

Proteomic analysis in allergy and intolerance to wheat products

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Owing to its extensive use in the human diet, wheat is among the most common causes of food-related allergies and intolerances. Allergies to wheat are provoked by ingestion, inhalation or contact with either the soluble or the insoluble gluten proteins in wheat. Gluten proteins, and particularly the gliadin fraction, are also the main factor triggering celiac disease, a common enteropathy induced by ingestion of wheat gluten proteins and related prolamins from oat, rye and barley in genetically susceptible individuals. The role of gliadin and of its derived peptides in eliciting the adverse reactions in celiac disease are still far from being completely explained. Owing to its unique pathogenesis, celiac disease is widely investigated as a model immunogenetic disorder. The structural characterization of the injuring agents, the gluten proteins, assumes a particular significance in order to deepen the understanding of the events that trigger this and similar diseases at the molecular level. Recent developments in proteomics have provided an important contribution to the understanding of several basic aspects of wheat protein-related diseases. These include: the identification of gluten fractions and derived peptides involved in wheat allergy and intolerance, including celiac disease, and the elucidation of their mechanism of toxicity; the development and validation of sensitive and specific methods for detecting trace amounts of gluten proteins in gluten-free foods for intolerant patients; and the formulation of completely new substitute foods and ingredients to replace the gluten-based ones. In this article, the main aspects of current and prospective applications of mass spectrometry and proteomic technologies to the structural characterization of gluten proteins and derived peptides are critically presented, with a focus on issues related to their detection, identification and quantification, which are relevant to the biochemical, immunological and toxicological aspects of wheat intolerance.

KEYWORDS: celiac disease • gluten detoxification • gluten-free foods • mass spectrometry • proteomics
• wheat allergy • wheat intolerance

Wheat, together with rice, is the most used cereal for human nutrition and represents one of the main world food resources for mankind, with a total production of about 600 million tons each year globally, 75% of which is destined to food use. In the Mediterranean diet, wheat is used for preparation of bread and pasta, fulfilling a third of the protein and energy daily requirements of an adult person (2400 kcal/day). Owing to its unique functional properties, wheat flour and derived fractions, such as starch and gluten, are also ingredients widely used by the food industry for obtaining products with optimal texture, sensory and functional properties. In particular, wheat proteins play an extremely important role in the determining both nutritional and functional properties of derived foods.

Based on their differential solubility, wheat proteins can be classified into water/salt-soluble albumins and globulins, and water/salt-insoluble gliadins and glutenins (GS; for a recent review see [1]). Gliadins, traditionally subdivided into α , β , γ and ω fractions, are alcohol-soluble components representing about half a gluten protein. GS are subdivided into both low-molecular-weight GS subunits (LMW-GS; in the molecular weight range 20–45 kDa) and high-molecular-weight GS subunits (in the molecular weight range 70–90 kDa) associated by interchain disulphide bonds [1]. LMW-GS can be further subdivided, on the basis of isoelectric point and molecular weight, into the B, C and D groups. Southern blot analysis of wheat DNA has suggested that the number of genes

encoding the α , β and γ -type gliadins is extremely high (more than 100 copies/haploid genome) [1].

The protein fraction is also among the most common causes of either allergy [2] or intolerance [3] related to wheat consumption. In particular, the gliadin fraction has been ascertained as the main factor triggering celiac disease (CD), a currently common enteropathy induced by ingestion of wheat gluten proteins and related prolamins from oat, rye and barley in genetically susceptible individuals [3]. The exact role and interactions of gliadin or its derived peptides in producing the adverse reactions in CD are still far from being clearly explained.

The development of methods for the purification and structural characterization of proteins has been of the utmost interest in food biotechnology research. Research on the structural and physicochemical properties of wheat proteins has been essential for providing clues about their structure/function relationship, which remains to be clarified for many aspects. The analytical capabilities of the proteomic approach are challenged by the complexity of the wheat seed proteome and particularly of the gluten protein fraction. Limited database entries available, complexity arising from sets of homologue proteins, large occurrence of repeated motifs, very low number of basic residues for tryptic hydrolysis (especially for gliadins and for LMW-GS) are some of the drawbacks that complicate the comprehensive proteomic cataloguing of the gluten proteins.

These challenging issues can only be addressed by use of integrated, up-to-date analytical approaches, which taken together constitute the platform of modern food proteomics, and where a pivotal role is played by mass spectrometry (MS). The application of MS to protein analysis has been revolutionized in recent years with the development of electrospray ionization and matrix-assisted laser desorption/ionization techniques, and more recently with the introduction of novel soft ionization techniques. Furthermore, the interfacing of MS with protein databases has resulted in totally new possibilities for protein sequencing, detection of chemical modification at high sensitivity (femtomole to attomole), characterization of protein conformations and protein–protein or protein–ligand interactions, and for proteomic studies in general. The potential of proteomics for research in food science is being increasingly recognized and MS approaches to food and nutrition topics are now emerging as irreplaceable tools for the assessment of quality and safety, and to elucidate the structure/function relationship of protein and peptides in foods.

Allergy & intolerance to wheat

Allergy is a hypersensitivity reaction, induced by immunological mechanisms, which produce a pathological condition characterized by reproducible and objective clinical symptoms, in response to environmental stimuli, normally well tolerated by healthy individuals [4]. Allergic reactions can be provoked by a variety of environmental challenges, including inhalation of dust and particles such as pollen, and by ingestion of foods. They are a consequence of inappropriate immune responses, which may result in inflammatory reactions and tissue damage and, in most cases, involve generation of an IgE antibody response towards the offending environmental agent, defined as allergen (FIGURE 1A). IgE-mediated

allergies develop in two phases: sensitization and subsequent reaction on re-exposure to an allergen. Both stages are triggered by allergens, which are almost always proteins. IgEs bind to the surface of histamine-containing mast cells that, in turn, bind the allergens, becoming cross-linked in the process. This event stimulates the release of mediators such as histamine from mast cells, causing acute inflammatory reactions that become manifested in a range of symptoms.

Wheat and other cereals have been found to trigger two types of IgE-mediated allergic disease. Occupational respiratory allergy (baker's asthma) and rhinitis are reactions to inhaled flour in workplaces such as bakeries. In addition, some individuals may react to wheat proteins as a result of prior sensitization to grass pollen; these peoples are serologically distinct from those who are exposed to flour in a work environment [5]. The second type is properly defined allergy to cereals.

The most recent studies indicate that allergy to wheat is provoked by several different protein allergens, and follows sensitization events that occur through three different ways: respiratory, digestive and through the skin. They contribute to produce different clinical patterns, depending on the route of allergen exposure, target organs and immunological mechanisms involved. The symptoms may manifest as a classical food allergy to ingested wheat proteins including skin, gastrointestinal and respiratory symptoms, or as a rare form of allergy called wheat-dependent exercise-induced anaphylaxis, a rare but very severe reaction, characterized by respiratory symptoms, urticaria, throat constriction and sudden decrease of blood pressure. The variety of responses and symptoms makes the diagnosis, treatment and prophylaxis of food allergy difficult.

Food intolerance is rather a reproducible reaction to some food component ingested by susceptible individuals, with symptoms which can sometimes take days to manifest themselves, differently from allergic reactions, whose symptoms appear very rapidly. Food intolerances may be immune mediated but also non-immune mediated. The most prevalent example of a clearly defined immune-mediated food intolerance is CD, a (non IgE-) immune-mediated enteropathy triggered by ingestion of gluten-containing foods in genetically susceptible individuals, which is thought to affect approximately 1–2% of the population in Western countries [3]. CD pathogenesis is the result of the interaction of a series of complex mechanisms involving genetic, immunological and environmental factors. Genetic factors play a basic role in CD development, as proven by the well-established familiar aggregation of diagnosed cases (5–15%) and by the concordance of approximately 85% in homozygous twins. Linkage studies clearly showed the association of CD with some defined alleles of the class II major histocompatibility complex (HLA). However, CD is also characterized by a strict connection between genes and the environment, as this intolerance is triggered only upon the introduction of glutencontaining foods in the diet of susceptible individuals. Ingested gluten proteins, after proteolytic degradation during intestinal digestion, give rise to several peptides that can stimulate either adaptive or innate immune responses (FIGURE 1B & C) [6].

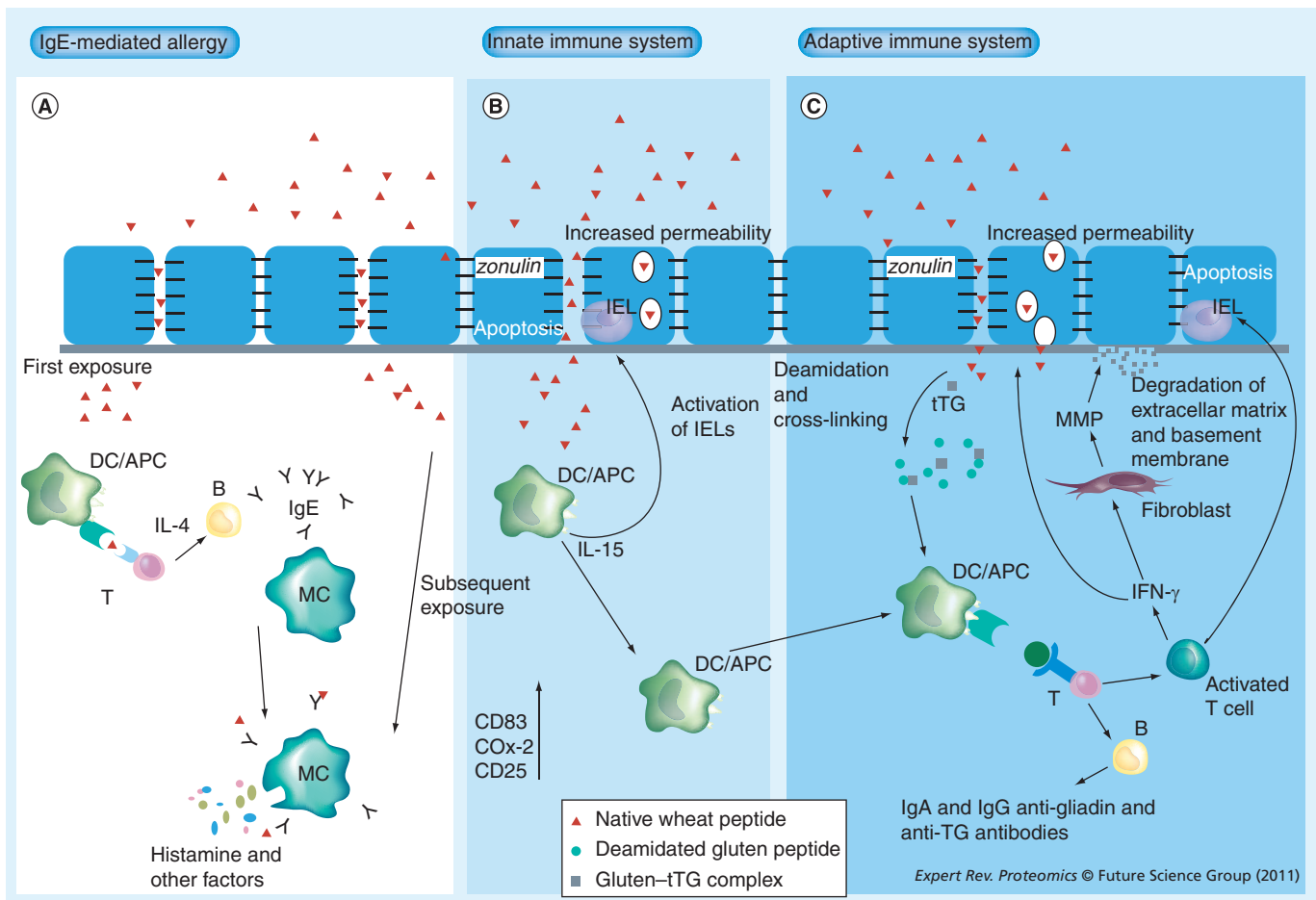


Figure 1. The pathogenesis of wheat-related hypersensitivity reactions and of the possible activation pathway of the innate and adaptive immune systems triggered by wheat-derived protein epitopes. Innate immunity and adaptive immunity are triggered by different gluten peptide sequences in the case of celiac disease. **(A)** Food-derived protein epitopes surviving gastrointestinal digestion can reach the gut epithelium and are endocytosed by APCs, activating naive antigen-specific CD4⁺ lymphocytes. At the first exposure, helper Th2 lymphocytes promote the production of specific IgE antibodies (Abs) by B cells, through the expression of IL-4. Once produced by B cells, IgE Abs bind specific receptors on the outer membrane surface of mast cells, which are found in many different tissues, and basophils, which are found in the blood. This so-called sensitization process does not result in any symptoms itself. A subsequent exposure to the allergen results in the allergen cross-linking with IgE Abs on the surface of the mast cell or basophil membrane. This interaction between the allergen and the allergen-specific IgE triggers the release of a host of chemical mediators of allergy (degranulation). A potential activation of eosinophils has been supposed in specific cases. **(B)** Both the adaptive and innate immune systems are recruited in response to gluten epitopes, as occurs in celiac disease. Gluten peptides reach the lamina propria either via the paracellular (as a consequence of an increased permeability of the intestinal epithelium due to an upregulation of zonulin) or via the transcellular pathways (by using enterocytic vesicles). **(C)** Intruding peptides are deamidated by tTG, enhancing the immunostimulatory potential of the epitopes. The gluten peptides may also covalently bind to tTG. The resulting deamidated peptides are presented in complex with HLA-DQ2 or HLA-DQ8 heterodimers of APCs, such as dendritic cells, macrophages or B cells, to T-cell receptors. This binding strongly activates CD4⁺ T cells, which become able to trigger enterocyte apoptosis and, by secreting proinflammatory cytokines, to stimulate cytotoxic T cells and fibroblasts to produce MMP pattern, which cause epithelial cell damage and tissue remodeling. Stimulated CD4⁺ T cells are also able to induce B-lymphocyte differentiation into plasma cells producing specific anti-gliadin and anti-tTG Abs. On the other hand, gliadin peptides can activate the innate immune system by the stimulation of both dendritic cells and lamina propria mononuclear cells to produce IL-15. This cytokine causes an upregulation of stress protein by enterocytes, which are recognized by the natural killer receptors present on intra-epithelial lymphocytes. APC: Antigen-presenting cell; DC: Dendritic cell; IEL: Intraepithelial lymphocyte; MC: Mast cell; MMP: Matrix metalloproteinase; tTG: Tissue transglutaminase.

Wheat allergies & allergens: state of the art

Food allergy is an increasingly emergent issue in food science and technology. Although this disease is limited only to categories of patients, the number of individuals who are interested has been steadily increasing over the last 10 years, making this field

of primary importance for clinicians, as well as for the food industry. Systematic analysis of cereal and legume species such as wheat, rice, pea, soy, peanut and maize using high-resolution separation techniques in combination with MS and multi-dimensional protein identification is enabling the detection and

identification of several previously uncharacterized allergenic proteins in seeds samples including wheat (FIGURE 2), thus demonstrating the potentiality of proteomic approaches to survey food samples with regard to the occurrence of allergens.

With the exception of CD, which has a *sui generis* pathogenesis and is classified as a food intolerance, non IgE-mediated hypersensitivity reactions to wheat seems to be of marginal relevance. The most recognized allergic reactions are IgE mediated (FIGURE 1A) [7]. IgE antibodies (Abs) specific to wheat albumins and globulins were found in the sera of patients with wheat allergy and with baker's asthma, which is still one of the most common causes of occupational asthma worldwide [8]. Thus far, little is known about the relevant allergens causing baker's asthma, with the exception of α -amylase inhibitors (α AI), thioredoxin and some gliadin components [9]. A classification of the most important groups of wheat allergens is presented in FIGURE 2.

An operating IgE-mediated mechanism of food allergy can be clinically evidenced by a positive IgE test, as well as by the measurement of chemical mediators released. Although detection of allergen-specific IgE in serum is the most straightforward screening method, double-blind placebo-controlled challenges

constitute the reference procedure for the diagnosis of allergy, due to a strong overlap between pollinosis and food allergy [10]. In any case, since the specificity of all diagnostic approaches depends on the purity of the allergens applied, attention of investigators has focused on the identification and employment of defined allergens in diagnosis. However, these determinations are mostly based on immunological evidence, whereas only few of the suspected causative wheat allergens have been structurally characterized or cloned to allow functional analysis [11–20] (a comprehensive list of cloned wheat allergens including database accession number is reported in TABLE 1). Therefore, the reliability of current diagnostic procedures is insufficient. Diagnosis of wheat allergy is further complicated by the low solubility of cereal seed storage prolamins in the dilute salt solutions routinely used in clinical diagnosis, which means that cereal allergy may remain undiagnosed.

Identification of allergens in wheat & derived foods: the proteomic approach

Although the search for clinically relevant allergens has drastically progressed, identification and characterization of allergens still require extensive effort in addition to a large amount of starting

Table 1. Main cloned wheat allergens, including original references and database accession number(s) in Protein Knowledgebase (UniProtKB)/TrEMBL at SwissProt.

Protein name	Database accession number(s) in UniProtKB/SwissProt (EMBL entry)	Entry name	Original ref.
<i>Proteins involved in respiratory allergy</i>			
Thioredoxin h1-type (Tri a 25. 13 kDa)	Q4W1F7 (AJ890020)	Q4W1F7_MAIZE	[11]
Thioredoxin H-type protein (Tri a 25. 13 kDa)	Q4W1F6 (AJ890021)	Q4W1F6_MAIZE	[11]
Thioredoxin h1-type protein (Tri a 25. 13 kDa)	64394 (X69915)	TRXH_WHEAT	[11,12]
D-type LMW glutenin subunits	B6ETR9 (FM212916)	B6ETR9_WHEAT	[13]
D-type LMW glutenin subunits	B6ETS1 (FM212928)	B6ETS1_WHEAT	[13]
LMW glutenin subunits	B6ETS0 (FM212927)	B6ETS0_WHEAT	[13]
LMW glutenin subunits	Q8H0J4 (AJ519838)	Q8H0J4_WHEAT	[13]
LMW glutenin subunits	Q8H0J5 (AJ519835)	Q8H0J5_WHEAT	[13]
LMW glutenin subunits	Q8GU18 (AJ519837)	Q8GU18_WHEAT	[13]
Putative LMW glutenin subunits	Q571Q5 (AJ937920)	Q571Q5_WHEAT	[13]
Thiol reductase (Tri a Bd. 27 kDa protein)	Q7Y1Z2 (AB085212)	Q7Y1Z2_WHEAT	[14]
ω 5-gliadin	Q40215 (AB181300)	Q40215_WHEAT	[15]
ω 5-gliadin (putative ω -gliadin)	Q571R2 (AJ937839)	Q571R2_WHEAT	[16]
α/β -gliadin A-III	Q41546 (X02540)	Q41546_WHEAT	[17]
Wheat serin protease inhibitor-like allergen	B3FHM6 (EU051824)	B3FHM6_WHEAT	[18]
Lipid transfer protein Tri a 14	Q8GZB0 (AJ852536)	Q8GZB0_WHEAT	[19]
<i>Proteins involved in food allergy</i>			
α/β -gliadin A-III	P04723 (M11076)	GDA3_WHEAT	[20]
γ -gliadin	P04853 (M16064)	GDB2_WHEAT	[20]
HMW glutenin subunit (y-type repetitive domain)	P10387 (X12929)	GLT0_WHEAT	[20]
HMW glutenin subunit DX5 (x- and y-type)	P10388 (X12928)	GLT5_WHEAT	[20]
LMW glutenin subunit	P10385 (X07747)	GLTA_WHEAT	[12,20]
LMW glutenin subunit 1D1	P10386 (X13306)	GLTB_WHEAT	[12,20]
EMBL: European Molecular Biology Laboratory; HMW: High-molecular-weight; LMW: Low-molecular-weight. Data from [20].			

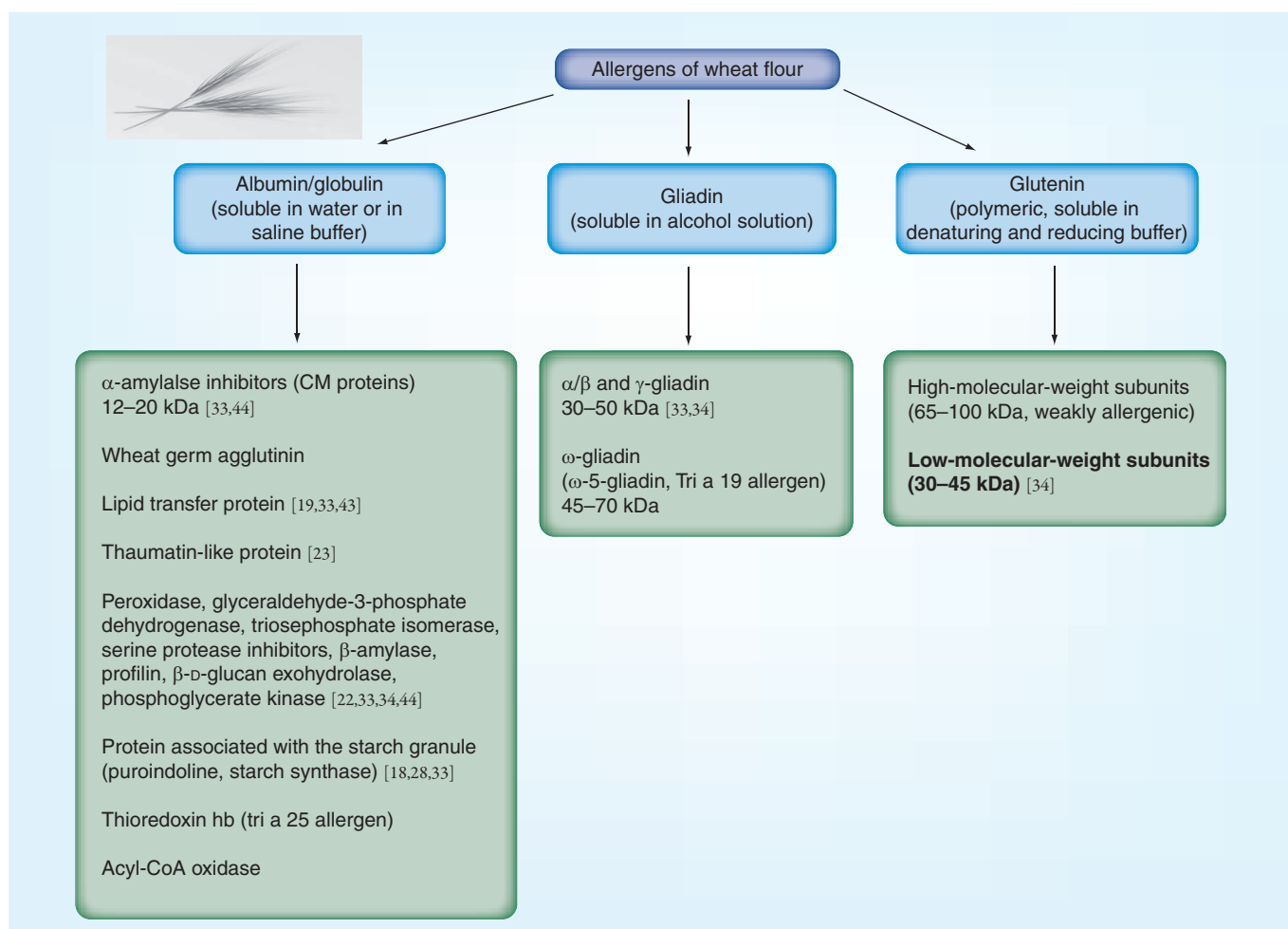


Figure 2. Classification of the different groups of allergenic proteins identified in wheat flour. The most important group is in bold. Many allergens have been identified through the application of proteomic strategies in combination with genetic, clinical and immunological approaches. The reference numbers are reported in those cases where the contribution of proteomic science to allergen definition has been fundamental.

material, which has been made possible only for those wheat allergens that have been successfully cloned (TABLE 1). In general, allergen characterization requires the combination of clinical, immunological, genomic and proteomic approaches. In this respect, the application of proteomic technologies to the identification of new allergens has been shown to be convenient in a number of cases (FIGURE 3) [21]. Usually, the proteomic analytical strategy consists of the 2D-polyacrylamide gel electrophoresis separation (PAGE) of the protein extracts and subsequent staining of the IgE-reactive spots by immunoblotting with sera of the allergic individuals. IgE-binding proteins can be identified by MS-based techniques after in-gel tryptic digestion (FIGURE 4).

The pioneer study of Sander *et al.*, combining PAGE, immunoblotting and MS to identify wheat water-soluble enzymes involved in respiratory allergy traced the successful proteomic strategy followed since then for allergen identification (exemplified in FIGURE 4) [22]. The molecular identities of purified wheat allergens in baker's asthma patients were characterized using 1D-sodium dodecyl sulfate PAGE combined with tandem MS, showing involvement of α AI, peroxidase I, and for the first time of two

proteins, thaumatin-like protein and lipid transfer protein (LTP) 2G [23], whose role in respiratory disorders still awaits to be elucidated. Nonspecific LTPs have been recognized as allergens in several plant species, among which maize, barley, peach, olive and several others are important in human nutrition, so that they are sometimes considered 'panallergens' [24]. The capability of wheat LTP and ω -gliadin to translocate across the intestinal epithelium either in native or hydrolyzed form has been recently demonstrated by using model Caco-2 cell monolayers [25].

The repertoire of IgE-binding components of wheat and maize in respiratory allergy has been investigated employing a combination of genomic and proteomic techniques. Wheat components detected by this approach included newly identified molecules with high cross-reactive potential [11,26,27]. Thioredoxins from distinct cereals such as wheat and maize have been confirmed to constitute a family of cross-reactive allergens [11,28], which show immunogenic potential similar to α AI.

As far as food allergy is concerned, it is now known that wheat can trigger either IgE-mediated or retarded, cell-mediated hypersensitivity reactions. Allergy to ingested wheat seems to be far more

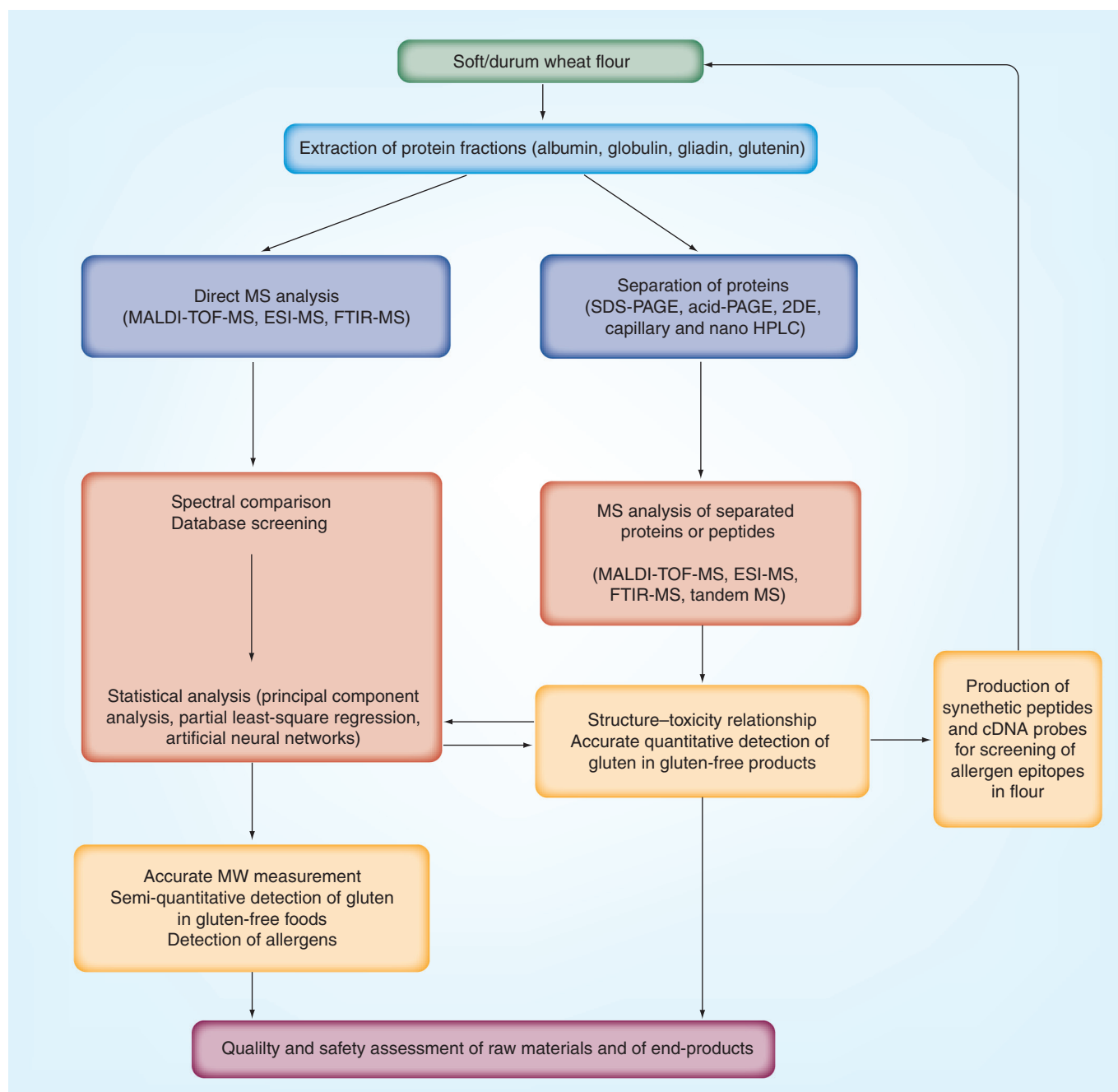


Figure 3. The proteomics approach as applied to wheat gliadin analysis can complement studies at the gene level. The protein profiles of gliadin and low-molecular-weight glutenin subunit components are very heterogeneous, as evidenced by SDS-PAGE and reverse-phase HPLC analysis, while a simpler protein profile is observed for the high-molecular weight glutenin proteins. The complexity of gluten protein can be attributed to the presence of two or three sets of homologous chromosomes in durum and bread wheat, respectively, and to additional polymorphisms related to mutation of gluten protein genes into many allelic forms. ESI-MS and MALDI-TOF-MS have partly resolved the gluten heterogeneity, becoming an important tool for gliadins and glutenin characterization and providing a basis to classify wheat varieties at the molecular level, for the identification of allergens and for trace detection of proteins in complex food matrices. ESI: Electrospray ionization; FTIR: Fourier transform infrared spectroscopy; MS: Mass spectrometry; MW: Molecular weight; PAGE: polyacrylamide gel electrophoresis; SDS: Sodium dodecyl sulfate.

widespread than was expected in the past [29], and concerns an elevated number of subjects comparable to CD. In allergy caused by wheat ingestion, insoluble gluten proteins are the main allergens, but recently several proteins of the soluble fractions have

been indicated as potential antigens. Furthermore, once ingested, wheat proteins undergo a series of modifications during the digestion process, by effect of the acidic stomach environment, action of digestive proteolytic enzymes (gastric pepsin, proteases and

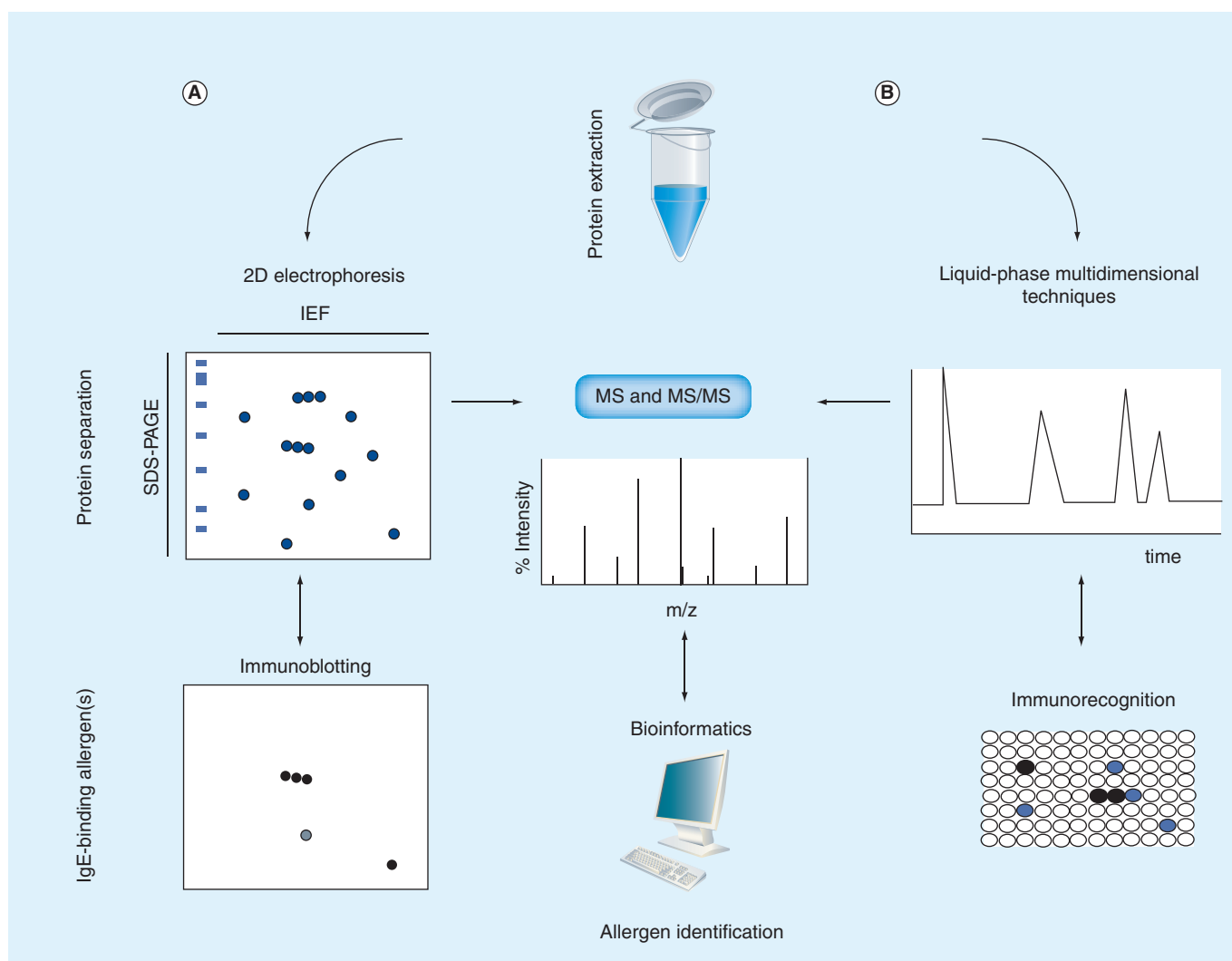


Figure 4. Proteomic platform for food IgE-binding allergen discovery, sometimes referred to as ‘allergenomics’ [21]. It combines a protein separation method (i.e., electrophoresis or chromatography) with IgE immunorecognition (i.e., immunoblotting or immunoarray) (A). Allergens are immunolabelled with serum of allergic individuals. Confirmation of the IgE-binding potential can be provided through immunoblot inhibition analysis. In this strategy, MS, supported by bioinformatic and database analyses, has the pivotal task for protein identification. The immunorecognition of chromatographically separated peptides arising from the digestion of allergic proteins (B) – or, alternatively, chemically synthesized peptides – are increasingly being used for B-cell epitope mapping. IEF: Isoelectric focusing; MS: Mass spectrometry; SDS: Sodium dodecyl sulfate.

peptidases in the intestinal lumen) and of tissue transglutaminase (tTG), which can modify the digestion products, giving rise to aggregates [30,31]. These reactions can greatly affect the solubility, immunogenicity and allergenicity of a protein. As recently demonstrated, low pH and treatment with enzymes such as pepsin may have a significant impact on solubility, immunogenicity, IgE binding and *in vivo* activities of various ingested allergens. Allergens resistant to digestive enzymes may be responsible for clinical symptoms [32]. Comparative studies to direct the use of defined allergens in serological and biological assays and their standardization are therefore required.

All these aspects have hardly been considered until the last few years, mainly owing to the extremely high complexity of these molecular systems. Furthermore, recent studies combining proteomics with immunochemistry according to Figure 4 have shown

that particularly important allergens are both gliadins and LMW GS [33,34], where the potentially allergenic epitopes have been investigated. Among these, the epitope QQQPP, repeated multiple times in the LMW glutenin sequence, has been shown to be the minimum IgE-binding sequence *in vitro* [35]. However, as observed by several authors, it should be carefully considered, that these *in vitro*-generated antibodies do not generally recognize the same epitopes recognized by antibodies elicited during natural exposure to correctly folded allergens, which actually contain 3D rather than linear epitopes [36,37].

These observations also point out that prediction of a protein’s allergenicity is an issue that cannot be addressed by proteomic analysis alone, but would require integration of clinical, immunological and biochemical approaches. As a matter of fact, our almost complete lack of knowledge about human IgE-binding

epitopes, due to the unavailability of monoclonal IgE antibodies, still makes proteomics and bioinformatics tools barely useful to face this issue.

A further complication is evidenced by the observation that sera with food allergy to wheat were also shown to contain IgE able to recognize soluble wheat proteins, such as the same members of the α AI family involved in baker's asthma [34]. Recognition observed in crude flour, however, is absent in proteins extracted from pasta or bread [38], suggesting that thermo-labile α AI allergens are degraded by the thermal processes necessary for preparation of baked foods [39]. On the contrary, the potential allergenicity of gluten proteins is apparently increased by cooking treatment, which induces the formation of protein aggregates also including sugars deriving from the Maillard reaction. These aggregates are resistant to hydrolysis by digestive enzymes and they are supposed to reach the intestines in an immunologically active form, determining the allergic response. This effect is more pronounced in bread crust than in crumb owing to the lower humidity level in the former [40].

These pieces of evidence suggest that we should carefully evaluate the differences existing among potential allergens in crude flour and transformed products, where physical treatments and interactions with other classes of molecules (polysaccharides, lipids and other proteins) can induce strong modifications in wheat

proteins, which require the application of proteomic strategies for structural characterization. A remarkable example is the case of beer. Malt derived from germinated barley (*Hordeum vulgare* L.) and, to a minor extent, from other cereals (wheat included), is the basic ingredient used to produce beer. Through the sequential processes of malting, mashing and brewing, the activated barley endogenous proteinases hydrolyse hordeins completely or partly, and also modify other proteins. It was believed that the very complex fermentation process would fully eliminate any allergen. However, very recently, application of proteomic technologies, following the scheme illustrated in FIGURE 2, proved that not only intact allergens such as nonspecific LTP and Z4 protein still survived intact in beer, but some proteolytic fragments were also produced (FIGURE 5) [41], whose allergenic potential is still to be defined with the application of appropriate immunological and clinical assays.

From a technical point of view, various modifications of 1D electrophoresis and 2D electrophoresis (2DE) were used for proteomic analysis of wheat allergens; among them, acetic acid/urea-PAGE (AU-PAGE) revealed LMW allergens that bind to IgE of patients with food allergy [42,43]. 2DE in immobilized pH gradient followed by AU-PAGE and immunodetection with patient sera coupled to MS was decisive to reveal allergenicity of α -amylase/trypsin inhibitors and LTP [43]. The system developed in this study is a useful

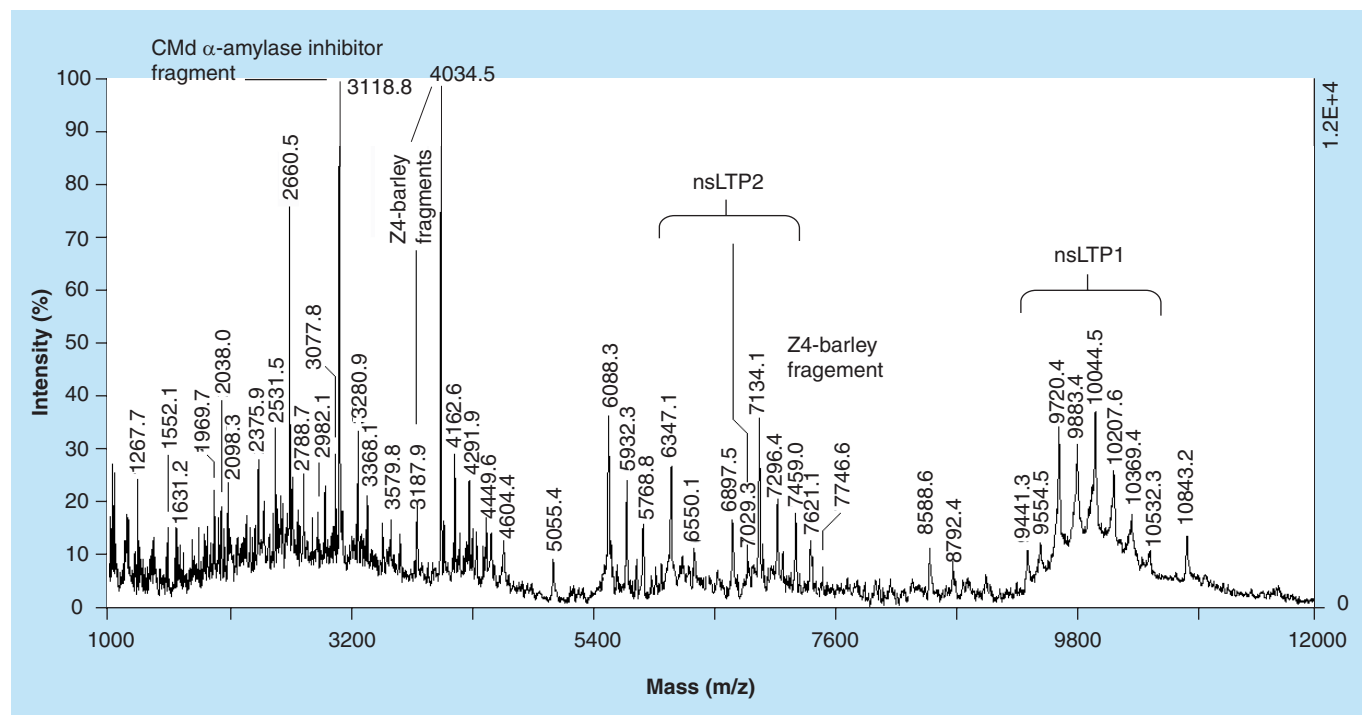


Figure 5. Proteomic analysis demonstrated that the technological treatments can induce strong modifications in wheat protein composition. In beer, a beverage produced from germinated barley (*Hordeum vulgare* L.) through the sequential processes of malting, mashing and brewing, the barley endogenous proteinases and yeast enzymes hydrolyze hordeins completely or partly, and modify other proteins. It was believed that the very complex fermentation process would fully eliminate any allergen. Application of proteomic technologies, following the scheme illustrated in FIGURE 2, proved that not only intact allergens such as nsLTP and Z4 protein still survived intact in beer, but proteolytic fragments were also produced, the allergenic potential of which is still to be defined. Some of the dominant signals are assigned. Mass differences of approximately 162 between adjacent signals are due to nonenzymatic glycosylation of polypeptides (Maillard reaction). nsLTP: Nonspecific lipid transfer protein.

standard protocol for the separation of LMW proteins. In a study aimed at characterizing salt-soluble wheat allergens, wheat proteins were separated using 2DE gel high-resolution techniques and IgE-binding components were detected by immunoblotting [44]. Proteins most frequently recognized by IgE of allergic patients, were identified using MS (MALDI-TOF, Q-TOF, ION-TRAP and liquid chromatography [LC]-MS/MS IT). In order to evaluate the diagnostic value of specific IgEs, an ELISA assay was applied to quantify levels of IgE against wheat extract and selected allergens. This strategy contributed to the identification of wheat allergens aimed to increase the specificity of serum IgE and cell activation diagnostic assays. In any case, it should be emphasized that detection of allergen-specific IgE in serum is the most straightforward method for the diagnosis of allergy, which must, however, be confirmed *in vivo* because a high incidence of false-positive results can occur [5]. In the case of food allergy, including wheat, double-blind placebo-controlled challenge remains the gold standard for the diagnosis of allergy.

Proteomics & food intolerance: CD

In last decade, MS techniques have been largely utilized in the study of CD, the most common food-sensitive enteropathy. Its relevance in biomedical research is such that the study of molecular mechanisms at the basis of this disease has been recently defined as 'coelionomics' [45]. CD is caused by an abnormal immune response to dietary gluten, which occurs in genetically susceptible individuals carrying the HLA-DQ2 and HLA-DQ8 haplotype. High levels of IgA and/or IgG antibodies against gliadin and autoantibodies to tTG (anti-tTG) and endomysial antibodies are present in CD patients (FIGURE 1C) [46].

Despite the progress in understanding the molecular mechanisms of mucosal lesions, it remains unknown how incoming gliadin peptides can activate the immunological pathway. Generally, food proteins are digested by gastric pepsin and pancreatic proteases and are further degraded by gut brush border membrane enzymes to yield single amino acids, dipeptides or tripeptides that are transported across the epithelial layer. However, gluten protein are characterized by proline-rich motifs [1], which make specific gliadin peptides resistant to human digestive protease. These gastrointestinal resistant peptides can reach high concentration levels in the gut epithelium, triggering two immunological pathways: an innate and adaptive immune response, as depicted in FIGURE 1.

The activation of the innate immune response in CD has recently caught the attention of the research community [47]. To date, a few gluten peptides have been identified as responsible for immune response activation (TABLE 2). By using a proteomic strategy, it has been shown that peptide 31–55 (LGQQQPFPPQQPYQPQPFPSQQPY) survived gastrointestinal digestion of both recombinant α/β -gliadin and whole gliadin extracted from a common wheat cultivar [48]. This peptide encrypts the sequence 31–43, which upregulates the expressions of IL-15, of the enzyme cyclooxygenase-2 (COX-2) and of the cell activation markers CD25 and CD83 on lamina propria (LP) macrophages, monocytes and dendritic cells, without stimulating CD4⁺ T cells [49]. On the other hand, the majority of studies on CD have been focused on the central role of the adaptive immunity (FIGURE 1C). A very high number of gliadin peptides have been reported to act as antigens by stimulation of CD4⁺ T lymphocytes isolated from the small intestinal mucosa of CD patients. A comprehensive list of toxic and immunogenic gluten peptides, identified by MS analysis [47,50–52], can be found in TABLE 2.

Table 2. Toxic and immunogenic peptide epitopes identified in gluten.

Gliadin	Swiss-Prot accession number	Position	Amino acid sequence	Toxic	Immunogenic
α -gliadin	P04721	44–45	PQPQPFPSQQPY	Yes	
α -gliadin	P04721	31–43	LGQQQPFPPQQPY	Yes	
α -gliadin	P04721	31–49	LGQQQPFPPQQPYQPQPF	Yes	
α -gliadin	PI8573	31–55	PGQQQPFPPQQPYQPQPFPSQQPY	Yes	
α -gliadin	P04721	31–55	LGQQQPFPPQQPYQPQPFPSQQPY	Yes	
α -gliadin	PI8573	57–89	LQLQFPQPQLPYQPQLPYQPQLPYQPQPF		Yes
α -gliadin	PI8573	58–69	QLQFPQPQLPY		Yes
α -gliadin	PI8573	63–76	PQPQLPYQPQLPY		Yes
α -gliadin	P04724	205–216	PSGQGSFQPSQQ		Yes
α -gliadin	P04724	206–217	SGQGSFQPSQQN		Yes
α -gliadin	P04722	57–73	QLQFPQPQLPYQPQP		Yes
α -gliadin	AJ133612.1	64–89	PQLPQLPQPYPQPQLPYQPQPF		Yes
α -gliadin	AJ133612.1	71–89	QPQYPQPQLPYQPQPF		Yes
γ -gliadin	P08453	222–236	VQGQGIIQPQPAQL		Yes
γ -gliadin	P08079	58–83	FLQPQQPFPPQQPYQPQQPFPQ		Yes
ω -gliadin	AF280605	102–118	QPQQPFPPQQPFPWQP		Yes

Data from [48–54].

The enzyme transglutaminase (TG) plays a key role in adaptive immune system, since it catalyzes deamidation of specific glutamine residues, generating gliadin peptides with high affinity to HLA-DQ receptors. The discovery that tTG was the main, although probably not the sole, target of the endomysium CD-specific autoantibodies assigned this enzyme a crucial regulator role in CD. Wheat glutenin and gliadin were demonstrated to be effective acyl donor substrates for tTG [53]. The possible modification of gliadins at the level of the gut enterocytes by tTG, through a coupling to acyl-acceptors or through self-cross-linking, has been thought to generate toxic gliadin epitopes and neoantigens, which are then presented to immunocompetent cells (FIGURE 1C).

Using LC-MS analysis, Shan *et al.* demonstrated that a 33-mer peptide (LQLQFPQPQLPYQPQLPYQPQLPYQPQPF), was released by proteolytic cleavage of recombinant α 2-gliadin, and was highly resistant to brush border membrane enzymes [54]. This peptide is considered the most immunogenic as it includes six overlapping epitopes [50]. Hence, it has been suggested that the 33-mer can reach the underlying LP and, following deamidation by tTG, trigger the pathogenic cascade of CD. By applying a similar proteomic strategy, another multivalent 26-mer peptide (FLQPQQFPQQPQQPYQPQQPQQPFPQ) generated from hydrolysis of recombinant γ -gliadin, was found to be a good substrate of TG and displayed markedly enhanced T-cell antigenicity [53]. The same authors also used *in silico* analysis of the gluten proteome for the identification of at least 60 putative peptides that share common structural and functional features of the 33-mer and the 26-mer peptides.

Peptidomics, being an efficient tool for the rapid identification of peptides in complex mixtures, can therefore facilitate identification of tTG-mediated modifications of peptides [52,53]. The resolution and specificity achieved when using the new generation of hybrid quadrupole-TOF instruments allows differentiation of peptides where a single deamidated Q residue is present, even in complex mixtures, such as those occurring in the enzymatic digests of gluten proteins. The ability of tTG to mediate modification of specific gliadin peptides was demonstrated on a gastric/pancreatic digest of gliadin. Mono-dansyl-cadaverine was chosen as fluorescent chemical label for the selective probing of susceptible Q residue. By this method, a restricted number of gliadin peptides sharing specific amino acid motifs for tTG recognition were selectively fished among a myriad of unmodified peptides. Some of these sequences actually stimulated intestinal T-cell responses after tTG deamidation [51].

In addition to catalyzing deamidation reactions, tTG may generate additional antigenic neoepitopes by cross-linking gliadin peptides serving as glutamine donors, with protein of the extracellular matrix or with TG itself as acyl acceptor [52]. In the last case, the tTG–gliadin complex would act as a hapten carrier, which could explain the typical antibody response against tTG in untreated CD patients [46]. Although tTG is recognized as the major target antigen of IgA antiendomysial antibodies, the existence of additional unique CD-specific autoantigens was also demonstrated. Stulík *et al.* applied a proteomic strategy to identify

the autoantigens recognized by serum Abs of patients with active CD. By 2DE/MS fingerprinting, the four autoantigenic determinants were identified as actin, ATP synthase β chain and two charge variants of enolase α [55].

Expert commentary

From identification of allergens to diagnosis of allergy

The data obtained to date show that the proteomic approach using 2D immunoblotting (FIGURE 4) may be a powerful strategy for the identification of allergenic proteins. Its main advantages are the low amount of sample needed, the analytical specificity and the ‘multiplex’ possibility to simultaneously screen the entire protein panel for discovering food allergens. Nevertheless, caution is necessary in the assessment of new allergens due to the substantial differences of the *in vivo* physiological state of proteins and to the extreme conditions of the electrophoretic process. In this last case, in particular, ‘false’ epitopes can be produced that do not correspond to any physiological situation [36,37]. After denaturation, proteins can expose reactive motifs that are usually buried in the native conformation or even in the environment of the digestive tract. Similarly, potential epitopes recognized upon the gel setting can be for the most part susceptible to degradation by the digestive proteases. Both these situations may lead to false positives being included in the list of allergens. For these reasons, investigations aiming to establish the associations between specific subpopulations of specific IgE, measured by the use of individual allergen components or parts thereof, and clinically relevant aspects of the allergic disease are required to confirm the actual involvement of potential allergens.

Furthermore, most patients only raise IgE antibodies to some allergenic components. Therefore, depending on the number of sera employed in the analysis, potential allergens can escape detection.

Regarding the diagnostic aspects, it should be highlighted that diagnosis of wheat allergy can only be effected by combining evidence from a patient’s clinical history, detection of wheat-specific IgE and results of elimination diet and oral challenges, or bronchial challenges in case of baker’s asthma. In routine diagnostic settings, extracts of natural allergens are used for *in vivo* (skin prick test) and *in vitro* (specific IgE Abs) tests are normally employed. The diagnostic accuracy of both approaches is still very unsatisfactory [56]. This poor capacity could be explained by the fact that commercial test reagents are mixture of water/salt-soluble wheat proteins that lack allergens from the insoluble gluten fraction. Moreover, cross-reactivity of water/salt-soluble wheat proteins with grasses increases the frequency of clinically insignificant positive test results. The increasing availability of allergen panels derived from several different sources is enabling detailed screening of the sensitization profile in individual patients to be practicable. In this respect, the frontier of diagnostics is now represented by allergen microarrays and especially by the immuno solid-phase allergen chip technology, which has been recently reviewed [57], which may simultaneously screen the IgE-binding capacity of many patients’ sera to many allergens, thus analyzing the IgE-binding profiles of

many allergens [58,59]. We can expect that these analytical devices might largely benefit from the structural information provided by the novel proteomic approaches [21].

Since most patients do raise IgE antibodies in response to only some wheat components, a complete definition of the potential allergens, by now achievable through the proteomic approach, is a key point for developing diagnostic measures with improved sensitivity and specificity. Currently, electrophoresis and MS combined with the immunochemical detection are bridging some of the main gaps that hamper a comprehensive classification of the wheat allergens, namely the lack of a standardized protocol to extract and characterize all of the wheat protein fractions and the lack of data about the IgE-binding properties, and especially about the differences between raw and cooked wheat-derived foods [35].

Another, not secondary, issue is related to the presence of potentially allergenic additives of non-wheat origin in baked products. The most important of these are the soy-derived products (soy protein isolates, soy lecithin, soy milk and others) but also eggs and milk. The low levels at which these ingredients may be present require the development of novel proteomic strategies to complement immunological, biochemical and clinical methods to achieve efficient and sensitive diagnosis of wheat food allergy cases.

Production of hypoallergenic, gluten-free & detoxified products & ingredients: multiple roles for proteomic sciences

Today, food allergies are treated symptomatically and through the strict exclusion of the injuring agent from the diet. However, serious nutritional, psychological and compliance-related implications arise from its substitution when the foods in question are important components of diet. Furthermore, owing to the increasing number of people affected by wheat allergy, efforts have been made to produce hypoallergenic foods, characterized by a limited allergen content, which should prevent sensitization and alleviate clinical symptoms.

There are basically two ways of producing hypoallergenic foods. The genetic approach is based on the selection or production of materials with reduced allergen content by identification of cultivar or other cereal species [4,60]. The use of kamut (*Triticum polonicum*) or some spelt cultivars has been proposed upon the hypothesis that these ancient *Triticum* species have a low allergen content. Genetic engineering techniques have been used to inhibit production of α -amylase-like allergen in rice, and the same strategy has been proposed for application in wheat [61].

The second approach is based on the application of chemical, physical or enzymatic treatments on wheat. Removal of allergenic characteristics of flour proteins has been attempted by deamidation, hydrolysis and disulphide reduction. The consequences of these (bio)chemical reactions on wheat protein immunogenicity and allergenicity can be evaluated only by the combination of proteomic, peptidomic and immunological approaches, for which the term 'allergomics' has recently been introduced [23].

As an example, proteomic analysis has recently shown that deamidation of glutamine residues produced contradictory results: deamidation of the allergenic epitope QQQPP lowered IgE reactivity in patients, whereas deamidation of wheat protein isolates commonly used as additives in food industry apparently induced the appearance of novel antigens [62,63]. Hydrolytic treatment with actinase suppressed allergic reactions in tested patients, whereas hydrolyzed wheat proteins, used as food additives, lost IgE-binding capacity when cleaved by acid treatment but not if obtained by enzymatic reaction [64].

The major technological problem faced in the development of hypoallergenic baked products is that modifications of potential allergens is likely to also induce a loss of the functional properties of dough. Efficient methods to produce a hypoallergenic flour also need to be supported by technological advances in order to obtain satisfying, if not optimal, sensory quality products. Probiotic preparation used as a starter culture allowed sourdough bread with elevated acceptability scores to be prepared [65,66]. In this specific case, MS was instrumental to ensure degradation of allergenic epitopes following the entire technological production process of bread and also to monitor protein modifications during pepsin and pancreatin digestion mimicking physiological-like conditions.

In the case of CD, new therapeutic approaches are now being investigated that would permit celiacs to 'peacefully' coexist with gluten, including a search for genetically modified wheat lacking toxic gluten peptides [67]. Very recently, by genetic engineering hexaploid *Triticum aestivum*, it has been demonstrated that the removal of the α -gliadin locus from the short arm of chromosome 6 of the D genome resulted in a significant decrease in the presence of CD4⁺ T-cell stimulatory epitopes. However, this also detrimentally affected the technological properties of flour. On the other hand, by removing the α -gliadin and ω -gliadin and LMW glutenin subunits loci from the short arm of chromosome 1 of the D genome, it was possible to almost completely abolish T-cell stimulatory epitopes from the wheat proteome while maintaining technological properties [68]. Based on the natural genetic variation of some wheat cultivars, the use of 'less toxic' bread wheat varieties suitable for consumption by CD patients had previously been proposed [69,70]. However, this proposal opened a broad discussion among researchers until it was eventually concluded that it did not eliminate any risk for CD patients.

Furthermore, two approaches are currently being tested to prevent or at least reduce gluten toxicity in wheat flour: masking of gliadin epitopes (possibly by use of tTG) or their proteolytic degradation, all fields where MS is playing an important role in validating results. On the side of degradation of toxic epitopes in CD, the main difficulty is that the gluten-derived T-cell epitopes are highly resistant to proteolytic degradation within the GI tract, which does not possess the enzymatic equipment to efficiently cleave proline-rich peptides driving the abnormal immune intestinal response in CD patients. For this reason, oral supplementation with exogenous prolyl-endopeptidases able to digest gluten produced for instance by *Aspergillus niger* has been proposed as an alternative treatment to the gluten-free diet. *In vitro* and *in vivo* studies driven by MS [71] confirmed in part the effectiveness of prolyl-endoprotease as an

oral supplement to reduce gluten intake in patients [72,73]. However, it has been correctly argued that excessively high concentrations of endoproteases are required for efficient epitope hydrolysis. Furthermore, it was found that some partly digested fragments of the α -gliadin still contain epitopes able to trigger pathogenic T-cell activation. These still potentially toxic gliadins fragments were finally able to cross the gut epithelium and to ultimately reach the LP at harmful concentrations [74].

It has been well assessed that gliadin-derived peptides, probably through a mechanism related to their toxicity for CD patients, agglutinate human K 562(s) cells, a line of chronic myeloid leukemia [75]. The MS-based identification of a peptide from durum wheat gliadins that was able to prevent the agglutination of K 562(s) cells induced by prolamins fragments, opened the way to set up a strategy to induce tolerance to wheat-derived products in celiacs. Over the years, this finding has stimulated studies aimed to establish a possible therapeutic strategy for CD, as recently reviewed [76]. Recently, two proteolitically stable peptides from durum wheat, able to inhibit CD-specific T-cell activation of gluten hydrolyzates, have been identified. Besides the prevention of the K 562(s) agglutination, these peptides downregulated inflammatory T-cell proliferation and IFN- γ production, also causing an increase in IL-10 secretion [75].

In any case, the rheological and sensory properties remain the main factors determining the acceptability of a food. Although the quality of life of CD patients can be considered normal from a strictly nutritional point of view, a series of disadvantages are still associated with the gluten-free diet, which can be summarized as costly, poor sensory quality and low durability. It is then clear that the production of fermented and baked products based on non-wheat materials with acceptable quality requires the optimization of technological processes depending on the particular food matrix. At present, the main raw materials employed in the production of a gluten-free dough are cereals including rice, maize, sorghum and millet, and pseudo-cereals such as amaranth, quinoa and buckwheat [77]. The phylogenetic tree of the main cereal species is shown in FIGURE 6. Rice and maize are cereals at the basis of the diet of very large populations worldwide. Nutritional aspects of the pseudo-cereals in the gluten-free diet have recently been reviewed [78].

Millet is considered the most ancient cereal, cultivated in Africa, Asia and East Europe, and includes several species, of which the most important are pearl millet (*Pennisetum glaucum*) and finger millet (*Eleusine coracana*) [79,80]. Teff (*Eragrostis tef*) is an Ethiopian endemic crop, used by local populations for producing traditional bread, such as injera. Millet and teff are examples of 'neglected crops' as they have received very limited research investments to update the productive processes, compared with major crops. Nevertheless, they are attracting renewed interest due to their capability of adapting to extreme climatic and soil conditions, in addition to tolerance of pests and diseases. Immunological analyses attested to the absence of immunodominant gluten-like peptides in teff, supporting it as a candidate for a safe alternative in the diet of celiacs [81]. On the other hand, this result was expected on the basis of the relative phylogenetic

distance of teff from Triticaceae (FIGURE 6). The proteomic characterization of the storage proteins of Teff has revealed the occurrence of essentially two protein populations (molecular weights ~18 and ~24–25 kDa, respectively). However, mass spectrometric data confirmed the lack of structural relationships between gluten and teff proteins (FIGURES 7–9). These evidences are attracting the attention of American and European industries for the production of hypoallergenic/gluten-free baked foods. Furthermore, in consideration of the relatively recent introduction of crops that are traditionally prerogative of 'Western' countries in the nutrition of African populations, the exposure to gluten foods could potentially induce CD to emerge on a large scale in individuals genetically predisposed and naturally observing a gluten-free diet for generations. The utilization of the endemic crops seems to be the only practicable strategy to prevent a large epidemiologic manifestation of CD in Africa.

To improve their rheological properties, in general, these products also require a gluten replacement to provide dough structure and gas-retaining properties. This can be obtained by the use of various texturizers such as hydrocolloids, or by the introduction of innovative fermentation processes. Enzymes are largely employed in the biotechnology of gluten-free products. Use of amylase and cyclodextrin glycosyl-transferase allows staling of rice flour-based products to be delayed by reducing starch retrogradation, and in the same matrix glucose oxidase improved protein linking. Proteomic approaches have been able to describe protein modifications in various food matrices after TG treatment [82]. Recently, microbial TG has been used with the aim of introducing covalent isopeptide bonds to promote bonding among proteins of various origin: milk, egg and soy proteins. In this way, soy, milk and egg proteins employed in the formulation of gluten-free dough can favor formation of a protein network to improve specific volume and reduce crumb gumminess.

The newest biotechnology innovation in production of gluten-free baked products on which both academic and industrial research are focused envisage the combination of gluten detoxification and gluten substitutes to obtain safe foods with optimal sensory characteristics. For both these aspects, the contribution of proteomic analysis has been proven essential. The enzymatic procedure of Gianfrani *et al.* prevents the immunostimulatory response though maintaining gluten structural integrity by a pretreatment of toxic peptides and of whole gliadin with bacterial TG [53]. The mechanism of action, revealed by proteomic analysis, consists of protein deamidation in the presence of amine group donors (lysine and lysine methyl ester), resulting in masking of gluten epitopes. Interestingly, from an industrial perspective, a similar action has also been observed by proteomic tools after incubation of wheat flour with microbial TG and amine group donors. A further example of strategy of gluten detoxification before assumption has been described very recently by Stenman *et al.*, who used a pool of proteases extracted from germinating wheat to remove toxic epitopes, including the 33-mer peptide, as assessed by MS [83].

Sourdough lactobacilli are also finding application in gluten-free products for their positive impact on aroma, bread volume and crumb structure [84,85] and durability [86], while MALDI

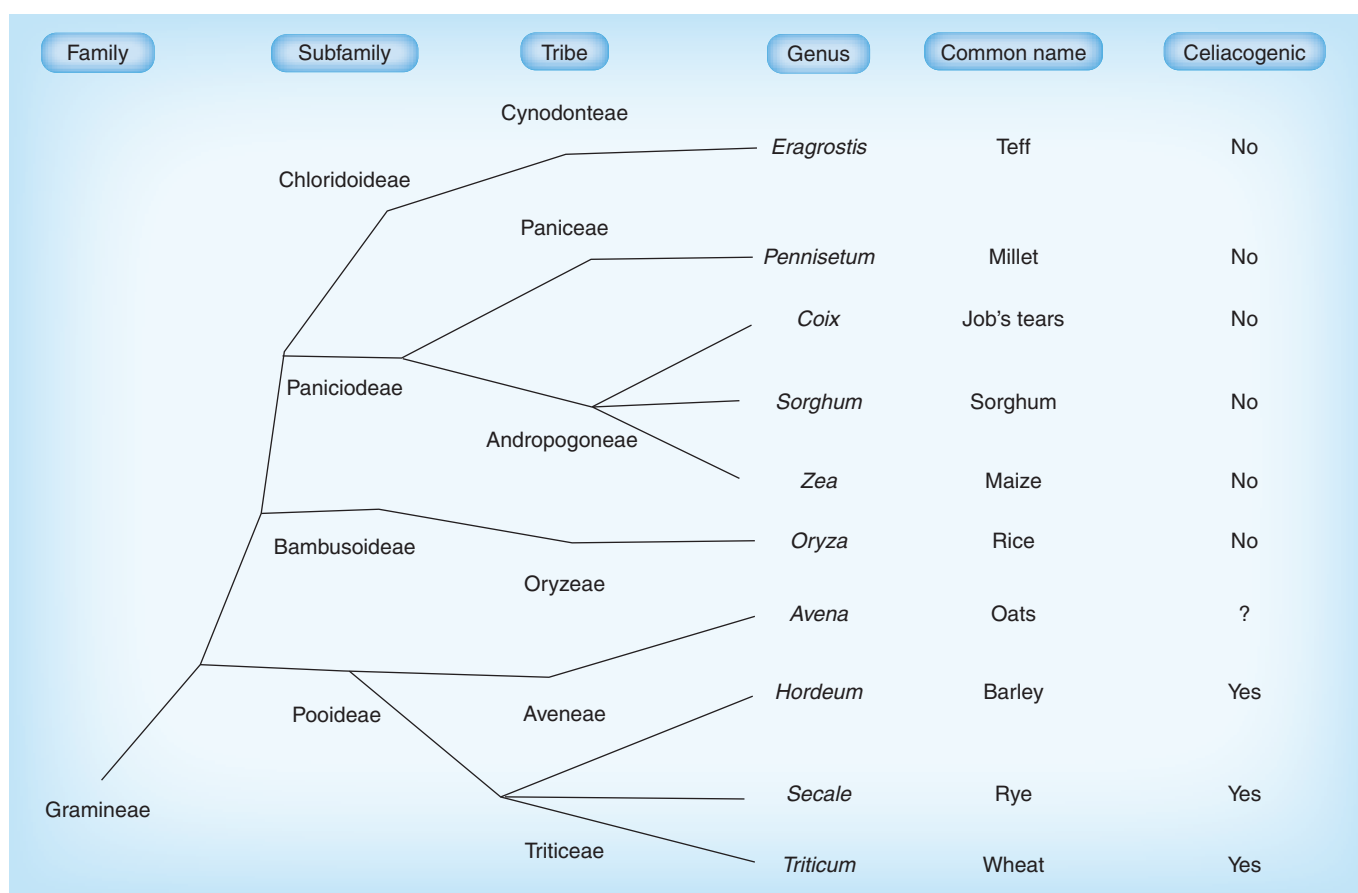


Figure 6. Taxonomy of the most common dietary grains. Wheat, barley and rye, which contain the celiac disease (CD)-activating proteins gluten, hordein and secalin, respectively, are derived from the *Triticeae* tribe of the grass (*Gramineae*) family. By contrast, oats, which are distantly related, contain (if any) CD-activating proteins in only minor amounts. Rice, maize, sorghum, millet, Job's tears and teff, phylogenetically far from wheat, are non-celiacogenic cereals. Today, the main raw materials employed in the preparation of wheat-free and gluten-free baked products are non-celiacogenic cereals, such as rice (*Oryza sativa* and *Oryza glaberrima*), maize (*Zea mays*), sorghum (*Sorghum bicolor* L. Moench) and millet (*Panicum miliacum*). For the production of gluten-free products for CD, the use of celiacogenic cereals containing gluten-forming proteins is not allowed. These include all species belonging to the *Triticum* genus), and related cereals such as barley (*Hordeum vulgare*), rye (*Secale cereale*) and oat (*Avena sativa*). For this last cereal, recent clinical studies indicated minimal levels or even absence of toxicity, suggesting a possible admittance in CD preparations. From the phylogenetic tree, the closest interspecies relationships can be inferred.

and electrospray ionization (ESI)-based MS techniques demonstrated removal of gluten potentially contaminating the dough at a level up to 300 ppm [87–90]. The action of sourdough lactobacilli on durum wheat for the production of pasta has been recently investigated by combining proteomic, immunologic, rheological and sensory analysis [66], proving that it is possible to produce pasta retaining good sensory quality while destroying CD epitopes, thus opening completely new ways for improvement of acceptability of CD foods. Italian-type pasta produced by the same kind of fermentation methods obtained higher scores for cooking and sensory attributes than conventional gluten-free products [91].

Proteomic, ELISA and cell culture analyses are showing that sourdough lactobacilli, normally utilized in the production of baked foods, might provide a dough with ELISA-responsive gluten below 10 ppm [65,90]. According to this biotechnology strategy, this dough can be used in bread-making after mixing

with either tolerated flour(s) (millet, teff, buckwheat) or with hydrocolloids (gums or modified starches), followed by yeast fermentation, obtaining a bread with acceptable rheological and sensory characteristics [90]. The same detoxified flour, mixed with butter, sugar and water, has been proposed for the production of cakes then employed for an *in vivo* challenge by daily administration of 100 g product to CD patients in remission with no adverse symptoms or alteration in clinical parameters. If these encouraging results could be confirmed by larger studies, flour detoxified by sourdough lactobacilli could become the starting material for the production of totally innovative fermented gluten-free foods. The same strategy has been also proposed for the detoxification of rye flour, another celiacogenic cereal [92]. By these innovative biotechnological approaches, several additional advantages can be envisaged: production of gluten-free foods by traditional processes without pharmacological agents; improved sensory and rheological quality of

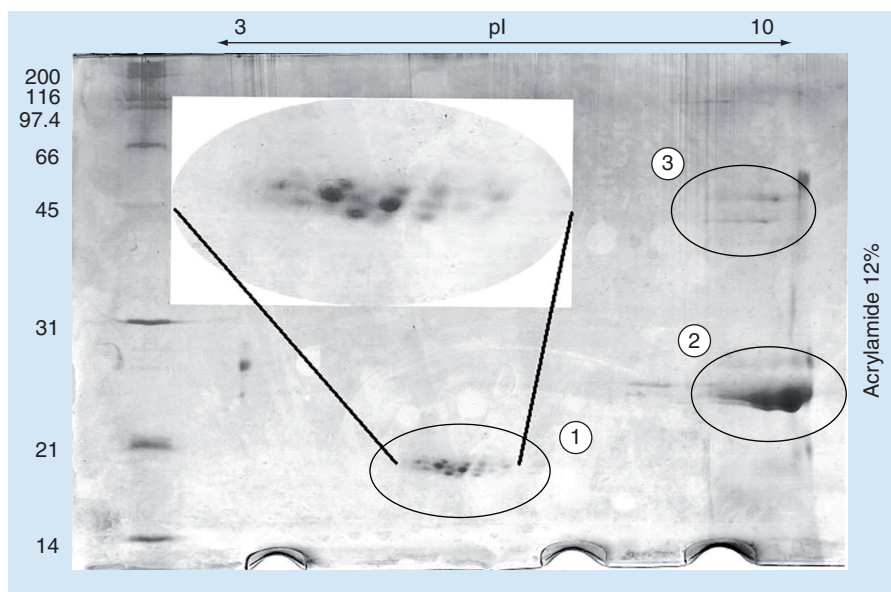


Figure 7. Proteomic analysis of teff storage proteins (I). Teff (*Eragrostis tef*) is a cereal closely related to millet that is traditionally used in Ethiopia for producing a thin bread called injera. In the last few years, it has attracted the attention of industries for production of hypoallergenic/gluten-free baked foods. Absence of either prolamins or other toxic proteins has been demonstrated by proteomic analysis. Teff seed proteins were extracted in denaturing/reducing buffer (50% 2-propanol/4 M urea/100 mM Tris HCl, 10 mM dithiothreitol; pH = 8.0) and analyzed by 2D-IEF/SDS PAGE in comparison with the protein extracts of other cereals. The proteomic characterization of the storage proteins of teff has revealed the occurrence of essentially two protein populations (molecular weights ~18 and ~24–25 kDa, respectively). Individual subsets of proteins as detected with different techniques are labeled with the numbers 1, 2 and 3. Each of these group consists of several protein isoforms. In the protein fraction of teff, similarly to maize, the major 30–40 kDa polypeptides of the celiacogenic cereals are missing.

the foods; and use of largely cultivated raw materials and of conventional industrial plants for CD patients. Even in this perspective, the issue of the risk of the contaminating gluten in foods still remains.

The analysis of gluten-free foods & beverages for the assurance of CD patient safety: all problems resolved?

The potential contamination always possible in gluten-free products, even from unsuspected sources, is probably the most challenging safety aspect. Although a toxic gluten-free diet is the only treatment for CD patients, a reliable *in vitro* or *in vivo* system to analyze toxicity in food samples to control diet is not yet available. The availability of an accurate, fast and cheap alternative to immunological ELISA may also facilitate research towards the development of novel drugs, therapies, prognostic tools and food-processing technologies to aid patients with gluten intolerance, and for gluten-free labeling and certification processes. MS is the only nonimmunological method currently available to detect high-specificity gliadins and related prolamins in flours and in food samples (FIGURE 10). A first analytical approach is based on LC-MS, monitoring 'signature' peptides of gluten proteins. A recent study was targeted to develop a LC-MS/MS method for quantitative detection of six unique physiologically relevant wheat gluten peptides in various foods

and consumer products [93]. Experimental results showed that the method was able to detect and quantify target peptides in food. Limits of detection and quantification for target peptides were in the range 1–10 ppb and 10–100 ppb, respectively. However, this intriguing analytical method still needs to be validated on a wide range of foods matrices and ingredients before it can be proposed to replace or complement immunological reference assays.

The MALDI-based approach (FIGURE 10) relies on the possibility of obtaining characteristic MALDI-TOF-MS profiles of unfractionated gliadin, hordein, secalin and avenin extracts [94]. Based on these four distinguishable mass patterns, prolamins from different cereals can be differentiated and also identified when simultaneously present in foods [95]. Sample preparation is relatively simple and analysis can be performed directly on the alcohol extract. With this information, a fingerprint can be obtained for gluten proteins. In particular, MALDI-TOF-MS has been applied to the characterization of wheat α -gliadins and high-molecular-weight GS, genetic variants and to varietal analysis through measurement of accurate molecular weight.

A remarkable application is in the analysis of the products of starch hydrolysis, such as glucose syrup, crystalline dextrose and maltodextrins (largely used as sweeteners, anticrystallisers and stabilizing agents), obtained industrially through chemical and/or enzymatic methods. In these products, gluten determination by immunological tests is made unreliable by a series of factors, including the low amount of gluten to be detected being dispersed in a very high amount of substances (low and high mass sugars, other byproducts of the process) which interfere with determination [96]. Gluten semiquantitative measurement in these products by combining procedures of extraction and isolation with MALDI-TOF-MS analysis made it possible to detect and identify low quantities of protein (estimated sensitivity 1–10 ppm), thus allowing researchers to verify whether these products exceeded the 20 ppm limit required for foods 'rendered' gluten-free. The pattern of proteins/peptides present in the samples was found to vary either qualitatively or quantitatively, depending on the sample type. This also meant that the MS approach may allow identification of the differences and quantification of the protein/peptide level in different industrial products of the same category.

The MALDI-based approach for the analysis of gliadin mixtures, although fast and specific, does not allow the identification of subtle structural differences among individual

gliadin components. In particular, the fact that gliadins can be modified by heating with a corresponding loss of resolution and a decrease in mass signal intensity suggests that MALDI-TOF-MS can only be used for semiquantitative measurements of gliadins. Therefore, depending on the complexity of the food matrix analyzed and on the efficiency of the gluten extraction procedure, the MALDI-based response needs to be definitively evaluated with particular caution as the operative conditions can significantly affect both reliability and repeatability of the analysis.

Therefore, in order to identify either the subtle structural differences among gliadins or glutenins or the variations induced by the technological processes of the food industry, the more resolutive ESI-MS or Fourier transform MS, and tandem MS need to be applied. The most powerful approach in the field, however, is obtained when the capability of MS in identifying protein structure is coupled with a high-resolution chromatographic or electrophoretic technique. This is exemplified by the analysis of fermented products. Malt beverages and traditional beers are interesting model study matrices to test the potential of MS in the determination of gluten-like epitopes. Barley malt beer contains an average 0.2–0.6 g/100 ml of protein/peptide material originating almost exclusively from barley proteins and to a minimal extent from yeasts. As anticipated earlier in this article, the polypeptide fraction is constituted by either native

or chemically modified proteins, as well as by peptides arising from hydrolytic events covering a broad range of molecular sizes [43,97]. Owing to the uncertainties associated with the determination of the content of hordeins and gliadin-like epitopes, beer is usually precautionarily excluded from the diet of CD patients. New findings, mainly supported by proteomic data, seem to disprove that hordeins occur in beer in a potentially harmful amount [98–100]. These observations are justified by the low solubility of hordeins in a low-alcohol solution such as beer (~5% ethanol), and by their precipitation and removal during the mashing and boiling stages. A rapid and almost complete degradation of the gluten-like CD toxic peptides by proteases of germinating cereals has already been demonstrated [101]. On the other hand, recent evidence suggests that beer should not yet be considered as being definitely safe for CD. In a very recent study several low-molecular-mass peptides deriving from γ 3- and β -hordein were identified in beer by nano-LC/ESI-MS/MS analysis [43], demonstrating the existence of potentially immunogenic epitopes generated from hydrolysis of the hordeins, including a 7-mer sequence PQQPYYPQ, which contains the two 4-amino acid motifs, QQPYP and QPYP, which are associated with CD toxicity. Owing to the high complexity of the peptide fraction of beer, further targeted studies are required, and in their accomplishment, MS will have undoubtedly have a pivotal position.

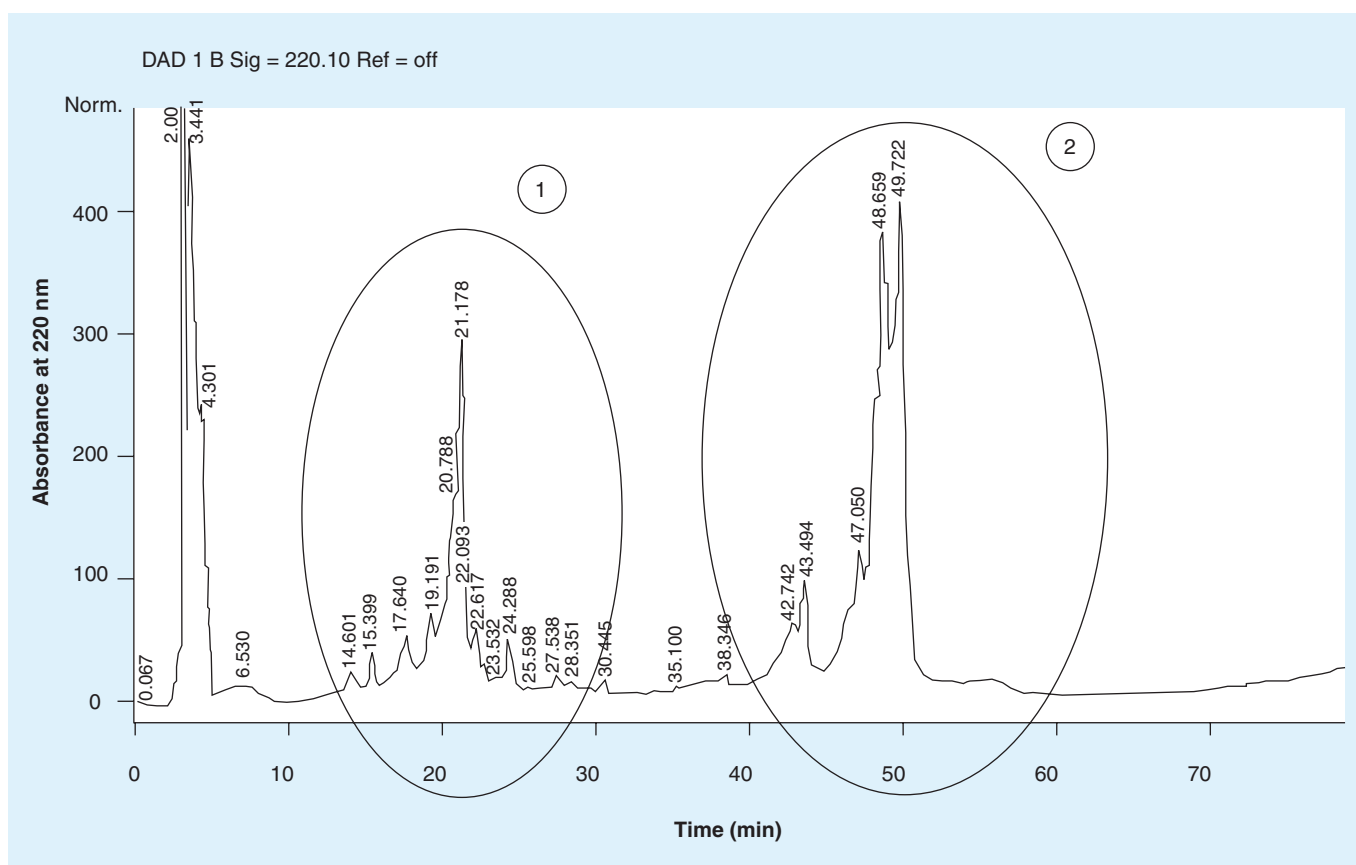


Figure 8. Proteomic analysis of teff storage proteins (II). C_8 Reverse-phase-HPLC chromatogram of teff proteins extracted in denaturing/reducing buffer. Numbering of protein subsets (1 and 2) refers to 2D-IEF/SDS PAGE separation in **FIGURE 7**.

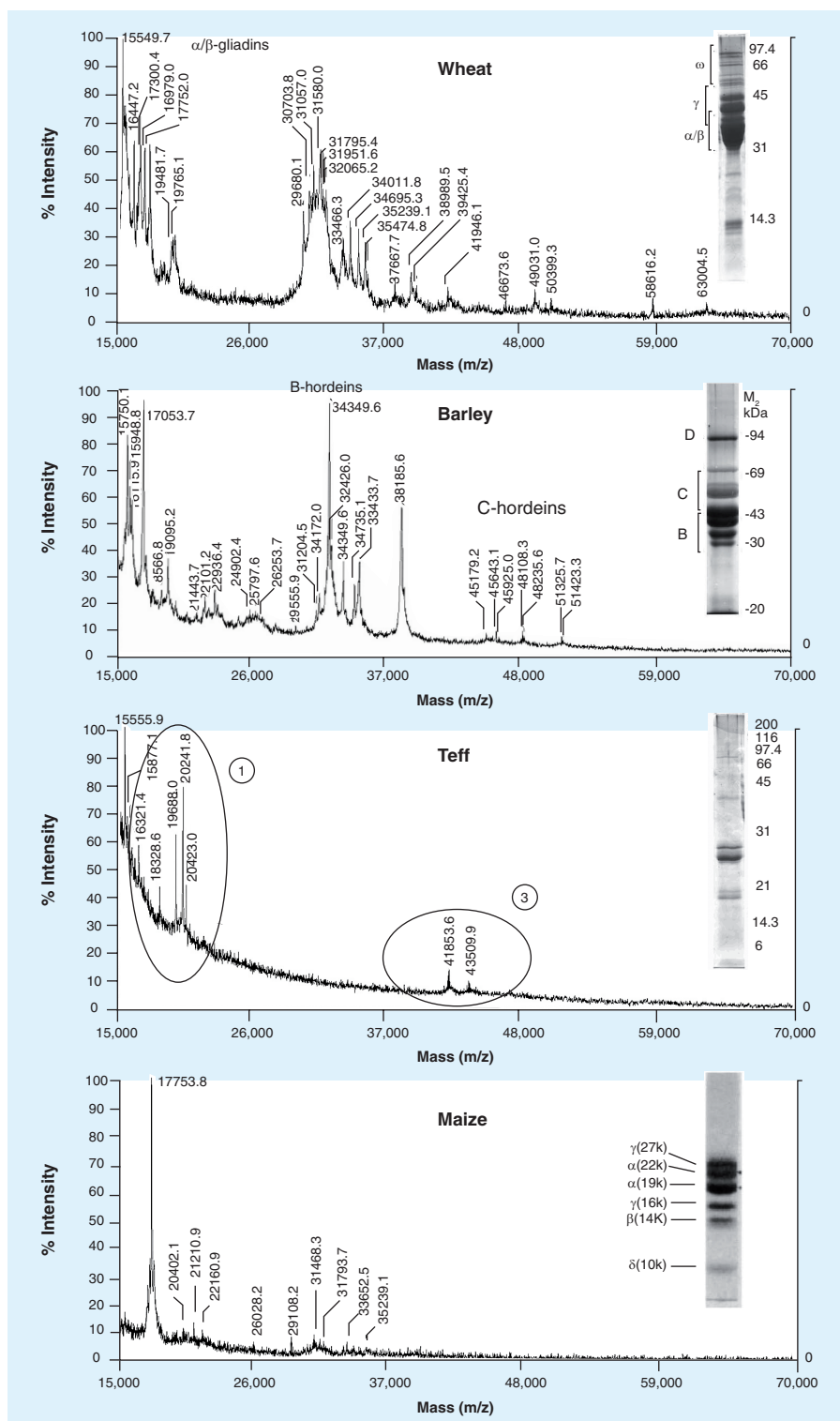


Figure 9. Proteomic analysis of teff storage proteins (III). Teff seed proteins extracted in denaturing/reducing buffer were analyzed by MALDI-TOF-mass spectrometry in comparison with the prolamin extracts of other cereals. Subsets of proteins labelled with numbers 1 and 3 correspond to those of the 2D-IEF/SDS PAGE analysis in **FIGURE 7**. Proteins of subset 2, despite their high abundance, were not detected, probably because of a low ionization efficiency. SDS-PAGE analysis of the respective protein extracts are shown in the insets. In the protein extracts of teff, similarly to maize, the major 30–40 kDa polypeptides of the celiacogenic cereals are missing.

From these examples, it can be deduced that in wheat-derived processed food, ingredients and beverages, even if considered free of gluten or of allergenic components, potentially harmful protein fragments generated by the technological treatments can still be present.

Five-year view

Nutrition plays a crucial role in health as well as disease. Despite its youth, food proteomics is already influencing multiple aspects of the food chain (agriculture, food production, food safety and quality assurance) and is delivering economic benefits and improving aspects of human nutrition and health. By dissecting the proteome or metabolome of their constituents, using the new technologies, specific markers can be identified to trace the complete productive processes from raw materials to end products, and structure/function links can be clarified.

Over the coming years, an integrated approach based on the various ‘-omics’ for food peptide and protein characterization, all of which rely on MS analysis, should accelerate the development of functional food products, as well as extend our knowledge in design of novel foods and ingredients. For example, the capacity of MS techniques to analyze supramolecular complexes is opening new scenarios in the understanding of protein–protein, protein–carbohydrate and protein–lipid interactions, which in turn determine the rheological and sensory properties of complex matrices such as wheat dough and their industrially designed substitutes for intolerant patients using tTG-modified non-gluten proteins [102].

Thus, in the wider perspective of studying what food components become after ingestion, proteomics will offer a relevant contribution to help disentangle some of the routes through which food components interact with one another to generate novel immunodeterminants or to potentiate those that already exist.

Food allergy and intolerance are fields in which MS is proving to be essential at various levels, including the controversial feature of the resistance to digestion of allergenic proteins or the efficiency of removal of epitopes from a food destined

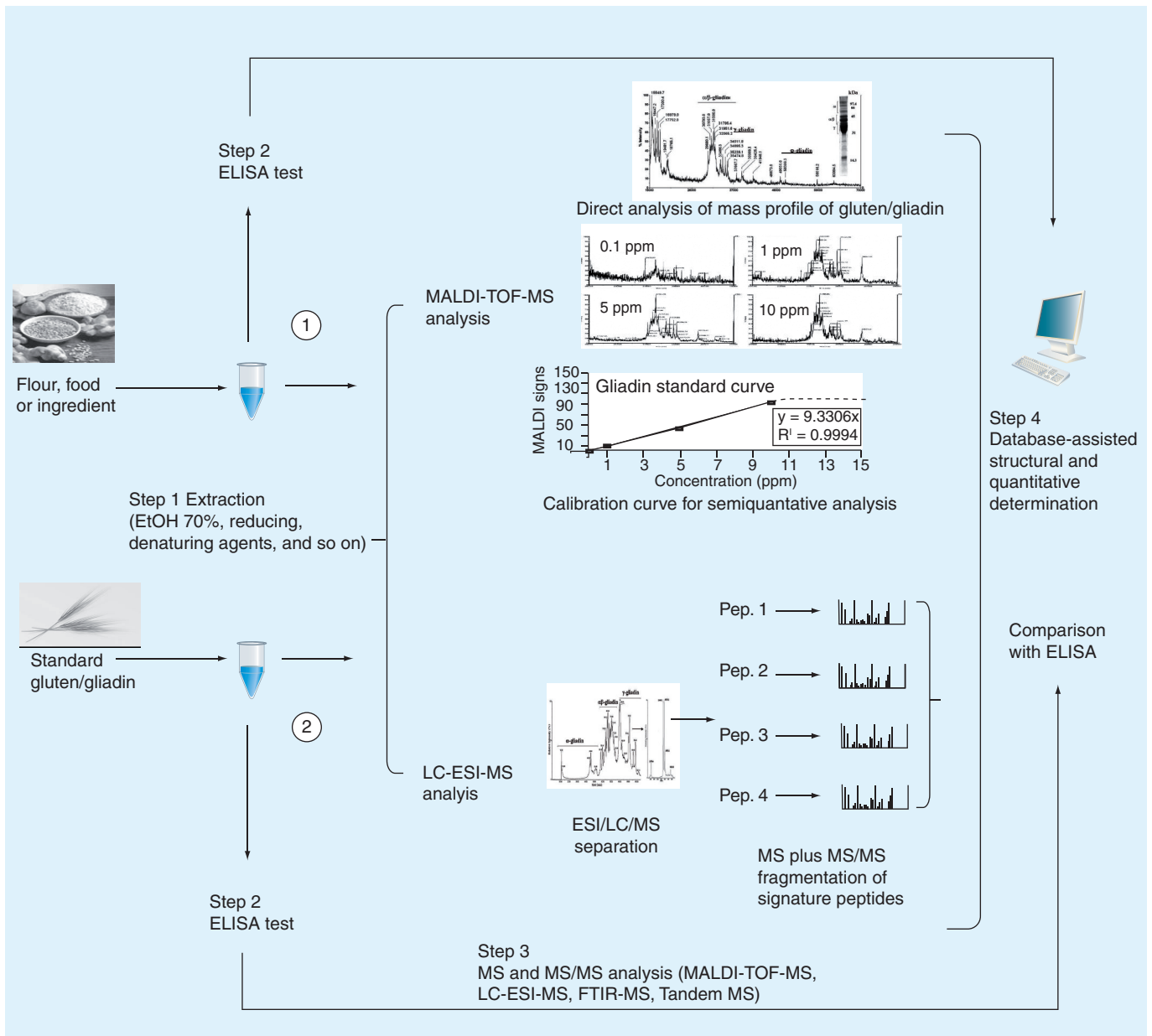


Figure 10. Issues in proteomic determination of gluten in foods. Mass spectrometry is the only nonimmunological method currently available to detect gliadins and related prolamins in flours and in food samples with high specificity. Extraction of gliadin/gluten proteins from the complex food matrix (step 1) is essential for successful qualitative and quantitative analysis (step 2). The MALDI-based approach (route 1) relies on the possibility of obtaining characteristic MALDI-TOF-MS profiles of unfractionated celiacogenic gliadin, hordein, secalin and avenin extracts. Based on these four distinguishable mass patterns, prolamins from different cereals can be differentiated and also identified when simultaneously present in foods. A second analytical approach (route 2) is based on LC-MS, based on the monitoring of 'signature' peptides of gluten proteins (limits of detection and quantification for target peptides are in the range 1–10 ppb and 10–100 ppb, respectively). Applications are in the analysis of the hydrolyzed starch products, or fermented beverages such as beer. ESI: Electrospray ionization; EtOH: Ethanol; LC: Liquid chromatography; MS: Mass spectrometry.

for patients. The proper MS techniques, selected from time to time from within the large spectrum of possible applications, can provide a means of identifying the immunodominant epitope(s) in the target food as a starting point for designing antibodies against specific epitopes, and to standardize the quantitative detection of trace amounts. Therefore, large cost- and

health-saving results are being achieved by application of the MS-based efficient monitoring procedures [21,58]. Furthermore, the advances and future developments of protein array technology, which will be largely driven by MS-derived structural information, will ensure more rapid and accurate detection of food composition for the safety of intolerant consumers. For

all these reasons, employment of novel MS techniques in food proteomics is expected to increase during the next few years, due to their emerging potentiality in the molecular characterization of food products. In this direction, the MS-based forthcoming advances in quantitative peptidomic analysis, such as isobaric tag for relative and absolute quantitation or targeted selected reaction monitoring [103], will play a key role.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Key issues

- Allergies to wheat are provoked by ingestion, inhalation or contact with either the soluble or the insoluble gluten proteins. Gluten is also the main factor triggering celiac disease, a severe enteropathy affecting susceptible individuals. The mechanisms through which wheat proteins and their derived peptides elicit adverse reactions in allergic or intolerant patients still remain to be completely explained. Elucidation of these key issues requires a combination of clinical, immunological, genomic and proteomic approaches.
- The recent developments in proteomics have already provided an important contribution to the disclosure of basic aspects of wheat protein-related diseases. These include: structural and functional information to assist the identification of proteins involved in wheat allergy or intolerance, including celiac disease, and their mechanism of activation of toxicity; the development of methods for detecting trace amounts of wheat proteins in foods; and the formulation of substitute foods and ingredients to improve the quality of life of patients.
- The major challenge for the development of acceptable gluten-free products is the poor sensory quality due to the lack of the gluten network. The use of structuring agents enabled the use of pre-hydrolyzed wheat flour to obtain breads with improved sensory characteristics. Mass spectrometry may ensure degradation of toxic epitopes following the entire technological production process of bread, as well as monitoring protein modifications responsible for rheological and sensory characteristics.
- Mass spectrometry is the only nonimmunological method for high-specificity detection of prolamins in foods. Once the appropriate sample handling and the correct operative conditions are provided, semi-quantitative determination of wheat proteins can be efficiently obtained by MALDI-TOF-MS analysis. Accurate liquid chromatography-tandem mass spectrometry methods are now available for quantitative measurement of gluten peptides in food and ingredients, with limits of detection and quantification in the range 1–10 ppb and 10–100 ppb, respectively.
- Proteomic analysis is making clear that even though in wheat-derived food and beverages are considered free of gluten or of allergenic components, harmful peptide sequences generated by the technological treatments can still be present.
- The availability of fast and accurate alternatives to immunological ELISA tests may also facilitate research towards the development of novel drugs, therapies and food-processing technologies to aid patients with gluten intolerance, and for gluten-free labeling and certification processes – all issues where the role of proteomic science is emerging.

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