses of different wheat cultivars is important.

Extracted proteins from a food matrix may need further purification depending on the sensitivity and specificity of the separation and detection methodology in use. In some cases, a subsequent enzymatic digestion of the extracted proteins is needed to be able to analyze them. In other cases, a set of chemical treatments of the extracted protein is required for further analysis. For example, the sample should contain peptides, not proteins, if the detection method is MS, and it is not necessary for the sample to be enzymatically digested for electrophoretic separation or HPLC analysis, where the reduction of polymeric subunits alone is sufficient using chemical treatments [DTT, 2-mercaptoethanol (2-ME), etc.]. Besides, there are possible factors—mainly due to sample handling, contamination, storage, etc.—that may affect the success of sensitive analytical measurements. These factors are often overlooked, and misleading explanations are given for unexpected results.

For HPLC, the protein extracts should be heated at 80°C for 2 min (174) to achieve stability for several days. Beyond heat treatment, the use of sterilized water at each step in the chromatographic sample preparation is recommended. Freeze-drying, usually carried out after elution from separation columns, causes irreversible loss of solubility of proteins due to the formation of polymeric protein aggregates in ACN solutions. Therefore, membrane concentration is recommended for chromatographic sample preparation rather than freeze-drying (158).

The chromatographic analysis of intact gluten proteins proved to be insufficient for identification purposes, and enzymatic digestion of proteins prior to MS/MS analysis was suggested. The general use of trypsin digestion failed in the case of gluten, which contains low levels of Arg and Lys, and a replacement by chymotrypsin was shown to effectively produce peptides suitable for chromatography (175).

To simplify protocols, the possibility of avoiding protein extraction from food prior to enzymatic digestion was investigated. Direct enzymatic digestion of food samples was developed for LC/MS/MS analysis (176). Pepsin, trypsin, and chymotrypsin were used to model gastric and duodenal conditions before chromatography. The procedure is a further development of the digestion procedure introduced by Marti et al. (177) but without the use of elastase and carboxypeptidase A. The digestion releases suitable marker peptides if gluten is present by applying a 100:1 protein: enzyme ratio and reaction times of 2 h with pepsin and 30 min with trypsin/chymotrypsin.

Critical steps in sample preparation for ELISA tests are the protein extraction from the food matrix, especially when the food is processed, and the subsequent resolubilization of the purified proteins. The AOAC ω-gliadin ELISA uses 40% ethanol for extraction of gluten (178). The R5 ELISA requires a cocktail for protein extraction and is capable of extracting prolamins from heated and unheated foods as well (179). The method involves guanidium hydrochloride (GdnHCl) and 2-ME for solubility enhancement of gluten (180, 181). This cocktail does not extract glutenins, and the ELISA test quantifies the gluten content by doubling the measured gliadin content.

A recent study aimed to replace 2-ME, as its reducing power was considered to be weak, with the nontoxic and odorless tris-2-carboxyethyl-phosphine in this cocktail (182). This sample preparation method was found to be as effective as the original cocktail for ELISA as well as for HPLC applications.

Based on the fact that Arg suppresses protein-protein interactions, it was assumed and proven that it increases the solubility of large protein molecules like gluten (183). The solubility profile of proteins extracted with Gdn HCl and Arg was the same, indicating equal efficiency in solubilizing gluten proteins, but the solvent concentration used was much less (2 M) for Arg than for GdnHCl (6 M).

A quantitative ELISA compatible extraction method of gluten from processed or nonprocessed food matrix

was suggested by Villar et al. (184). The method uses an extraction cocktail (GdnHCl or urea as a dissociating agent, phosphate buffered saline or Tris as a buffer, and 2-ME as a disulfide bond reductor), which does not affect ELISA quantification. With this method, the entire toxic fraction of the gluten can be dissolved because the cocktail ingredients open the conformation of proteins. Consequently, insoluble protein formed aggregates during heat processing in food making. These aggregates can be extracted with 50–70% ethanol–water solution and quantified by ELISA (184).

Competitive R5 ELISA is compatible with ethanol extraction, which can extract only the native proteins and not the processed ones (181). However, the combination of competitive R5 ELISA and enzymatic digestion of prolamins provides a reliable quantitative determination of partially hydrolyzed gluten (185).

The solubility of proteins determines their extractability for any analysis. The use of different salts showed the solubility reduction effect of NaCl and the opposite effect in the case of $MgCl_2$ (183). Therefore, the use of NaCl during extraction should be replaced by another cationic salt.

Immunological Methods

Protein-based methods usually involve immunological techniques that use either human antibodies like serum IgE of allergic patients or antibodies raised in animals against purified allergens or allergenic food extracts (mixtures of proteins). Immunological approaches in gluten detection appear so far to be the most sensitive methodology available. They are widely used, but results show poor reproducibility due to the use of different reference standards, extraction protocols, and antibodies (145, 186, 187).

Several ELISA test kits on the market claim to determine the gluten content in food products (188, 189). Quite often the composition of extraction chemicals, the nature of the antibodies, and the calibration material in commercially available test kits are not disclosed. Some methods are specifically for detecting wheat, while others detect only rye or other toxic cereal proteins (178). Antibodies with the ability to recognize the presence of all toxic cereal species were also developed and are now widely used (190).

ELISA methods mostly use either gliadins or hordeins as reference standards, and antibodies specific for gliadins, e.g., Prolamin Working Group-gliadin or certain gliadin epitopes (e.g., R5; 9). Consequently, these methods do not consider other possible toxic proteins, e.g., glutenins (176, 191), and provide an estimation of the total gluten content in foodstuffs using a conversion factor equal to 2. This prediction is based on an assumption that gliadins provide approximately 50% gluten proteins (192). Besides the use of certain reference standards, the unique solubility properties of some molecules could also cause erratic results for the gluten content as determined by ELISA tests via an inappropriate extraction step. A good example of this is the alcohol-insoluble toxic glutenin content in wheat gluten that remains insoluble during alcoholic extraction (176). Only a few studies were done to compare different types of ELISA methods (143, 179, 188), and, thus, the uncertainty of such measurements has not yet been revealed (193).

Antibodies Used in Immunological Methods

ELISA tests yield different gluten amounts depending on the antibody and reference material used for raising the antibody as well as for calibrating the assay (193). Several commercially available test kits are based on the monoclonal mouse antibody (mAb) 401.21 raised against ω-gliadins of the Australian wheat variety Timgalen (194). The advantage of using this antibody is that this gliadin fraction does not denature during heating.

The PN3 antibody was raised against a synthetic 19-mer peptide (LGQQQPFPPQQPYPQPQPF) of α-gliadins (195). It reacts to a similar degree with gliadins, LMW-GS, hordeins, avenins, and secalins, but it does not react with HMW-GS. Antibodies were developed for certain LMW-GS types, not particularly targeting toxic sequences but providing the ability to detect LMW-GS in any applications (196).

A relatively new mAb called R5 was developed against an ω-secalin extract (197), and the assay based on this antibody recognizes gliadins, hordeins, and secalins to a similar degree but does not recognize avenins (190). The key motifs of recognition are the heat resistant QQPFP, QQQPFP, LQPFP, and QLPFP epitopes (181). QQPFP is present twice in α-gliadins and 11 times in γ-gliadins (198), and it is also present in barley hordeins and rye secalins (190). This antibody does not cross-react with proteins from inherently GF grains.

The G12 mAb was raised against the toxic 33-mer from α-gliadin as a detection antibody together with antibody A1 as a capture antibody. The advantage of these antibodies is the capability of recognizing oat avenins (199). The individual specificity of antibodies stresses the importance of validating immunochemical methods for gluten detection (193).

Commercial ELISA Kits for Gluten Testing

A test kit based on an immunological reaction consists of a reference material, one or more specifically developed antibody solutions, and other chemicals like buffers and extraction cocktails. There are several commercially available test kits on the market for gliadin and/or gluten detection: RIDASCREEN Gliadin ELISA kit (R-Biopharm, Darmstadt, Germany), Haven Gluten Assay Kit (Allmark, Chester UK), Gluten Check Assay

(Diagnostic Innovations, St. Asaph, UK), Gluten ELISA Kit (Tecna, Trieste, Italy), Gliadin ELISA kit (IM3717, Immunotech, a Beckman Coulter Co., Prague, Czech Republic), BioKits Gluten Assay Kit (Tepnel BioSystems Ltd, Manchester, UK), INGEZIM GLUTEN (Ingenasa, Madrid, Spain), etc. (*Note*: This information may be incomplete and does not constitute endorsement by the authors.)

Gliadins are considered so far to be the best markers to measure traces of gluten in food. For a quantitative method, an accepted standard that is used for calibration should be available (145, 193). A European gliadin reference material was developed by the Working Group on Prolamin Analysis and Toxicity. It was developed from 28 European wheat varieties and was evaluated and characterized by Denery-Papini et al. (200), van Eckert (201), and van Eckert et al. (202). Based on the findings and suggestion of Konic-Ristic et al. (120) and due to the fact that the amounts of gliadins and glutenins vary among wheat samples, there is a need to develop a new representative mixture of gliadins isolated from local wheat species used for bread production that may be used as a standard antigen and for calibration purposes. As glutenins can also be CD toxic, newly developed assays should be able to detect gliadins and glutenins. A reliable glutenin reference material together with the mAb 401.21 antibody, which detects both gliadins and glutenins, may help to fill this gap (193).

Using the approach of Skeritt and Smith (194), a barley specific ELISA was developed and commercialized in the early 1990s (178, 203). This method became approved as AOAC *Official Method*^{SM} **991.19** but only for gluten levels above 160 mg/kg. This method was unable to detect hordeins. The applied mAb 401.21 antibody has been used in commercial assays like Cortecs (now BioKits Gluten Assay Kit, Tepnel BioSystems Ltd); RIDASCREEN Gluten Kit R6101 (formerly a kit of R-Biopharm); and Transia Plate Gluten (Transia GmbH, Ober-Mörlen, Germany). This antibody recognizes mainly HMW-GS subunits, presumably LMW-GS, ω-gliadins, and, to a small degree, α- and γ-gliadins. This leads to a miscalculation of the total gliadin and, thus, the gluten content, mainly due to large variations (6–20%) of the ω-gliadin percentage in different wheat varieties (188). For the same reason, the ω-gliadin ELISA does not quantify accurately barley prolamins (190), and underestimates gliadins from durum wheat and overestimates prolamins from triticale and rye (179). There is also cross-reactivity to various gluten-containing grains (204).

The PN3 antibody (195) reacts to a similar degree with gliadins, LMW-GS, hordeins, avenins, and secalins, but does not react with HMW-GS. The R5 antibody-based ELISA test was developed by Valdes et al. (190) and validated by Mendez et al. (205). The R5 ELISA has been endorsed by the Codex Committee on Methods of Analysis and Sampling as a type 1 method for determination of the gluten content in GF foods (Codex Standard for Foods for Special Dietary Use for Persons Intolerant to Gluten; 206, 207). R5 is used in commercial kits such as RIDASCREEN Gliadin and INGEZIM GLUTEN. The R-Biopharm gluten test kit has been certified by AOAC to perform as stated by the manufacturer. Gliadins are detected with an R5 ELISA in the range of 2–5 mg/kg food, having an LOD of 3 ppm gluten (1.5 ppm gliadin) and an LOQ of 5 ppm (208). The assay sensitivity for R5 ELISA is 0.78 ng/mL for gliadins and 0.39 ng/mL for hordeins and secalins. The system proved insensitive to the nonceliac-toxic cereals maize, rice, and oats, and it was noncultivar dependent. It was also able to detect gliadins and hordeins in unprocessed and heat-processed wheat- and barley-based products, and to estimate the gluten content of hydrolyzed foods. Contamination of oat samples with wheat gliadins or barley hordeins can be detected by sandwich R5 ELISA using either gliadins or hordeins as standards (172). Although there is no cross-reaction with inherently GF grains, sandwich R5 ELISA overestimates hordeins especially in barley contaminated oats, unless a hordein standard is used for the tests (209).

In the sandwich format, R5 ELISA fails to detect some forms of gluten due to overlooking the glutenin fraction (188, 210). These publications are again drawing attention to the need for reference materials other than gliadins (211).

The G12 mAb was applied in both sandwich and competitive ELISA tests with high sensitivity, reproducibility, and repeatability (RSD > 15%). LOD for gliadins was 0.6 ppm in the sandwich format where G12 and A1 antibodies were used together [this is 1/3 of the LOD of the method of Valdes et al. (190)] and 0.44 ppm in the competitive format. Consequently, the LOD for gluten in the competitive assay was less than 1 ppm, and the method is applicable for both native and partially hydrolyzed cereals (199). Gliadin from Sigma (St. Louis, MO) and the European gliadin reference material were used as standards in these ELISA tests.

Prolamins in processed foods can be partially or totally hydrolyzed, e.g., in malted barley. This represents a limitation of ELISA tests that cannot recognize hydrolyzed gluten proteins. Neither the ω-gliadin ELISA nor the sandwich R5 ELISA is able to quantify hydrolyzed gluten (208). Sandwich ELISAs require two epitopes (or two antibody binding sites), which is not always the case when proteins are hydrolyzed (179). The R5 antibody-based competitive ELISA was specifically developed to overcome this limitation and so hydrolyzed gluten can be detected (212, 213). The combination of the competitive R5 ELISA and peptic-tryptic digestion of prolamins was shown to be suitable for the determination of partially hydrolyzed gluten in fermented cereals with LOD 2.3 and LOQ 6.7 ppm (185). The validation of this method is planned but not yet published. One drawback

of the competitive R5 ELISA is that it is compatible only with ethanol extraction, which is unsuitable for detecting heated proteins (181).

Deamidation can also occur during food processing, and this has an influence on food safety (214). Again, the R5 ELISA is not capable of detecting deamidated gluten, which is a risk for CD patients (4). Based on the fact that the R5 antibody recognizes gliadin epitopes, other T-cell stimulatory epitopes such as those present in LMW-GS cannot be detected (173). For an accurate quantification of the T-cell stimulatory epitope load, several specific antibodies would be necessary. Consequently, ELISA tests on their own do not provide safety for people having gluten intolerance. These methods are not clinically validated as an indicator of food toxicity toward CD patients (211). The declaration of a product as GF is highly dependent on what method was used to measure the gluten content and what food matrix was analyzed (179).

Proteomic Approach for the Identification of Gluten Allergens

The development of any detection method relies on the identification of a target analyte. In the case of food allergens this is not so straightforward. A particular allergenic food can often contain a number of proteins that can cause allergic reactions. Proteomic research is usually based on two components: a separation of proteins and an identification of individual proteins (215–220).

In cereal analysis, proteomics is an essential tool to separate, identify, and characterize individual proteins and relate them to cereal quality (159, 221) and to the research of specific health issues like CD. Proteomics workflow involves a high resolution separation technique [e.g., two-dimensional (2D) electrophoresis or chromatography]; a microanalytical process (N-terminal sequencing or MS) for single protein identification; characterization of post-translational modifications; and database searching [National Center for Biotechnology Information (http://ncbi.nlm.nih.gov), Universal Protein Resource (http://uniprot.org)] with protein database search engines such as Protein Lynx (Waters, Manchester, UK) or Mascot Distiller (Matrix Science, London, UK).

Proteomics has been proven to be effective in general protein identification and due to this fact its application for identifying cereal toxicity is increasing. The level of confidence in the identification of certain proteins is determined by the technique used, and therefore, can hide or reveal differences between proteomes. These differences may be tiny, e.g., point mutations in HMW-GS Ax 2*B in wheat cv. Bánkúti 1201 (222, 223), or insertion of a couple of base pairs into the DNA sequence coding the protein. An example for the latter is the frequently investigated Bx7 HMW-GS sequence in various wheat cultivars. The Glu-1 Bx7 HMW glutenin subunit of different cultivars was first reported to have differences in the DNA sequence (224), and later the presence or absence of an 18 bp insertion in different wheat cultivars was proven (223, 225–227). At the level of proteins, these differences can be identified based on SDS-PAGE mobility; however, no differences can be found by RP-HPLC (228, 229). Similarly, gene-derived and MALDI-TOF data showed contradicting results regarding the presence of this insertion into the coding region of the HMW-GS gene in a certain cultivar (41). Consequently and analogously, identification of toxic sequences in gluten proteins needs to be highly sensitive and, therefore, requires high resolution and high sensitivity methods.

Electrophoretic Techniques for Cereal Protein Separation

The separation and identification of cereal storage proteins began with the application of moving boundary electrophoresis, slab gels, and HPCE and has been further developed to provide high resolution (HR) separation with techniques such as SDS-PAGE, A-PAGE, IEF, free zone capillary electrophoresis (FZCE), and 2D electrophoretic techniques (230, 231), even in specific combinations like HR-2D-HPLC-HPCE (45). In proteomics 2D IEF×SDS-PAGE is used for protein separation followed by tryptic digestion and MS peptide mapping for identification (156, 159, 216, 220, 232, 233). Further 2D electrophoresis combinations have been reported in cereal proteomic studies, like A-PAGE ×SDS-PAGE (234–236).

The efficiency and specificity of electrophoresis always depends on the applied extraction methodology, which can explain possible overlaps among protein fractions during electrophoretic analysis (45). Sequential extraction protocols are in use to avoid this problem and improve the resolution of protein profiles as well (171, 173). From the quantification point of view, appropriate measures should be taken in 2D gel analysis because, for example, ω-gliadins can diffuse during destaining and storage (156). Several factors (salts, proteases, and the presence of polysaccharides, nucleic acids, lipids, phenols, and chemical residues) related to sample preparation can have disadvantageous effects on the electrophoretic resolution.

SDS-PAGE is based on separation by size and is widely used for qualitative characterization of all cereal proteins (237) due to quantification issues (238, 239). The separation of HMW-GS of wheat proteins (240–242), barley proteins (160, 243, 244), and oat and rice prolamins (160, 245, 246) is primarily carried out using SDS-PAGE, but there are specific protocols for LMW-GS as well (247). For more hydrophobic prolamins such as sorghum kafirin and maize zein, SDS-PAGE can also be used with the application of urea in the gel (27, 29, 87). For large polymers like wheat glutenins, a special SDS-PAGE technique, multistacking electrophoresis, was developed, and a better separation was achieved (248).

A-PAGE is based on differences in protein charge

density and is used for fingerprinting, especially in cultivar identification studies (243, 249). Wheat cultivar identification is usually based on the differences found in gliadin composition by A-PAGE (250). A-PAGE can be used for the separation of α-, β-, γ-, and ω-gliadins (160, 251, 252); for barley hordeins (244); for oat avenins and rice prolamins (245, 253); and for glutenins in the presence of the solubility enhancing agent urea (236); both at the analytical and preparative scales (254).

IEF is based on the different isoelectric points of protein fractions, separating them in an immobilized pH gradient. It has been used for the separation of storage proteins. Strong solubilizing agents, so-called chaotrops like urea and thiourea, used in this technique as protein solubility can be difficult to maintain during IEF separation (160, 255).

FZCE is based on the differences in proteins' charge density, producing analogous separations as A-PAGE with same migration order (42, 256–258). At least four different FZCE methodologies have been used for cereal protein separation with various problems, limitations, and improvements (45).

Chromatographic Analysis of Cereal Proteins

One-dimensional HPLC methods are most widely used for the separation of cereal proteins (259). SE- and RP-HPLC methods have been used to characterize size distribution, protein polymorphism, and biochemical characteristics based on the differences in molecular size distribution and hydrophobicity of cereal storage proteins (165, 166, 260–263). The apparent unextractable polymeric protein (UPP) content (%) proved to be a good indicator of changes in molecular size distribution of cereal polymeric proteins and is in use in many applications dealing with protein quality (223, 264–266). RP-HPLC hydrophobicity profiles of cereal proteins together with gel electrophoresis profiles provided a reasonable means for characterization of cereal proteins, especially when they are part of a complex sample matrix such as food. HPLC and CE alone have been used for cereal protein characterization from flour, but not from complex food. On the other hand, the analysis of proteins from such matrixes requires special protein extraction methods prior to chromatography. Up-to-date research projects are using chromatography for the separation of proteins on the analytical and preparative scales and applying high-throughput detection methods such as MS for identification. Ultra high-performance LC (UHPLC) can be used either coupled with medium resolution (such as triple quadrupole MS, QQQ) or high resolution (e.g., TOF-MS) detection techniques. Compared to HPLC/MS systems, UHPLC/MS produces a significant increase in peak resolution and spectral quality. The extra resolution reveals new information about the samples, and, in addition, the extra speed provided by the UHPLC system reduces chromatographic run time significantly.

MS and Coupled Techniques

In proteomics, high-throughput protein identification MS methods are replacing traditional methodologies like N-terminal sequencing. Soft ionization techniques are usually used for protein mass spectrometry, i.e., MALDI or electrospray ionization (ESI; 267).

The principle of MALDI is that ions are generated from solid-phase samples in a high vacuum by short laser pulses (268–271), and are accelerated by an electric field into a TOF mass analyzer. The flight time is proportional to the m/z of the analyte; therefore, the instrument can be calibrated with analytes of known mass. Other than TOF mass spectrometers, MALDI can be coupled also with ion trap and quadrupole analyzers (272–274). A more sensitive method for protein identification, MALDI-MS-peptide mass fingerprinting, involves enzymatic digestion of proteins resulting in a unique set of peptides that are characteristic in mass for the protein and provides its fingerprint (275–279). MS sequencing is performed in MALDI-MS by post-source decay (PSD; 280, 281). The choice of matrix has a big influence on the sequence coverage in peptide mapping (282, 283).

The direct identification and exact molecular mass determination of cereal proteins without prior separation has been achieved by MALDI-TOF-MS (35, 38, 40, 267, 284, 285). Quantification of gliadins in processed and unprocessed foods can also be carried out by MALDI-TOF-MS (135, 286, 287) together with screening for the presence of other toxic cereal prolamins (284, 288). Protein structural studies and verification of the gene-derived sequence of gluten proteins (36, 37, 41, 289, 290), and the verification of possible post-translational modifications of a protein, were also carried out using MALDI-TOF-MS (283). Prolamins of different plant origins (gliadin, hordein, secalin, and avenin) can be selectively differentiated with MALDI-TOF-MS detection, even if they are present simultaneously in a complex food matrix (291).

ESI is based on a liquid phase ion generation process following residual solvent evaporation or field desorption (292, 293). Nano-ESI-MS is an improvement of the basic ESI technology that has a reduced sample flow rate into the MS instrument, thus allowing more efficient peptide ionization (294). Coupled techniques like ESI-MS/MS or ESI-Q-TOF-MS are also in use (295). MS sequencing is performed in ESI-MS by collision-induced dissociation (CID; 296).

Gluten proteins yield relatively few peptides of a size suitable for MS/MS analysis when digested with trypsin (291). Combining MS techniques with gel electrophoresis and/or predigestion, preferably with more than one enzyme, or a specific sample preparation procedure, enhances the efficiency and specificity of proteomic identification methods, thus allowing more comprehensive protein structural measurements. The coupling of separation

techniques [2D gel electrophoresis (2DE) or RP-HPLC followed by SDS-PAGE] to MS/MS was suggested to be an efficient method to test gliadin identity (62, 297, 298). Similarly, the identification of barley gluten peptides was carried out using PAGE/MS/MS (289, 299). The actual molecular masses of ω-gliadins of different wheat species (e.g., winter wheat, spring wheat, wheat-rye hybrid, spelt, durum wheat, emmer, and einkorn) were studied by MALDI-TOF-MS and SDS-PAGE. Molecular masses obtained from the MS measurements were by far lower than the values derived from SDS-PAGE mobility (300). Certain HMW-GS of selected wheat cultivars were sequenced by tryptic peptide mapping and MALDI-TOF-MS (282, 283).

Gluten protein markers were identified with capillary LC/Q-TOF-MS in the nanospray ESI+ mode following a tryptic digestion (301) of various beer products made from gluten-containing and GF raw materials (302). Complementary analysis with ELISA and MS/MS showed that some samples contained gluten detectable with MS but not ELISA, despite the label claiming nongluten beer. Coupled LC/MS/MS techniques can be even more effective if capillary LC is used for separation. Nonhydrolyzed gliadins and their peptic digests were analyzed by capillary LC/ESI-Q-TOF-MS, and some toxic epitopes like the 33-mer were used for calibration (303). This technique was successfully used for the detection of gliadin epitopes from fermented foods (303–305). To monitor and characterize the tTG2 deamidation of gluten peptides, a quantitative, MS-based approach was developed (17). The kinetics of tTG2-mediated deamidation of gliadin-derived, DQ2-restricted epitopes was shown to have large variations in the degree of deamidation between different peptides and between individual glutamine residues within each peptide.

A novel combination of direct enzymatic digestion and LC/MS/MS was developed for the quantification of gluten traces in native and processed food samples, which is based on the detection of six gluten marker peptides (176). The optimized method could detect these gluten marker peptides in the range of 0.01–100 ppm with LOD 0.001–0.03 ppm and LOQ 0.01–0.1 ppm. To minimize the matrix effects and improve the selectivity, a 60 min run time and 2.7 µm superficially porous silica C18 column (Ascentis Express C18, Supelco, Bellefonte, PA) was found to be optimal in the HPLC separation of peptides.

Identification of proteins by MS/MS is based on searching protein sequence databases against spectra generated from proteolytic cleavages of proteins of interest. Databases of allergenic proteins were developed in the early 1990s, and since then this interactive bioinformatics tool is an essential part of proteomics (306, 307). The predictability of potentially allergenic proteins can be carried out since 2004 when an algorithm was developed for that purpose (308).

Gluten marker peptides suitable for ELISA and proteomic studies were identified with the help of such databases and used in several method developments. These peptides are suitable to detect celiac toxic gluten due to the fact that they are all present in wheat, rye, and barley and absent in nonimmunogenic grains. The most important ones, the 33-mer of α-gliadin (53) and the 26-mer of α-gliadin (106), were used to develop antibodies against them and as marker peptides (309–311). Further, seven marker peptides were identified by Sealey-Voyksner et al. (176) that were chosen from a pool of potential markers of 25 peptides. If the protein is not present in databases, MS sequencing is used to allow protein or expressed sequence tag (EST) databases to be searched (312). The NCBI nonredundant database currently (end of 2010) contains almost 16 000 protein records for wheat and related species (62), but obviously not all of them are toxic sequences. The wheat α-gliadins, however, represent a challenge in the identification process by MS/MS due to difficulties of their separation from other gliadins and LMW-GS (291). MS/MS analysis of γ-gliadins of a single wheat cultivar proved that current databases were inadequate for identification, and a complementary investigation of gene expression was strongly suggested (298). According to this, such complete analysis of α-gliadins was carried out, and several new types of α-gliadins were revealed (62). The introduction of multistage and hybrid analyzers provides the ability to generate de novo amino acid sequence information. The interfacing of MS with protein databases allowed completely novel ways of protein characterization (high-sensitivity mapping, post-translational, and other modifications, protein conformations, protein–protein interactions, etc.; 297).

Genomic Approach

Currently, various technical approaches are designed to detect the presence of offending foods used as ingredients in food products. Several methods do not target a specific toxic protein but rather a marker indicative of the presence of the offending food. Any component that is specific for the offending food can serve as a marker to detect its presence. DNA-based methods rely on the amplification of specific DNA fragments by PCR in which specificity is achieved by the use of primers that only facilitate amplification of DNA originating from the offending food. The target DNA is generally species-specific and functions as a marker for the presence of a particular food ingredient.

The basis of the method is a set of oligonucleotides or primers. These primers can anneal to complementary single-stranded DNA, which can be obtained by heat denaturation of normal double-stranded DNA. The enzyme polymerase can add extra nucleotides to the primer by using the genomic DNA as a template. Subsequent heat denaturation and annealing of the second primer to the

newly synthesized single-strand DNA allows synthesis of a complementary strand of DNA. Several cycles of denaturation—annealing—extension result in the amplification of the target DNA fragment that is bordered by the primers used in the PCR. The amplified product can be visualized by staining after gel electrophoresis. This can also provide information on the size of the amplified product; however, it does not prove the identity of the PCR product. Southern blotting, in which the amplified product is detected on the basis of hybridization to a labeled version of the target DNA, provides a means for identification of the amplified product, while DNA sequencing allows a complete identification of such a PCR product.

Another PCR-based method is provided by real-time PCR. This technique requires expensive laboratory equipment, but it provides an accurate method for quantification of the target DNA. In contrast to conventional PCR, real-time PCR does not require post-PCR detection of the amplified product by means of gel electrophoresis. Instead it utilizes detection in real time. For this purpose, the tube in which the PCR takes place also contains a target-specific oligonucleotide probe with a fluorescent reporter dye and a quencher attached to it. The proximity of the quencher to the dye prevents the detection of fluorescence, but, when the probe hybridizes to the amplified target DNA, the 5' exonuclease activity of the polymerase cleaves the probe and, thereby, separates the quencher from the dye that is displaced by the newly synthesized DNA strand. The fluorescence of the free reporter dye can then be measured, and the increase in fluorescence is proportional to the amount of target DNA present in the sample.

Cereal genomes are very large due to polyploidy and local gene duplications (221, 313). The first stage in annotating any genome is to verify the range of gene products, the polypeptides synthesized in a specific situation. Characterizing gene expression can be done via either messenger RNA (mRNA) or protein analysis. However, mRNA characterization may not be a representative indicator of protein expression and often does not show a correlation with expressed proteins present in tissue (314).

Sandberg et al. (315) used real-time PCR methods for the specific discrimination of wheat, rye, barley, and oats in food samples. Specific primers targeting cereal prolamin genes were chosen for the amplification. The methods were applied for detection of toxic cereal contamination in oat samples. The results of the analysis were compared with those obtained with an established ELISA for gluten analysis. The PCR methods were then used as confirmatory methods in food analysis of GF and naturally GF foods.

Olexova et al. (316) optimized a PCR method for the detection of gluten-containing cereals in flours and GF bakery products, and an intralaboratory validation was carried out. By the analysis of model samples of soya flour and cakes, an LOD of 0.1% (w/w) of fine wheat flour was determined. The method was successfully applied to four samples of flours and biscuits designated GF, out of which two flours and one brand of biscuits were found positive for gluten-containing cereal DNA.

Real-time PCR assays using TaqMan probes were applied by Zeltner et al. (317) to detect gluten-containing cereals. Homologous target sequences encoding HMW glutenin were chosen to detect wheat, kamut, spelt, and rye. The sensitivity of the systems was determined by testing different matrixes. In vegetable food matrixes, 2.5 mg/kg wheat and 5 mg/kg wheat in meat products were detectable. The oat- and barley-specific systems resulted in a sensitivity of 10 mg/kg. The genomic approach for the detection of gluten is a good supplementary tool to detect wheat DNA in foodstuffs.

Conclusions

Limitations of immunological methodologies in CD-toxic protein detection and the more widespread availability of MS equipment will advance the more extensive use of this highly sensitive and selective method. The proteomics approach, including the search of peptide biomarkers, is likely the most successful way to develop a widely applicable method for the sensitive detection of gluten in unprocessed and processed foodstuffs. However, the reliable quantification of gluten by an MS-based approach is still in its infancy. Several currently ongoing projects target in a harmonized way various research topics—development of reference materials, protein database building, method comparisons, analysis of different food matrixes, etc.—to generate reliable and generally applicable method protocols applicable for both food producers and analytical laboratories, while at the same time supporting the related legislations and protecting the well-being of consumers suffering from CD.

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