CEREALS FOR DEVELOPING GLUTEN-FREE PRODUCTS AND ANALYTICAL TOOLS FOR GLUTEN DETECTION

Cristina M. Rosell[1*], Francisco Barro[2], Carolina Sousa[3], Mª Carmen Mena[4]

[1] Institute of Agrochemistry and Food Technology. CSIC. Avenida Agustin Escardino, 7. Paterna 46980. Valencia. Spain.

[2] Institute for Sustainable Agriculture. CSIC. Alameda del Obispo s/n, 14080-Córdoba, Spain.

[3] Departamento de Microbiología y Parasitología, Facultad de Farmacia, Universidad de Sevilla, Sevilla, Spain.

[4] Proteomics Facility, Centro Nacional de Biotecnología. CSIC. Calle Darwin 3, Cantoblanco, 28049 Madrid, Spain.

Address for correspondence: *crosell@iata.csic.es

Abstract

Recently, gluten free foods have attracted much research interest motivated by the increasing market. Despite the motivation for developing gluten-free foods it is necessary to have a scientific basis for developing gluten-free foods and the tools for detecting the peptide sequence that could be immune-toxic to some persons. This review will be focused primarily on the cereal-based commodities available for developing gluten free blends, considering those naturally gluten free cereals besides the controversial oats, and the recent transgenic approaches for developing cereals free of immunotoxic gluten. Secondly, the biochemical tools for mimicking gluten network viscoelastic properties will be presented. Finally, special emphasis will be put in compiling the available techniques for gluten detection and quantitation.

Key words: gluten free cereals, breeding, analytical tools, prolamins

1. Introduction

Wheat, barley and rye are temperate cultivated cereals from the grass tribe triticeae. These cereals are major suppliers of calories to the human diet as the main component of the grain is starch. They also contain 8-15% of protein, which mainly correspond to the gluten proteins. Those proteins are important because of their impact on grain processing properties, for the production of bread, pasta and noodles from wheat, and for malting, brewing and distilling of barley. Gluten is a complex mixture of proteins comprising the gliadins and glutenins in wheat and equivalent proteins in barley and rye, representing 80% of total grain proteins. However, gluten proteins also have negative impacts on human health, in relation to allergies and intolerances. Gluten is defined differently depending on the discipline (biochemistry, food technology, nutrition). In the gluten-free intolerance context the most appropriate

definition is that provided by the European Commission where “gluten means a protein fraction from wheat, rye, barley, oats or their crossbred varieties and derivatives thereof, to which some persons are intolerant and which is insoluble in water and 0.5 M sodium chloride solution” (Commission of the European Communities, 2009).

In recent decades, gluten has attracted great attention due to the increasing number of diagnosed patients with intolerance to this protein fraction, relating to the improved sensitivity of the detection methods and the increasing awareness of the existence of the disease. Three pathologies are associated with gluten intake (Figure 1), which appear to be increasing in importance: i) food allergy that affects 0.2-0.5% of the population but has stronger clinical implications (Zuidmeer et al., 2008), ii) coeliac disease (CD), which is an autoimmune disorder caused by the ingestion of gluten not only from wheat, but also rye, barley and some varieties of oats (Comino et al., 2011) affects both children and adults throughout the world at various frequencies (from 0.1% to >1.6%), specifically 1% of the Werstern world (Abadie et al., 2011), and iii) gluten sensitivity, a pathology of intolerance to gluten that has been recently rediscovered (Sapone et al., 2011), which excludes CD and wheat allergy, with an estimated prevalence of 6% for the USA population.

There is evidence that there are peptides that are responsible for celiac disease and gluten allergy. However, the intolerance's and non-celiac gluten sensitivity have been related to other components present in bread. Much research has been focused on determining the peptide sequence promoting bowel inflammation, although very much remains unknown. CD is an inflammatory disease of the upper small intestine caused by glutamine- and proline-rich peptides from cereal storage proteins (gluten) with a minimal length of nine amino acids, but innate immunity is also involved in the damage produced by these cereals (Bernardo et al., 2012). In people with a genetic susceptibility, such peptides are insufficiently degraded by gastrointestinal enzymes; they permeate the lymphatic tissue, are bound to celiac-specific,

antigen-presenting cells, and stimulate intestinal T-cells. Till now, the only effective therapy is based on a gluten-free diet (GFD) long-life, which explains the interest in developing safe, nutritionally balanced and palatable gluten free foods. This poses challenges for the use of cereals to develop gluten free-products. Nevertheless, this challenge has been met by agronomic and food technology approaches, which will be discussed in the next sections. This review will be focused on cereal-based commodities available for developing gluten-free blends and the biochemical tools for mimicking gluten network viscoelastic properties. Special emphasis will be put on describing the available techniques for gluten detection and quantitation.

2. Gluten free cereals

2.1 Cereals reported to be gluten free

Although the name wheat is always associated with gluten and with toxicity for coeliac patients, some reports have stated that some wheat cultivars are safe for this population. In fact, Spaenij-Dekking et al. (2005) investigated whether wheat varieties exist with a natural low number of T-cell-stimulatory epitopes by analysing gluten protein sequences present in public databases. In their study, wheat accessions from diploid (AA, SS/BB, and DD genomes), tetraploid (AABB), and hexaploid (AABBDD) *Triticum* species were also tested for the presence of T-cell-stimulatory epitopes in gliadins and glutenins by both T-cell and monoclonal antibody-based assays. The authors identified gluten proteins that lack one or more of the known T-cell-stimulatory sequences suggesting that it would be possible to select and breed wheat varieties that contain low amounts of T-cell-stimulatory sequences. One example is *Triticum monococcum*, the oldest and most primitive cultivated wheat. Pizzuti et al. (2006) evaluated the toxicity of this wheat by using an *in vitro* distal duodenum biopsies culture system, which were subjected to conventional histological examination and

immunohistochemical detection. This revealed that *T. monococcum* gliadin shows a lack of toxicity.

Cereals that are always considered gluten free are rice (*Oryza sativa L.*), maize (*Zea mays L.*) and sorghum (*Sorghum bicolor (L.) Moench*), which are distant relatives of wheat and are known to be safe for coeliacs. In addition, a number of species of millets, the Ethiopian cereal teff, and a range of pseudocereals are also available for providing gluten free flours, even increasing the nutritional pattern of those products in the case of pseudocereals (Alvarez-Jubete et al., 2010). Nevertheless, rice and corn are low in protein, fibre, and folate (Table 1); whereas teff, quinoa, amaranth and buckwheat show a favourable fatty acid composition and they are high in protein (Hager et al., 2012). In particular, amaranth and teff are high in calcium, magnesium and iron, although those contents are greatly dependent on the region of cultivation.

Rice flour is the most suitable commodity for bakery applications due to its bland taste, white colour, digestibility and hypoallergenic properties. Other attributes such as the low content of protein and sodium and the presence of easily digested carbohydrates are additional benefits. In rice, the ratio of albumin-globulin-prolamin-glutelin is unique among the cereals, having a high concentration of glutelins and low prolamins prolamins (Hamaker, 1994). As in the other cereals, rice proteins are deficient in essential amino acid lysine, but as a consequence of the ratio of protein fractions, rice has higher content of lysine than the other cereals, which is shared by oats. It is important to note that although the amino acid pattern of the proteins could be similar in different rice varieties, significant differences have been reported in protein and starch digestibility among varieties (Acquistucci et al., 2009).

Immunochemical, molecular and *in vitro* and *in vivo* studies support the conclusion that sorghum does not contain peptides that are toxic for coeliac patients. The analysis of

aqueous/alcohol-soluble prolamins (kafirins) from different sorghum varieties provides molecular evidence for the absence of toxic gliadin-like peptides in sorghum (Pontieri et al., 2013). A similar study carried out with amaranth revealed that all of the amaranth samples studied showed similar binding affinities for both specific anti-gliadin antibodies and human IgAs, and the molecular characterisation of amaranth proteins suggests that it is safe for coeliacs to consume (Ballabio et al., 2011).

Teff, a low risk cereal for coeliacs that is grown in Ethiopa and other countries, has an excellent amino acid composition (including all 8 essential amino acids for humans), very high fibre content and high nutrient content in general, being suitable for gluten-free applications (Gebremariam et al., 2012).

2.2 The oats controversy: selection of oat varieties with no toxicity in coeliac disease

There is an on-going debate concerning the presence or absence of proteins in oats that could be toxic for coeliac patients. Traditionally, treatment with a gluten free diet GFD has excluded not only wheat, barley, and rye, but also oats. Oats, like rice, differs from other cereals in their prolamin content. The percentage of proline and glutamine (amino acids abundant in toxic regions) in avenins is lower than in prolamins from toxic cereals (Figure 2). However, there is still some debate about the safety of oats (Pulido et al., 2009). Several *in vivo* and *in vitro* studies have indicated that the majority of coeliac subjects could tolerate moderate amounts of pure oats. Some countries permit the use of oats in “gluten-free” products, e.g. Gluten Free Oats®. According to the Codex Alimentarius Commission (2008), oats can be tolerated by most, but not all, people who are gluten-intolerant. Therefore, the acceptance of oats that are not contaminated with wheat, rye, or barley in foods covered by this standard may be determined at the national level. Moreover, according to the Commission of the European Communities (2009), a major concern is the contamination of oats with wheat, rye, or barley that can occur during grain harvesting, transport, storage, and processing. Therefore, the risk

of gluten contamination in products containing oats should be taken into consideration with regard to labelling of products. Some cross-reactivity with gliadin-specific antibody has been attributed to wheat contamination in oat-based food (Pulido et al., 2009).

However, other authors have reported clear evidence suggesting that avenins have the ability to induce the activation of mucosal T-cells, causing gut inflammation and villous atrophy (Pulido et al., 2009). Arentz-Hansen et al. (2004) described the intestinal deterioration suffered by some CD patients following the consumption of oats while on gluten free diet. Avenins can trigger an immunological response in these patients similar to the response produced by the gluten of wheat, rye, or barley. The monitoring of 19 adult coeliac patients who consumed 50 g/day of oats over twelve weeks showed that one of the subjects was sensitive to oats. Therefore, it is critical to clarify either qualitatively or quantitatively the potential immunotoxicity of oats to coeliac patients (Arentz-Hansen et al., 2004; Pulido et al., 2009).

Comino et al. (2011) showed using the anti-33-mer antibodies (G12 and A1) raised against the toxic fragment that oat immunogenicity for CD patients varies according to the cultivar. The intensity of the signal obtained with the antibody was proportional to the potential damage caused to CD patients, thereby providing a rational explanation of why some oats trigger immunological response, and a solution to avoid the presence of such varieties in gluten-free diets.

3. Transgenic approaches for developing cereals free of immunotoxic gluten

The identification and/or breeding of wheat cultivars or close relatives naturally deficient in immunogenic prolamins is very appealing. However, none of the tested materials (including landraces, cultivars, and deletion lines) was completely nontoxic for CD patients (Spaenij Dekking et al., 2005; van den Broeck et al., 2009; Comino et al., 2012a). The high level of

complexity of gliadin genes and the fact that they may contain multiple and different T-cell epitopes (van Herpen et al., 2006), make conventional breeding approaches to obtain wheat varieties with reduced content of immunotoxic sequences very difficult.

The use of genetic engineering to down-regulate gene expression is now routinely used in many crops, including all the major cereals, and is therefore an attractive opportunity for reducing the immunotoxic components of gluten and, therefore the incidence of gluten-related allergies and intolerance in cereals. Most of the examples listed below of down-regulation of gluten proteins in wheat and other cereals are based on RNA interference (RNAi) technology (Table 1). Although not all of them were specifically aimed to develop low-gluten cereals for human allergies and intolerance, they prove that the down-regulation of the different gluten fractions related to the CD can be achieved using this technology.

RNA silencing is a sequence-specific RNA degradation system that is conserved in a wide range of organisms, and termed post-transcriptional gene silencing and RNAi, in plants and animals, respectively. RNAi is a post-transcriptional process triggered by double-stranded RNA (dsRNA), which leads to gene silencing via a two-step mechanism (Watanabe, 2011).

Becker et al. (2006) reported the use of hpRNA constructs to knock down the α-gliadins in cv Florida. They produced a range of transgenic plants with the α-gliadins silenced. Flours from transgenic and wild type lines were analysed by RP-HPLC, showing a 63% reduction of the α-gliadins in the transgenic lines (Becker et al., 2012) (Table 1). The loss of α-gliadins was compensated by the increase of albumins/globulins (34%), ω-gliadins (23%), γ-gliadins (20%), HMW-GS (16%) and glutenin-bound ω-gliadins (8%). In contrast, LMW-GS were not affected. Overall, the total contents of gliadins and gluten were reduced, respectively, by 16 and 9%. Interestingly, the crude protein content was similar in transgenic lines and in the wild type.

Lange et al. (2007) introduced an antisense construct against C-hordeins (the sulphur-poor

storage proteins of barley which are homologous to the ω-gliadins of wheat) into barley cv 'Golden Promise'. The reduction of the target proteins ranged from 29 to 40% depending on the transgenic line. Other storage proteins were affected by the down-regulation of C-hordeins; the relative amount of γ/B-hordeins was increased in the transgenic lines while there were also minor relative increases in the amounts of D-hordeins (homologous to the HMW-GS of wheat). The increase in the sulphur-rich γ/B-hordeins and D-hordeins was related to significant higher expression levels of the corresponding genes (Hansen et al., 2007). Interestingly, this re-distribution in the proportions of storage proteins resulted in a more balanced amino acid composition; the five antisense lines had lower amounts of proline, glutamic acid/glutamine, and phenylalanine (up to 12%, 6%, and 9% reduction), while the lysine, threonine, and methionine contents were increased by up to 16%, 13% and 11%, respectively.

Gil-Humanes et al. (2008) and Piston et al. (2011) also used two hpRNA constructs to silence the γ-gliadins in two genotypes of the bread wheat cv 'Bobwhite'. They reported 18 transgenic lines with reductions in the γ-gliadin fraction. However, the down-regulation was variable, ranging from 65 to 97% depending of the transgenic line (Piston et al., 2011). The reduction of γ-gliadins was also accompanied by an increase in other storage proteins, in particular, the ω- and α-gliadins (Table 2). However, the total gliadin content did not differ significantly in the transgenic lines relative to the wild type. Likewise, the transgenic lines showed increases in the HMW-GS, LMW-GS and total glutenin contents. Overall, the gluten content was increased by an average of 50% and the total protein content was slightly higher in most of transgenic lines, but more significantly for lines of one of the genotypes. They carried out an *in silico* analysis of the amino acid composition of gluten proteins in transgenic lines with down-regulated γ-gliadins and control lines, showing that the overall proportions of amino acids were not significantly modified in the transgenic lines relative to the wild type

(Piston et al., 2011).

The ω-gliadin fraction has also been targeted for silencing by RNAi (Altenbach and Allen, 2011). Analysis of proteins in grain from transgenic plants demonstrated that the ω5-gliadins were either absent or substantially reduced relative to the amounts in non-transformed controls while other gliadins, including ω-gliadins from genomes A and D, were not affected. The effects of silencing of ω5-gliadin on other gluten or non-gluten proteins were not reported.

Several groups have taken advantage of the possibilities that the RNAi technology offers for the silencing of multigene families, and have also addressed the down-regulation of more than one group of gliadins and/or glutenins.

Gil-Humanes et al. (2010) reported two hpRNA constructs designed using a chimeric fragment of 361 bp, encompassing sequences highly conserved among α-, ω-, and γ-gliadins. They used both hpRNA constructs, single or in combination, to produce a range of transgenic plants from two genotypes of the bread wheat cv Bobwhite. They showed that the chimeric fragment was able to effectively down-regulate the expression of genes from all three gliadin groups. Interestingly, one line (BW2003) was found to carry the translocation T1BL.1RS and expressing the ω-secalins from rye, which were also silenced by the chimeric fragment (Gil-Humanes et al., 2010). The gliadin composition, determined by RP-HPLC, showed a significant reduction in the gliadin content in all of the transgenic lines, ranging from 70 and 88%. The down-regulation of gliadins also resulted in changes in other grain proteins. The HMW-GS were increased in some transgenic lines compared with wild types. In contrast, the LMW-GS were unaffected in most of the lines, and only four lines showed a significant decreased in the LMW-GS content relative to the wild-type line. Overall, the gluten proteins were decreased by up to 56% while the non-gluten proteins albumins and globulins were increased in some transgenic lines (Gil-Humanes et al., 2011), as consequence, the total

nitrogen content of the grain was not significantly affected.

A different approach was followed by Wen et al. (2012) to down-regulate gliadins and LMW-GS in wheat. They reported the transcriptional suppression of wheat DEMETER (DME) homologs using RNA interference in the bread wheat cv "Brundage 96" (Wen et al. 2012). The results of qRT-PCR showed suppression in DME transcript abundance ranging from 3.0 to 85.2% in different transgenic plants. The level of reduction of LMW-GS and gliadins based on the HPLC chromatograms ranged from 45.2 to 76.4%, depending of the transgenic line. However, not all gliadin and glutenin fractions were equally affected, and the transgenic lines showed different profiles of elimination and/or reduction of specific gliadin and/or glutenin family members. In most of the transgenic lines all three gliadin fractions and LMW-GS were down-regulated, and HMW-GS were likewise slightly decreased. They also reported lines in which a slight increase in the amount of ω-gliadins and a significant increase in the amount of HMW-GS was also observed (Wen et al., 2012).

3.1 Effects of RNAi silencing on baking properties and immunotoxicity

In all the above examples, the silencing of a given gliadin fraction is compensated by an increase in another group of gliadins, glutenin subunits or even albumins/globulins, so that the total content of gliadins and/or gluten in the grain is not greatly affected. There are two important points to consider: 1) how quality is affected by the silencing of gliadins, or other gluten proteins, and 2) whether the deficiency of these gluten fractions results in an effective reduction of immunotoxicity for the transgenic lines to be used in foodstuff tolerated by many patients with CD or other gluten-related pathologies.

Becker et al. (2012) reported the rheological and gluten properties, and baking quality of wheat lines with reduced α-gliadin content. Due to the strong reduction in α-gliadins and the lower ratio of gliadins to glutenins, the rheological properties of glutens from the transgenic

flour and the wild type flour differed strongly. In contrast, the rheological properties of the doughs were almost identical. They also showed that the deficiency of α-gliadins did not substantially affect the baking performance of wheat flour, although breads made using flour from lines with silenced α-gliadins had lower volumes (-11%) compared to those of wild type breads (Wieser et al., 2006). Piston et al. (2011) determined the mixing properties of 18 transgenic lines from two genotypes with the down-regulated γ-gliadins by using the mixograph and sodium dodecyl sulphate sedimentation (SDSS) test. They showed that most of the parameters were not affected by the down-regulation of the γ-gliadins, and only peak width (PW1), mixing time (MT), and SDSS volumes were affected in some lines. These observations were later confirmed by the introgression of the silencing of γ-gliadins into commercial lines of bread wheat (Gil-Humanes et al., 2012). They concluded that the reduction of γ-gliadins seems not to have a direct effect on the mixing and bread-making properties of wheat dough, but the compensatory effect on the synthesis of the other prolamins can provide stronger doughs with improved resistance to overmixing. It is not known whether the silencing of all three groups of gliadins (Gil-Humanes et al., 2010), or gliadins and LMW-GS (Wen et al., 2012) will have effects on wheat quality. Preliminary results were reported by (Gil-Humanes et al., 2010) based on the SDSS test. They showed that most of transgenic lines with down-regulated ω-, γ- and α-gliadins had SDSS volumes comparable with those of wild type lines, and five lines had SDSS volumess significantly lower than those of wild type lines. However, the SDSS volumes of these five lines were still comparable with those of the medium-quality bread wheat.

The transgenic lines described above are deficient in one or more groups of gluten proteins (α-, γ-, ω-gliadins and glutenin subunits), which are the known sources of immunogenic epitopes. Thus, these lines themselves hold good potential to be tested for immunotoxicity by monoclonal antibodies and T-cell assays. At present, only transgenic lines deficient in γ-

gliadins (Gil-Humanes et al., 2008) and in all three gliadin fractions (Gil-Humanes et al., 2010) have been tested by monoclonal antibodies and T-cell assays. They reported that for transgenic lines with silenced γ-gliadins, the gliadin content (ppm) did not decrease significantly but increased for some lines as consequence of the compensatory effects on other gliadins, specifically on α- and ω-gliadins (Gil-Humanes et al., 2008; 2010). However, when gluten proteins from transgenic lines deficient in all three groups of gliadins were tested, there was a significant reduction in the gliadin content in all of the transgenic lines, with an average reduction of 92.2% and a range between 89.7% and 98.1% (Gil-Humanes et al., 2010).

According to the "Codex Alimentarius standard for gluten-free food" the gliadin content is taken as 50% of gluten so that the gluten content has to be calculated by multiplying the gliadin content by a factor of 2. The gluten content was calculated (Table 3) for lines reported by Gil-Humanes et al. (2010) and the predicted tolerable daily intake of gluten estimated on the basis of results by Catassi et al. (2007). In this study, a daily intake of 50 mg gluten was established as the maximum dose in order to avoid damage to the small intestine of CD patients during a prolonged exposure of 90 days. This means that for the wild type lines the tolerated amount of flour would be less than 0.4 g (Table 3). However, for the line E82 a coeliac individual could safely ingest around 20.4 g per day of flour, and for line D793 the tolerated amount would be of up to 10.7 g. This amount of gluten could be reduced even more by mixing flour from the transgenic lines with flours from non-toxic cereals such as rice or corn.

Acceptance of transgenic plants, as raw material for food depends upon the advantages of these crops are clearly perceived by the end consumers. Until now, GM crops commercialized mainly offer pest or herbicide resistance, for which the end consumer benefits are not obvious. For consumers, large companies and/or farmers, are the major beneficiaries of GM

crops, and the benefit that they may have on agriculture and the environment is very diluted as a result of heavy campaigning by anti-GMO groups in developed countries. The development of wheat lines suitable for coeliacs and other gluten intolerances could be a major turning point: it aims to solve a health problem that directly affects a large proportion of consumers, also in developed countries, and with which the other consumers are very sensitive.

4. Leavened Gluten free products: challenges for miming gluten

Gluten free diets are characterised by an unbalanced intake of different nutrients, with very low contributions to the recommended daily protein intake, and high contributions to the carbohydrate and fat dietary reference intake (Matos and Rosell 2011). This highlights the importance of designing nutritionally balance or enriched gluten free products. Recently, gluten-free foods have been the subject of massive research motivated by three global trends: the increased number of coeliac patients, which is related to the enhanced sensitivity of the detection techniques, the market trends because gluten free foodstuffs are viewed as a niche market for expansion, and the association between gluten-free foods and health living. Irrespective of the motivation for developing gluten-free foodstuffs it is necessary to have a scientific basis for developing gluten free foods, which has been a very active area of research. Recently, Zannini et al. (2013) reviewed the technological challenges and strategies for developing gluten-free bread and the importance of foodomics studies (comprising genomics, proteomics, metabolomics and materiomics) to overcome the limitations due to the inability of gluten-free cereal flours to retain carbon dioxide in fermented products. As a consequence, the addition of different components, by different flour and dough treatments or by changing the method of baking is required to improve quality in gluten-free bread production (Houben et al., 2012).

The functional properties of cereals and cereal-based flours play a major role when developing fermented gluten-free products. For instance, the type of rice milling and the resulting particle size of the flour define the suitability of the rice or corn flours for breadmaking (Song and Shin, 2007; Brites et al., 2010). Particle size fractionation of rice flour may be advisable for selecting specific physico-chemical properties (De la Hera et al., 2013). In fact, De la Hera et al (2012) stated that coarser corn flours (>180 μm) provide breads with higher volume and softer crumb due to their ability to retain carbon dioxide during proofing. Considering the thermal properties, it has been reported that initial pasting and setback viscosities decrease with decreasing particle sizes, with wet milled rice flours that have higher apparent viscosity and smaller particle sizes than dry-milled rice flour showing better properties for making gluten-free rice bread (Song and Shin, 2007). Rice cultivars with intermediate amylose contents, which also are sticky and soft, give bread with high volume (Iwashita et al., 2011). In addition to the cultivar type, germination is also of interest for increasing the nutritional pattern of cereals and pseudocereals (Omary et al., 2012) and because proteolytic enzymes produced during germination could decrease the toxicity of some cereals. A concrete example is the hydrolysis of rye secalin peptides by cereal enzymes from germinating barley. These released short fragments and reduced the toxicity of the rye (Stenman et al., 2010).

Nevertheless, some additional structuring agents are always necessary to counteract the lack of viscoelasticity associated with the absence of gluten. Leaving aside the addition of structuring ingredients (hydrocolloids, proteins, and so on), which has been a very active area of research, and without trying to be exhaustive, some attempts to create internal networks in cereal flours are discussed. Oxidizing or crosslinking enzymes have proved to be good alternatives for improving dough performance and the quality of the gluten-free breads. For example, the addition of cyclodextrin glycosyl transferase to the rice bread formulation led to

rice loaves with very soft crumb and extended the shelf life (Gujral et al., 2003a, b). Other useful enzymes in rice bread formulation are glucose oxidase and transglutaminase (Gujral et al., 2004a, b) that catalyse the formation of inter and intramolecular crosslinks between the cereal proteins leading to a protein network (Marco and Rosell 2007, 2008). Protein crosslinking catalysed by transglutaminase was confirmed by the decrease in the amount of free amino groups and the disappearance of numerous protein bands as a consequence of the formation of large protein polymers, linked by isopeptidic and disulphide bonds, with reduced solubility (Marco et al., 2007).

Numerous alternatives have been reported recently to improve nutritionally gluten free products; it is not the intention of this review to list all of them, only to point out the most significant ones. The use of whole grains mainly to increase the fibre content or the addition of bran has been proposed to improve the nutrient profile of gluten-free products (Sairam et al., 2011). Making blends of cereal and pseudocereal flours such as buckwheat flour may give to gluten-free breads with enhanced contents of total phenolic compounds, rutin and quercetin contents and antioxidant activity (Sakac et al., 2011). Pregerminated brown rice is another alternative bread ingredient because it is more nutritious after germination with increased free gamma amino butyric acid (Charoenthaikij et al., 2010; 2012).

5. Gluten analysis

Gluten may trigger immune responses of different kinds in predisposed individuals. Since the only effective treatment is the avoidance of gluten containing foods, it is necessary to have reliable methods for gluten determination. Gluten proteins have unique compositions with approximately 15% proline and 35% glutamine residues (Alaedini and Green, 2005). Although most dietary proteins are digested into simple amino acids, dipeptides, and tripeptides by gastrointestinal proteases, the high content of proline residues makes the gluten

peptides resistant to degradation by digestive proteases (Bethune and Khosla, 2008; Fasano, 2009). About 10% of gluten appears to be made up of potentially toxic gliadin peptides (Khosla et al., 2005), thus it is desirable to quantify the amounts of these peptides, so that the toxicity of the gluten present in foods can be established more precisely. Nevertheless, Although α- and ω–gliadin-derived peptides are immunodominant in adults, responses to the LMW glutenins and γ-gliadins have been frequently observed in children and occasionally in adults (van de Wal et al., 1999; Vader et al, 2002).

The presence of the numerous gluten components, the variation in the extraction efficiency, and the lack of reference materials are some issues that hinder the implementation of equivalent laws at national level and the comparison of data across the different methods (Diaz-Amigo et al., 2012).

5.1. Factors affecting gluten analysis

Proteins are modified to improve their functionally and increase their usage in different applications to form and stabilise structures. Proteins can be modified in various ways as deamidation, transamidation and degradation. All of these modifications can also happen naturally due to enzymes in cereal seeds.

Hydrolysis is widely used in the preparation of wheat products in the food industry. Hydrolysis may decrease the toxicity of gluten (Greco et al., 2011) but also the fragmentation of peptides can make it more difficult to analyse gluten in these foods (Mena et al., 2012).

Deamidation of gluten proteins is a widely used process in the food industry that leads to increased charge density on the protein, causing changes in protein conformation due to electrostatic repulsion. The analysis of deamidated gluten is more difficult as there is a decrease in the affinity and recognition of antibodies to gluten proteins and peptides, which

may lead to underestimation when immunoassays are used to the quantify gluten content of foods (Kanerva et al., 2011).

An important factor in prolamin and glutelin analysis is complete extraction from the samples. The extraction of gluten from wheat, barley, rye and oat standards is easier than from processed foods. During the processing of some foods, proteins are treated at high temperatures in a dry state at a neutral pH, forming isopeptide bonds between the ε-amino groups of lysine and the β- or γ-carboxamide groups of asparagine and glutamine residues. Furthermore, the heat-treatment of cooked and baked products leads to the formation of protein aggregates in an insoluble matrix that makes analyses even more difficult. Therefore it is necessary to use an extraction system giving complete recovery of both prolamins and glutelins, to guarantee that products marketed as suitable for coeliacs are really gluten-free.

The so-called cocktail solution combines reducing and disaggregating agents to extract gluten proteins completely as this ensures that protein aggregates are disrupted. (Garcia et al., 2005). However, it is not compatible with all the techniques used for gluten analysis because β-mercaptoethanol interferes with the specific binding of the antibodies used in some types of immunoassays, giving false results. More recently, another combination of reagents called UPEX (universal prolamin and glutelin extractant solution) leads to a complete extraction and it is compatible with all gluten analysis procedures (Mena et al., 2012). This solution includes the odourless reducing agent, Tris (2-carboxyethyl)-phosphine (TCEP) that is more specific for breaking disulphide bridges and less toxic than the other reducing agents commonly used (Han, 1999) and the disaggregating agent N-Lauroylsarcosine, widely used in plant cell lysis, which contributes to opening polypeptide chains and is even more efficient than guanidine hydrochloride (Schlink and Reski, 2002).

In addition, a novel universal gluten extraction solution (UGES) has recently been described (Biomedal Diagnostics, Seville, Spain). The components of this gluten extraction solution are

a reducing agent, a solubilising agent (arginine) and an antiseptic agent in ethanolic solution (patent WO 201231612; Muñoz-Ruano et al., manuscript in preparation). The UGES procedure gave high extraction efficiency from both simple and complex matrices even if they had heat-processed.

Another of the critical points in gluten analysis is the use of a correct standard. This standard should be as representative as possible of the gluten proteins to be analysed. The Working Group on Prolamin Analysis and Toxicity (PWG) gliadin standard is the standard most used internationally for gluten analysis. This standard is obtained from a mixture of 28 wheat cultivars representative of the European wheat-producing countries (van Eckert et al., 2006). Nevertheless, cereals contain a greater number of proteins than those present in the PWG standard. Some authors have suggested that it would be more correct to use a hydrolysed standard combined with a competitive assay to quantify peptides of partially hydrolysed gluten in fermented wheat, rye, and barley products (Gessendorfer et al., 2009). Commercial foods usually have only partial hydrolysis and, when the proteins are exhaustively hydrolysed, the toxicity for coeliac patients of the peptides generated usually disappears (Stepniak et al., 2006). Comparing the intact PWG gliadin standard with a partially enzimatically digested gliadin standard it has been demonstrated that the resulting curves are similar in each case when applying a competitive immunoassay and therefore the intact PWG gliadin standard can be used as a more accessible gold standard as it is more difficult to perform a reproducible hydrolysed standard (Mena et al., 2012). However, other strategies based on the use of immunotoxic peptides of gluten as standard are being developed for the analysis of samples with gluten hydrolysate This standard presented a high degree of repeatability, reproducibility and stability and the results obtained were correlated with the potential relative immunotoxicity of gluten (Comino et al., 2012b; 2013)".

5.2. Immunological techniques

Many methods for detecting prolamin proteins have been developed over the years. The complexity of prolamin proteins, however, creates an enormous challenge in accurately quantifying the total prolamin content. Some methods including the polymerase chain reaction (PCR), HPLC and MS, may be used simultaneously with immunological methods. One of the most recent methods developed for gluten analysis is a potentiometric electronic tongue, which was able to detect 1-2 mg/Kg of gliadin from different matrices (Peres et al., 2011). The most practical methods for food analysis is based on immunological analysis. An ideal antibody for gluten analysis in foods should be not only a reliable indicator of the presence of prolamins from cereal species known to be toxic to CD patients but also should recognise the specific intramolecular regions responsible for such toxicity. Nevertheless, there are many such regions and even today not all have been identified.

5.2.1. Antibodies testing for gluten-free foods

Several antibodies (Ab) have been raised against different prolamin epitopes. The anti ω-gliadin Ab is used in a sandwich format that was approved as an official method by AOAC (Association of Official Agricultural Chemist, Skerrit and Hill, 1990, 1991). Other antibodies were raised against different epitopes of α-gliadin, such as PN3 (residues 31-49) for the toxic 19-mer peptides (Sturgess et al., 1994; Ellis et al., 1998), CD5 (residues 51-75), Abs against T-cell stimulatory peptides present in gluten (Mitea et al., 2008), and R5 which recognises highly repeated peptide sequences present in wheat, barley and rye grains. This antibody is a monoclonal antibody (Valdés et al., 2003; Mendez et al., 2005) that reacts with the epitope QQPFP and other motifs such as QLPFP, LQPFP, and QQQFP present in coeliac-toxic

sequences that occur repeatedly in gliadins, hordeins, and secalins being highly sensitive towards these prolamins (Valdes et al., 2003).

Recent reports recommend updating the concept of "gluten detection" to "potential relative immunotoxicity of gluten" for the safety of coeliac consumers. Two monoclonal antibodies, G12 and A1 (anti-3-mer antibodies), were raised against the main immunogenic epitope of the α-gliadin 33-mer. These antibodies were able to detect the presence of gliadin 33-mer-related epitopes in prolamins from wheat, barley, rye and various oats varieties (Morón et al., 2008a, b; Comino et al., 2011), and G12is a reliable tool for detecting oat varieties potentially safe for patients with celiac disease. These antibodies have proved to be very useful for the detection of toxic peptides in food samples and for the enzymatic detoxification of gluten in clinical research (Shan et al., 2002; Morón et al., 2008a; 2008b; Ehren et al., 2009).

5.2.2. Techniques

A standardised method of analysis is required to quantitatively determine the gluten content of food and provide the basis for enforcing regulations controlling the use of the term "gluten free" in food labelling. People with CD should feel confident that foods labelled "gluten-free" have been analysed for gluten using the "the best available" methodology.

In January 2009, the European Commission published a new European Regulation concerning the composition and labelling of foodstuffs suitable for people intolerant to gluten; this regulation states that foods may display the term "gluten-free" if the gluten content does not exceed 20 mg/kg as sold to the final consumer (Commission of the European Communities, 2009). Food containing between 20 ppm and 100 ppm of gluten is considered to be very low in gluten content according to the EU laws, but there is no full agreement among countries about the term "gluten-free food". Consequently, methods for gluten analysis must be sensitive enough to quantify these levels of gluten in foods.

5.2.2.1. Enzyme-linked immunosorbent assays (ELISAs)

ELISAs have become the recommended methods in gluten analysis. Immunological methods are based on the antibodies raise against the different prolamin fractions or specific sequences found in prolamins. The requirement for the assays is that they should measure the harmful proteins and peptides, regardless of the type of food or manufacturing process (Denery-Papini et al., 1999).

The sandwich R5 ELISA is the most common enzyme immunoassay formats used in detection of gluten proteins. It is highly sensitive and is especially useful for the quantification of antigens when their concentration is low, when they are contained in samples with a large amount of contaminating proteins, or both. This assay uses two antibodies that bind to different sites on the antigen. Nowadays, it is the usual method for determining gluten content in gluten-free foods internationally accepted by the Codex Alimentarius Commission (Codex Alimentarius Commission, 2006) combined with what is known as the cocktail extraction solution. This ELISA is based on the R5 antibody and has a limit of quantification of 1.56 ppm of gliadins (Valdes et al., 2003). In hydrolysed foods the quantification of gluten by the sandwich R5 ELISA would yield lower values than the true gluten content.

A sandwich ELISA using the monoclonal G12 and A1 antibodies gave very promising results for gluten analysis across a range of samples (Morón et al., 2008a; Halbmayr-Jech et al., 2012). This method had a detection limit for wheat, barley, and rye prolamins of <1ppm. Furthermore, the reactivities of these antibodies were correlated with the potential immunotoxicity of those dietary grains from which the proteins were extracted, thereby providing a rational explanation for why some cereal varieties trigger immunological response, and enabling the presence of such varieties to be avoided in gluten-free diet (Comino et al., 2012 a).

The competitive R5 ELISA, based on the R5 monoclonal antibody, leads to an accurate quantification of both intact and fragmented gluten because it uses only one antibody and requires only one epitope. In addition, the competitive system is cheaper and faster than the sandwich system ELISA (Mena et al., 2012). The Codex Alimentarius Commission states that a modification of the R5 assay (competitive ELISA) has to be applied for the detection of hydrolysed gluten (Codex Alimentarius Commission, 2006). However, the Food and Drug Administration (FDA) comments that analysts should be free to use any method that provides comparable results and that other methods may be equivalent. Another comment urged FDA to remain flexible as to the method of test validation and added that not specifying analytical methods would permit a more rapid development of dependable and affordable technologies for testing gluten (Federal Register / Vol. 78, No. 150 / Monday, August 5, 2013 / Rules and Regulations).

The competitive technique is not compatible with the cocktail extraction solution, but the combination of this technique with the UPEX solution leads to complete gluten analysis. The limits of detection and quantification of the competitive R5 ELISA are 0.36 and 1.22 ng/ml of gliadins, respectively, being lower in liquid samples. Recently, a collaborative study has confirmed that the two R5 antibody-based ELISA test kits are able to detect gliadin at the lower level of the limit of detection with good reproducibility and repeatability (Immer and Haas-Lauterbach, 2012).

A competitive ELISA method was also developed for the detection of toxic gluten peptides in hydrolysed foods based on G12 antibody. This assay is highly sensitive and reproducible with a detection limit of 0.44 ppm gliadin. This ELISA system showed high reproducibility and repeatability (Morón et al., 2008b; Ehren et al., 2009).

5.2.2.2. Western blot

Single and two-dimensional gel electrophoresis (both SDS-PAGE and A-PAGE) have been employed by different authors in order to characterise wheat, barley and rye proteins (Shuaib et al., 2007; Salmanowicz and Nowak, 2009). Nevertheless, these techniques have not enough sensibility for quantifying gluten in gluten free foods. In order to solve this problem, the separated proteins after one-dimensional SDS-PAGE are electrotransferred onto a polyvinylidene difluoride membrane where the proteins are adsorbed. A specific antibody is the added, such as the R5 antibody (Garcia et al., 2005), G12 antibody (Morón et al., 2008a, b) or anti cells T gliadin alpha-20 antibody (van den Broeck et al., 2009). These immunological western blot techniques can be used for the confirmation of gluten content in foods.

5.3. Non-immunological techniques

The quantitative analysis of prolamins is mainly based on immunological methods, but mass spectrometric and chromatographic techniques have also been used (Wieser et al., 1998; Sealy-Voyksner et al., 2010). The use of complementary and alternative non-immunological systems to confirm the results of the immunological methods is very important especially in certain complex foods.

5.3.1. Proteomic techniques

Applying proteomics to this field is of great interest to complement other techniques but the analysis of gluten-free foods, where gluten proteins are in low amount comparing with the other major proteins presents more difficulties (Mamone et al., 2011). Mass spectrometry (MS) is one of the more important physical methods used nowadays due to its high sensibility, for identification, characterisation and quantification of proteins and peptides. The bases of these methods are an ionisation of molecules to study, separation of ions in basis to

their relation mass/charge and detection of the separated ions. Depending on the different method of ionisation, separation and detection, there are several MS techniques, used in different applications. MALDI-TOF MS was the first technique used to identify toxic prolamins involved in coeliac disease. Even though MALDI-TOF gluten analysis is very useful (Hernando et al., 2003), identifying gluten based on the analysis of intact proteins is not sufficient, due to extensive sequence similarities among gluten proteins; the results for hydrolysed gluten are also insufficiently accurate. For unambiguous gluten protein identification, a proteomic approach involving tandem mass spectrometry (MS/MS) or multistage MS experiments would be very promising.

5.3.2. Quantitative real-time PCR (Q-PCR)

Several polymerase chain reaction (PCR) based methods for the detection and quantification of DNA of gluten-containing cereals have been described, but there are very few studies of their application to the analysis of gluten in foods. Recently, a Q-PCR system for reliable and rapid quantification of wheat DNA in gluten-free foods and in raw materials has been developed based on the fluorescent dye SYBR Green I and a modified SDS/Guanidine–HCl/Proteinase K DNA extraction protocol. This is a highly specific and sensitive system with a quantification limit of 20 pg DNA/mg; thus it can be used as a non-immunological tool in order to confirm, by the ‘‘DNA pathway’’, the presence of wheat in food not only for coeliacs but also for individuals with wheat allergy (Mujico et al., 2011). However, in very processed foods, such as those subjected to hydrolysis, this type of techniques are not appropriated as they cannot identify the hydrolysed DNA and there is not possible to establish a correlation between the amount of DNA and gluten.

Other authors have developed a DNA-based allergen-multiplex ligation-dependent probe amplification method for the determination of gluten in foods which might constitute a complementary method to the traditional protein-based methods (Mustorp et al., 2011).

Conclusions

The pathologies associated with gluten have increased in recent years worldwide. Although epitopes that trigger the CD have been described in gliadins and glutenins, the majority of them reside in the gliadin fraction of gluten. Nature offers a variety of cereals that are free of toxicity for gluten intolerant individuals, which include rice, corn, sorghum, millet, teff, and possibly some specific wheat varieties. In addition, pseudocereals such as quinoa and amaranth are safe to be consumed by coeliacs. A current topic of debate is the potential toxicity of oats, although there is recent evidence that of toxicity of some oat some cultivars.

The structural complexity and polymorphism of gliadin proteins make it difficult to identify varieties naturally devoid of toxicity or to develop such varieties through breedings. Simultaneous silencing of the full complement of gliadins results in effective reduction of the epitopes recognised by T cells in CD, which shows that it is possible to eliminate or reduce the toxicity of wheat. Flour from these varieties properly labeled, can serve as starting material for the production of staple foods not only for CD but also for all gluten-related allergies or intolerance.

The development of gluten-free leavened products from gluten-free cereals is still a challenge for food technologist. Great advances have been made in assessing the impact of the flour functionality and properties on bread quality and in creating protein networks in gluten-free flours, as well as in determining the effects of processing in bread production. Nutrition is now becoming the focus of food technology research aimed at developing safe and nutritionally-balanced gluten free foods.

Safety of gluten free foods can be only ensured by providing reliable methods of gluten detection and quantitation. The numerous gluten components released after processing make extraction efficiency and detection very difficult. There is currently an internationally accepted method for gluten determination, the competitive ELISA based on the R5 antibody, while some other antibodies such as the monoclonal G12 and A1 are also very effective for detecting the presence of epitopes related to the gliadin33-mer in prolamins. In addition, different immunological and non-immunological techniques are being applied to increase the sensitivity and provide supplementary information on gluten protein identification.

Acknowledgements

The authors acknowledge the financial support of Spanish Scientific Research Council (CSIC), the Spanish Ministry of Economy and Competitiveness (AGL2010-19643-C02-02; AGL2011-23802), and the Generalitat Valenciana (Project Prometeo 2012/064).

References

Abadie, V., Sollid, L.M., Barreiro, L.B., Jabri, B., 2011. Integration of Genetic and Immunological Insights into a Model of Celiac Disease Pathogenesis. Annual Review of Immunology 29, 493–525.

Acquistucci, R., Francisci, R., Bucci, R., Ritota, M., Mazzini, F., 2009. Nutritional and physicochemical characterisation of italian rice flours and starches. Food Science and Technology Research 15, 507-518.

Alaedini, A., Green, P.H.R., 2005. Narrative review: celiac disease: understanding a complex autoimmune disorder. Annals Internal Medical 142, 289–299.

Altenbach, S.B., Allen, P.V., 2011. Transformation of the US bread wheat "Butte 86" and silencing of omega-5 gliadin genes. GM Crops 2, 66–73.

Alvarez-Jubete, L., Arendt, E.K., Gallagher, E., 2010. Nutritive value of pseudocereals and their increasing use as functional gluten-free ingredients. Trends in Food Science and Technology, 21 (2), 106-113.

Arentz-Hansen, H., Fleckenstein, B., Molberg, Ø., Scott, H., Koning, F., Jung, G., Roepstorff, P., Lundin, K.E., Sollid, L.M., 2004. The molecular basis for oat intolerance in patients with celiac disease. PLoS Medicine 1, 84–92.

Ballabio, C., Uberti, F., Di Lorenzo, C., Brandolini, A., Penas, E., Restani, P., 2011. Biochemical and immunochemical characterization of different varieties of amaranth (Amaranthus L. ssp.) as a safe ingredient for gluten-free products. Journal of Agricultural and Food Chemistry, 59 (24), 12969-12974.

Becker, D., Folck, A., Knies, P., Lörz, H., Wieser, H., 2006. Silencing the α-Gliadins in Hexaploid Bread Wheat, in: Lookhart, L.G., Ng, W.P.K. (Eds.), Gluten Proteins. AACC

International, St Paul, MN, pp. 86–89.
Becker, D., Wieser, H., Koehler, P., Folck, A., Mühling, K.H., Zörb, C., 2012. Protein composition and techno-functional properties of transgenic wheat with reduced α-gliadin content obtained by RNA interference. Journal of Applied Botany and Food Quality 85, 23-33.
Bernardo, D., Pena, A, S., 2012. Developing strategies to improve the quality of life of patients with gluten intolerance in patients with and without coeliac disease. European Journal of Internal Medicine 23, 6-8.
Bethune, M.T., Khosla, C., 2008. Parallels between pathogens and gluten peptides in celiac sprue. PLoS Pathogens 4, e34.
Brites, C., Trigo, M.J., Santos, C., Collar, C., Rosell, C.M., 2010. Maize-based gluten-free bread: Influence of processing parameters on sensory and instrumental quality. Food and Bioprocess Technology, 3 (5), 707-715.
Catassi, C., Fabiani, E., Iacono, G., D'Agate, C., Francavilla, R., Biagi, F., Volta, U., Accomando, S., Picarelli, A., De Vitis, I., Pianelli, G., Gesuita, R., Carle, F., Mandolesi, A., Bearzi, I., Fasano, A., 2007. A prospective, double-blind, placebo-controlled trial to establish a safe gluten threshold for patients with celiac disease. American Journal of Industrial Medicine 85, 160–166.
Charoenthaikij, P., Jangchud, K., Jangchud, A., Prinyawiwatkul, W., Tungtrakul, P., 2010. Germination conditions affect selected quality of composite wheat-germinated brown rice flour and bread formulations. Journal of Food Science, 75, S312-S318.
Charoenthaikij, P., Jangchud, K., Jangchud, A., Prinyawiwatkul, W., No, H.K., 2012. Composite wheat-germinated brown rice flours: Selected physicochemical properties and bread application. International Journal of Food Science and Technology, 47, 75-82.
Codex Alimentarius Commission, 2006. Joint Food and Agriculture Organization of the United Nations/World Health Organization Food Standards Program. Report of the Twenty- Seventh Session of the Codex Committee on Methods of Analysis and Sampling. ALINORM 06/29/23.
Codex Alimentarius Commission, 2008. Joint Food and Agriculture Organization of the United Nations/World Health Organization Food Standards Program. Food for special dietary use for persons intolerant to gluten, CODEX STAN118-1979 (revised 2008, http://www.codexalimentarius.net/web/more_info.jsp?id_sta=291).
Comino, I., Real, A., de Lorenzo, L., Cornell, H., López-Casado, M.A., Barro, F., Lorite, P., Torres, M.I., Cebolla, A., Sousa, C., 2011. Diversity in oat potencial immunogenicity: basis for the selection of oat varieties with no toxicity in coeliac disease. Gut 60, 915-922.
Comino, I., Real, A., Gil-Humanes, J., Piston, F., de Lorenzo, L., Moreno, M.L., López-Casado, M.Á., Lorite, P., Cebolla, Á., Torres, M.I., Barro, F., Sousa, C., 2012a. Significant differences in coeliac immunotoxicity of barley varieties. Molecular Nutrition and Food Research 56, 1697–1707.
Comino, I., Real, A., Vivas, S., Siglez, M.A., Caminero, A., Nistal, E., Casqueiro, J., Rodríguez-Herrera, A, Cebolla, A., Sousa, C. 2012b. Monitoring of gluten-free diet compliance in celiac patients by assessment of gliadin 33-mer equivalent epitopes in feces. The American Journal of Clinical Nutrition 95, 670-677.
Comino, I., Real, A., Moreno, M.L., Montes, R., Cebolla, A., Sousa, C. 2013. Immunological determination of gliadin 33-mer equivalent peptides in beers as a specific and practical analytical method to assess safety for celiac patients. Journal of the Science of Food and Agriculture 15, 933-943.
Commission of the European Communities, 2009. Commision Regulation (EC) No 41/2009 of 20 January 2009 concerning the composition and labelling of foodstuffs suitable for people intolerant to gluten. Official Journal of the European Union, pp. 16/13-16/15.

de la Hera, E., Gómez, M., Rosell, C.M., 2013. Particle size distribution of rice flour affecting the starch enzymatic hydrolysis and hydration properties. Carbohydrate Polymers, DOI 10.1016/j.carbpol.2013.06.002.

de la Hera, E., Talegón, M., Caballero, P., Gómez, M. 2012. Influence of maize flour particle size on gluten free breadmaking. Journal of Science of Food and Agriculture, 93 (4), 924-932.

Denery-Papini S., Nicolas Y., Popineau, Y., 1999. Efficiency and limitations of immunochemical assays for the testing of gluten-free foods. Journal of Cereal Science 30, 121-131.

Diaz-Amigo, C., Popping, B., 2012. Gluten and gluten-free: issues and considerations of labeling regulations, detection methods, and assay validation. Journal of AOAC International, 95 (2), 337-348.

Ehren, J., Morón, B., Martin, E., Bethune, M.T., Gray, G.M., Khosla, C., 2009. A food-grade enzyme preparation with modest gluten detoxification properties. PLoS One 4, e6313.

Ellis, H.J., Rosen-Bronson, S., O'Reilly, N., Ciclitira, P.J., 1998. Measurement of gluten using a monoclonal antibody to a coeliac toxic peptide of A-gliadin. Gut 43, 190-195.

Fasano, A. Surprises from celiac disease. 2009. Sci Am 301, 54–61.

Garcia, E., Llorente, M., Hernando, A., Kieffer, R., Wieser, H., Mendez, E., 2005. Development of a general procedure for complete extraction of gliadins for heat processed and unheated foods. European Journal of Gastroenterology and Hepatology 17, 529-539.

Gebremariam, M.M., Zarnkow, M., Becker, T., 2012. Teff (Eragrostis tef) as a raw material for malting, brewing and manufacturing of gluten-free foods and beverages: a review. Journal of Food Science and Technology, 1-15.

Gessendorfer, B., Koehler, P., Wieser, H., 2009. Preparation and characterization of enzymatically hydrolyzed prolamins from wheat, rye, and barley as references for the immunochemical quantitation of partially hydrolyzed gluten. Anal Bioanalytical Chemistry 395, 1721-1728.

Gil-Humanes, J., Piston, F., Giménez, M.J., Martin, A., Barro, F., 2012. The Introgression of RNAi Silencing of γ-Gliadins into Commercial Lines of Bread Wheat Changes the Mixing and Technological Properties of the Dough. PLoS ONE 7, e45937.

Gil-Humanes, J., Piston, F., Hernando, A., Alvarez, J.B., Shewry, P.R., Barro, F., 2008. Silencing of γ-gliadins by RNA interference (RNAi) in bread wheat. Journal of Cereal Science 48, 565–568.

Gil-Humanes, J., Pistón, F., Shewry, P.R., Tosi, P., Barro, F., 2011. Suppression of gliadins results in altered protein body morphology in wheat. Journal of Experimental Botany, 62, 4203–4213.

Gil-Humanes, J., Pistón, F., Tollefsen, S., Sollid, L.M., Barro, F., 2010. Effective shutdown in the expression of celiac disease-related wheat gliadin T-cell epitopes by RNA interference. Proceedings of the National Academy of Sciences of the United States of America 107, 17023–17028.

Greco, L., Gobbetti, M., Auricchio, R., Di Mase, R., Landolfo, F., Paparo, F., Di Cagno, R., De Angelis, M., Rizzello, C.G., Cassone, A., Terrone, G., Timpone, L., D'Aniello, M., Maglio, M., Troncone, R., Auricchio, S., 2011. Safety for patients with celiac disease of baked goods made of wheat flour hydrolyzed during food processing. Clinical Gastroenterology Hepatology 9, 24-29.

Gujral, H.S., Guardiola, I., Carbonell, J.V., Rosell, C.M., 2003a. Effect of cyclodextrin glycosyl transferase on dough rheology and bread quality from rice flour. Journal of Agriculture and Food Chemistry 51, 3814-3818.

Gujral, H.S., Haros, M., Rosell, C.M., 2003b. Starch hydrolyzing enzymes for retarding the

staling of rice bread. Cereal Chemistry 80, 750-754.
Gujral, H.S., Rosell, C.M., 2004a. Improvement of the breadmaking quality of rice flour by glucose oxidase. Food Research International, 37, 75-81.
Gujral, H.S., Rosell, C.M., 2004b. Functionality of rice flour with a microbial transglutaminase. Journal of Cereal Science, 39, 225-230.
Hager, A.-S., Wolter, A., Jacob, F., Zannini, E., Arendt, E.K., 2012. Nutritional properties and ultra-structure of commercial gluten free flours from different botanical sources compared to wheat flours. Journal of Cereal Science, 56 (2), 239-247.
Halbmayr-Jech, E., Hammer, E., Fielder, R., Coutts, J., Rogers, A., Cornish, M., 2012. Characterization of G12 sandwich ELISA, a next-generation immunoassay for gluten toxicity. J AOAC Intern 95, 372-376.
Hamaker, B.R., 1994. The influence of rice proteins in rice quality. In: Rice Science and Technology (eds. W.E Marshall & J.I.Wadsworth), pp. 177-194. Marcel Dekker Inc. New York.
Han, J., 1999. Tris[2-carboxyethyl]phosphine – A reducing agent with versatile applications including cleavage of disulfide bonds and quantitation of numerous oxidants. Previews 2, 16-21.
Hansen, M., Lange, M., Friis, C., Dionisio, G., Holm, P.B., Vincze, E., 2007. Antisense-mediated suppression of C-hordein biosynthesis in the barley grain results in correlated changes in the transcriptome, protein profile, and amino acid composition. Journal of Experimental Botany 58, 3987–3995.
Hernando, A., Valdes, I., Mendez, E., 2003. New strategy for the determination of gliadins in maize- or rice-based foods matrix-assisted laser desorption/ionization time-of-flight mass spectrometry: fractionation of gliadins from maize or rice prolamins by acidic treatment. Journal of Mass Spectrometry 38, 862-871.
Houben, A., Hochstotter, A., Becker, T., 2012. Possibilities to increase the quality in gluten-free bread production: An overview. European Food Research and Technology, 235 (2), 195-208.
Immer, U., Haas-Lauterbach, S., 2012. Gliadin as a measure of gluten in foods containing wheat, rye, and barley-enzyme immunoassay method based on a specific monoclonal antibody to the potentially celiac toxic amino acid prolamin sequences: collaborative study. J AOAC Int 95, 1118-1124.
Iwashita, K., Suzuki, K., Miyashita, K., Okunishi, T., 2011. Effects of rice properties on bread made from cooked rice and wheat flour blend. Food Science and Technology Research 17, 121-128.
Kanerva, P., Brinck, O., Sontag-Strohm, T., Salovaara, H., Loponen, J., 2011. Deamidation of gluten proteins and peptides decreases the antibody affinity in gluten analysis assays. Journal of Cereal Science 53, 335-339.
Khosla, C., Gray, G.M., Sollid, L.M., 2005. Putative efficacy and dosage of prolyl endopeptidase for digesting and detoxifying gliadin peptides. Gastroenterology 129, 1362-1363.
Lange, M., Vincze, E., Wieser, H., Schjoerring, J.K., Holm, P.B., 2007. Suppression of C-Hordein Synthesis in Barley by Antisense Constructs Results in a More Balanced Amino Acid Composition. Journal of Agricultural and Food Chemistry 55, 6074–6081.
Mamone, G., Picariello, G., Addeo, F., Ferranti, P., 2011. Proteomic analysis in allergy and intolerance to wheat products. Expert Rev Proteomics 8, 95-115.
Marco, C., Pérez, G., Ribotta, P., Rosell, C.M., 2007. Effect of microbial transglutaminase on the protein fractions of rice, pea and their blends. Journal of the Science of Food and Agriculture, 87, 2576-2582.
Marco, C., Rosell, C.M., 2008. Breadmaking performance of protein enriched gluten free

breads. European Food Research and Technology, 227, 1205-1213.
Matos, M.E., Rosell, C.M., 2011. Chemical composition and starch digestibility of different gluten free breads. Plant Foods for Human Nutrition 66, 224-230.
Mena, M.C., Lombardia, M., Hernando, A., Mendez, E., Albar, J.P., 2012. Comprehensive analysis of gluten in processed foods using a new extraction method and a competitive ELISA based on the R5 antibody. Talanta 91, 33-40.
Mendez, E., Vela, C., Immer, U., Janssen, F.W., 2005. Report of a collaborative trial to investigate the performance of the R5 enzyme linked immunoassay to determine gliadin in gluten-free food. European Journal of Gastroenterology and Hepatology 17, 1053-1063.
Mitea, C., Havenaar, R., Drijfhout, J.W., Edens, L., Dekking, L., Koning, F., 2008. Efficient degradation of gluten by a prolyl endoprotease in a gastrointestinal model: implications for coeliac disease. Gut, 57, 25-32.
Morón, B., Bethune, M.T., Comino, I., Manyani, H., Ferragud, M., López, M.C., Cebolla, A., Khosla, C., Sousa, C., 2008b. Toward the assessment of food toxicity for celiac patients: characterization of monoclonal antibodies to a main immunogenic gluten peptide. PloS One, 3, e2294.
Morón, B., Cebolla, A., Manyani, H., Alvarez-Maqueda, M., Megías, M., Thomas, M. del C., López, M.C., Sousa, C., 2008a. Sensitive detection of cereal fractions that are toxic to celiac disease patients by using monoclonal antibodies to a main immunogenic wheat peptide. American Journal of Clinical Nutrition 87, 405-414.
Mujico, J.R., Lombardía, M., Mena, M.C., Méndez, E., Albar, J.P., 2011. A highly sensitive real-time PCR system for quantification of wheat contamination in gluten-free food for celiac patients. Food Chemistry 128, 795-801.
Mustorp, S.L., Dromtorp, S.M., Holck, A.L., 2011. Multiplex, quantitative, ligation-dependent probe amplification for determination of allergens in food. Journal of Agriculture and Food Chemistry 59, 5231-5239.
Omary, M.B., Fong, C., Rothschild, J., Finney, P., 2012. Effects of germination on the nutritional profile of gluten-free cereals and pseudocereals: A review. Cereal Chemistry, 89 (1), 1-14.
Peres A.M., Dias L.G., Veloso, A.C.A. Meirinho S.G., Morais J.S., Machado A.A.S.C., 2011. An electronic tongue for gliadins semi-quantitative detection in foodstuffs. Talanta 83, 857-864.
Piston, F., Gil-Humanes, J., Rodríguez-Quijano, M., Barro, F., 2011. Down-Regulating γ-Gliadins in Bread Wheat Leads to Non-Specific Increases in Other Gluten Proteins and Has No Major Effect on Dough Gluten Strength. PLoS ONE 6, e24754.
Pizzuti, D., Buda, A., D'Odorico, A., D'Inca, R., Chiarelli, S., Curioni, A., Martines, D., 2006. Lack of intestinal mucosal toxicity of Triticum monococcum in celiac disease patients. Scandinavian Journal of Gastroenterology, 41, 1305-1311.
Pontieri, P., Mamone, G., De Caro, S., Tuinstra, M.R., Roemer, E., Okot, J., De Vita, P., Ficco, D.B.M., Alifano, P., Pignone, D., Massardo, D.R., Del Giudice, L., 2013. Sorghum, a healthy and gluten-free food for celiac patients as demonstrated by genome, biochemical, and immunochemical analyses. Journal of Agricultural and Food Chemistry, 61 (10), 2565-2571.
Pulido, O., Gillespie, Z., Zarkadas, M., Dubois, S., Vavasour, E., Rashid, M., Switzer, C., Goderfroy, S.B., 2009. Introduction of oats in the diet of individuals with celiac disease: A systematic review, In: Advances in Food and Nutrition Research, Steve L. Taylor (Ed.), pp. 235-285, USA.
Sairam, S., Krishna, A.G.G., Urooj, A., 2011. Physico-chemical characteristics of defatted rice bran and its utilization in a bakery product. Journal of Food Science and Technology-

Mysore 48, 478-483.
Sakac, M., Torbica, A., Sedej, I., Hadnadev, M., 2011. Influence of breadmaking on antioxidant capacity of gluten free breads based on rice and buckwheat flours. Food Research International 44, 2806- 2813.
Salmanowicz, B.P., Nowak, J., 2009. Diversity of monomeric prolamins in triticale cultivars determined by capillary zone electrophoresis. Journal of Agriculture and Food Chemistry 57, 2119-2125.
Sapone, A., Lammers, K.M., Casolaro, V., Cammarota, M., Giuliano, M.T., De Rosa, M., Stefanile, R., Mazzarella, G., Tolone, C., Russo, M.I., 2011. Divergence of gut permeability and mucosal immune gene expression in two gluten-associated conditions: celiac disease and gluten sensitivity. BMC Medicine 9, 23.
Schlink, K., Reski, R., 2002. Preparing high-qualitty DNA from moss (Physcomitrella patens). Plant Molecular Biology Reporter 20, 423a–423f.
Sealey-Voyksner J.A., Khosla C., Voyksner R.D., Jorgenson J.W., 2010. Novel aspects of quantitation of immunogenic wheat gluten peptides by liquid chromatography–mass spectrometry/mass spectrometry. Journal of Chromatography A 1217, 4167-4183.
Shan, L., Molberg, Ø., Parrot, I., Hausch, F., Filiz, F., Gray, G.M., Sollid, L.M., Khosla, C., 2002. Structural basis for gluten intolerance in celiac sprue. Science 297, 2275-2279.
Shuaib, M., Khan, I., Ali, Z., Ali, W., Ahmad, T., Zeb, A., 2007. Evaluation of different wheat varieties by SDS-PAGE electrophoresis. Pakistan Journal of Biological Science 10, 1667-1672.
Skerritt J.H., Hill A.S., 1990. Monoclonal antibody sandwich enzyme immunoassays for determination of gluten in foods. Journal of Agriculture and Food Chemistry 38, 1771-1778.
Skerritt, J.H., Hill, A.S., 1991. Enzyme immunoassay for determination of gluten in foods: collaborative study. Journal Association of Official Analytical Chemists. 74, 257-264.
Song, J.Y., Shin, M., 2007. Effects of soaking and particle sizes on the properties of rice flour and gluten-free rice bread. Food Science and Biotechnology 16, 759-764.
Spaenij Dekking, L., Kooy Winkelaar, Y., van Veelen, P., Wouter Drijfhout, J., Jonker, H., van Soest, L., Smulders, M.J.M., Bosch, D., Gilissen, L.J.W.J., Koning, F., 2005. Natural Variation in Toxicity of Wheat: Potential for Selection of Nontoxic Varieties for Celiac Disease Patients. Gastroenterology 129, 797–806.
Stenman, S.M., Lindfors, K., Venalainen, J.I., Hautala, A., Mannisto, P.T., Garcia-Horsman, J.A., Kaukovirta-Norja, A., Auriola, S., Mauriala, T., Maki, M., Kaukinen, K., 2010. Degradation of coeliac disease-inducing rye secalin by germinating cereal enzymes: diminishing toxic effects in intestinal epithelial cells. Clinical and Experimental Immunology, 161,242-249.
Sturgess R., Day P., Ellis H.J., Lundin K.E.A., Gjertsen H.A., Kontakou M., Ciclitira P.J., 1994. Wheat peptide challenge in coeliac disease. Lancet 343, 758-761.
Vader, W., Kooy, Y., Van Veelen, P., De Ru, A., Harris, D., Benckhuijsen, W., Pena, S., Mearin, L., Drijfhout, J.W., Koning, F., 2002. The gluten response in children with celiac disease is directed toward multiple gliadin and glutenin peptides. Gastroenterology 122, 1729-1737.
Valdes, I., Garcia, E., Llorente, M., Mendez, E., 2003. Innovative approach to low-level gluten determination in foods using a novel sandwich enzyme-linked immunosorbent assay protocol. European Journal of Gastroenterology and Hepatology 15, 465-474.
van den Broeck, H.C., van Herpen, T.W., Schuit, C., Salentijn, E.M., Dekking, L., Bosch, D., Hamer, R.J., Smulders, M.J., Gilissen, L.J., van der Meer, I.M., 2009. Removing celiac disease-related gluten proteins from bread wheat while retaining technological properties: a study with Chinese Spring deletion lines. BMC Plant Biology 9, 41.

van de Wal, Y., Kooy, Y.M.C., van Veelen, P., Vader, W., August, S.A., Drijfhout, J.W., Pena, S.A., Koning, F., 1999. Glutenin is involved in the gluten-driven mucosal T cell response. European Journal of Immunology 29, 3133-3139.

van Eckert, R., Berghofer, E., Ciclitira, P.J., Chirdo, F., Denery-Papini, S., Ellis, H.J., Ferranti, P., Goodwin, P., Immer, U., Mamone, G., Méndez, E., Mothes, T., Novalin, S., Osman, A., Rumbo, M., Stern, M., Thorell, L., Whim, A., Wieser, H., 2006. Towards a new gliadin reference material–isolation and characterisation. Journal of Cereal Science 43, 331-341.

van Herpen, T., Goryunova, S., Van Der Schoot, J., Mitreva, M., Salentijn, E., Vorst, O., Schenk, M., van Veelen, P., Koning, F., van Soest, L., 2006. Alpha-gliadin genes from the A, B, and D genomes of wheat contain different sets of celiac disease epitopes. BMC Genomics 7, 1.

Watanabe, Y., 2011. Overview of Plant RNAi, in: Kodama, H., Komamine, A. (Eds.), Methods in Molecular Biology, Methods in Molecular Biology. Humana Press, Totowa, NJ, pp. 1–11.

Wen, S., Wen, N., Pang, J., Langen, G., Brew-Appiah, R.A.T., Mejias, J.H., Osorio, C., Yang, M., Gemini, R., Moehs, C.P., Zemetra, R.S., Kogel, K.-H., Liu, B., Wang, X., Wettstein, von, D., Rustgi, S., 2012. Structural genes of wheat and barley 5-methylcytosine DNA glycosylases and their potential applications for human health. Proc. Natl. Acad. Sci. U.S.A. 109, 20543–20548.

Wieser H., Antes S., Seilmeier W., 1998. Quantitative determination of gluten protein types in wheat flour by reversed-phase high-performance liquid chromatography. Cereal Chemistry 75, 644-650.

Wieser, H., Koehler, P., Folck, A., Becker, D., 2006. Characterization of Wheat with Strongly Reduced α-Gliadin Content, in: Lookhart, L.G., Ng, W.P.K. (Eds.), Gluten Proteins. AACC International, St Paul, pp. 13–16.

Zannini, E., Kingston, W., Arendt, E.K., Waters, D.M., 2013. Technological challenges and strategies for developing low-protein/protein-free cereal foods for specific dietary management. Food Research International, DOI: 10.1016/j.foodres.2013.03.001.

Zuidmeer, L., Goldhahn, K., Rona, R.J., Gislason, D., Madsen, C., Summers, C., Sodergren, E., Dahlstrom, J., Lindner, T., Sigurdardottir, S.T., McBride, D., Keil, T., 2008. The prevalence of plant food allergies: A systematic review. Journal of Allergy and Clinical Immunology 121, 1210–1218.e4.

Figure Captions

Figure 1. Pathologies associated with gluten.

Figure 2. Taxonomy and basic characteristics of the prolamines of oats in relation with other cereals. A. Taxonomy of oats in relation with other cereals. B. Basic characteristics of the prolamins of wheat, oats, and rice.

Figure 1.

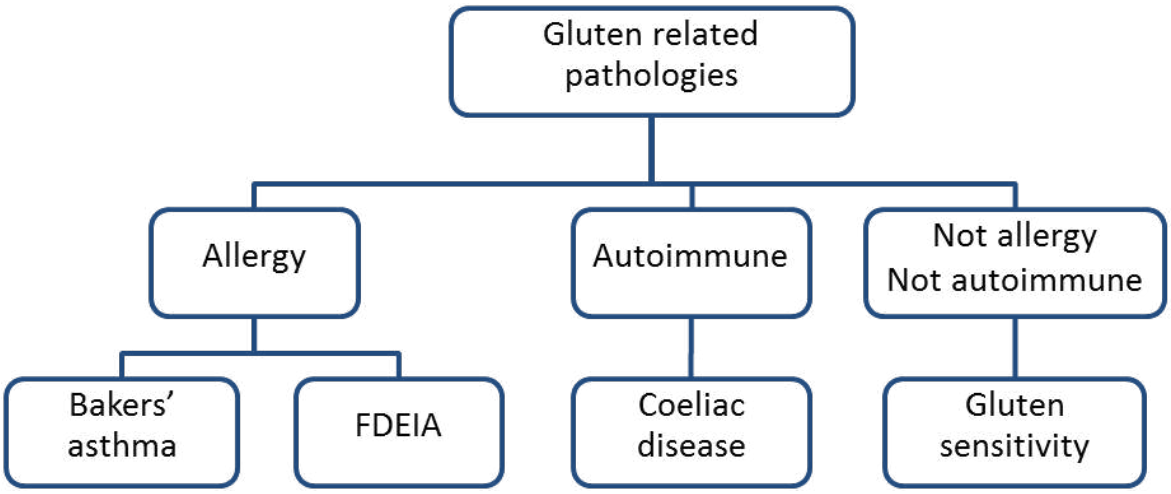
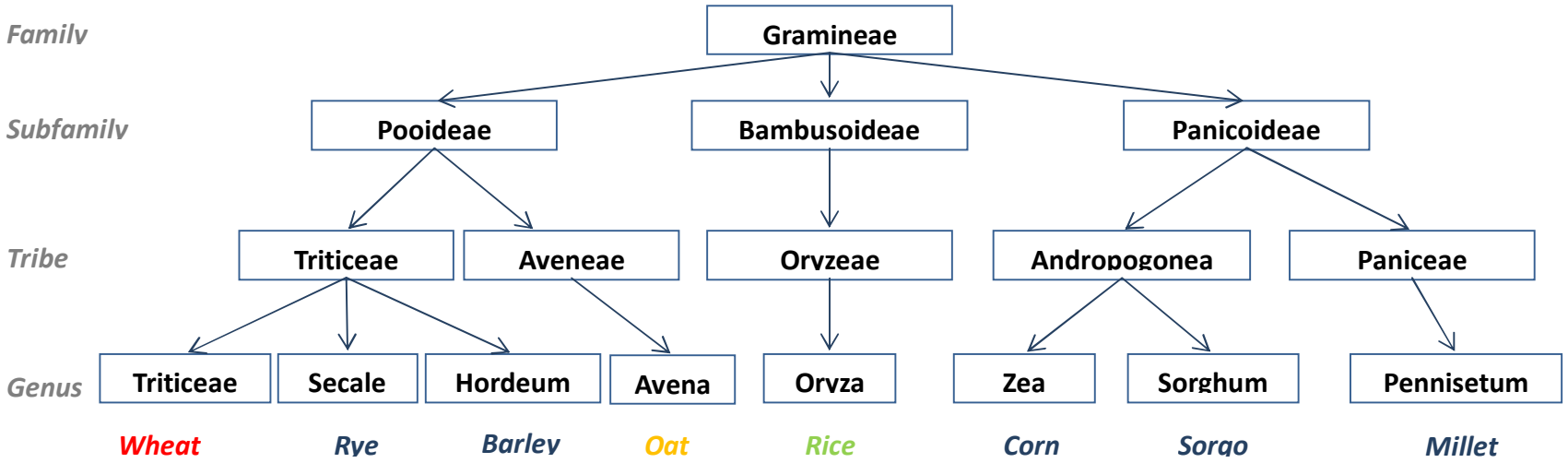

Gluten related pathologies
Allergy
Autoimmune
Not allergy Not autoimmune
Bakers' asthma
FDEIA
Coeliac disease
Gluten sensitivity

Figure 2.

A



B

WHEAT	OAT	RICE	Basic characteristics of prolamins
>100	8-25	34 (transcritos 21)	**Number of genes**
20-40 kDa	19-31 kDa	10-16 kDa	**Molecular weight**
Gln (35%) Pro (25%)	Gln (30%) Pro (10%)	Gln (22%) Pro (<other cereals)	**Prevalence of amino acids**
40-50%	10-20%	25%	**Percentage of prolamins based on the total seed proteins**

1

2