CHAPTER 16

Analytical Tools for Gluten Detection. Policies and Regulation

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Abstract

Gluten proteins are mixture of two groups of proteins named prolamins and glutelins. Many of these proteins are resistant to digestive enzymes and therefore after ingestion of gluten containing foods, there may be immunological potentially toxic peptides in small-bowel mucosal for celiac disease predisposed individuals. Since the only effective treatment of celiac disease is the avoidance of gluten containing foods, and taking into account the high prevalence of this disease, is mandatory to have reliable methods for gluten determination to ensure that consumption of labeled “gluten-free” food is safe for celiacs. Several factors may affect the results in gluten analysis such as the modifications of proteins produced during manufacturing of foods, the interference of the mixture of ingredients, and the use of the appropriate standard for gluten analysis. There are different available techniques for gluten analysis in foods. The most widely used are those based in the classical immunological techniques using different antibodies mainly enzyme-linked-immunosorbent assays, western blot, and lateral flow devices dipsticks. In addition, biosensors technologies can be applied to gluten analysis. Regarding the non-immunological tools, the most useful ones are the proteomics techniques and real time quantitative PCR. In most of the countries, regulations concerning the composition and labeling of foodstuffs suitable for people intolerant to gluten states that limit values for “gluten-free” foods and foods “specially processed to reduce the gluten content” are 20 and 100 mg/Kg of gluten respectively. Therefore any technique used must have at least a sensitivity to reach this lower limit.

Keywords

Gluten-free foods, ELISA, prolamins, glutelins.
1. Introduction

Gluten proteins are an extremely polymorphic mixture of two different groups of proteins named prolamins and glutelins, which are present as either monomers or as oligomers and polymers linked by interchain disulphide bonds. Many of these proteins are resistant to digestive enzymes and therefore after ingestion of gluten containing foods, there may be immunological potentially toxic peptides in small-bowel mucosal for celiac disease (CD) predisposed individuals. Traditionally only prolamins were considered as immunotoxic in adults, but in children and certain adults, there has been proved an immune response to glutenins. Prolamins are the major class of storage proteins in wheat, rye, barley and oats and their function is to store nitrogen, carbon and sulfur in the grain endosperm. They belong to the prolamin superfamily together with several plant food allergens such as 2S albumins, nonspecific lipid transfer proteins and cereal alpha-amylase/tripsin inhibitors. Osborne was the first to suggest the name prolamins for this group of cereal proteins because of their high content of proline and amide nitrogen. Osborne characterized cereal prolamins as freely soluble in relatively strong ethyl alcohol, but insoluble in absolute alcohol, slightly in water, and easily soluble in very dilute acids and bases.

Prolamins are distinct from other proteins in their high content in the amino acids proline (Pro or P) and glutamine (Gln or Q) that comprise 15 and 35 % of the gluten proteins, respectively. A special characteristic of proline is its ability to make \( \beta \)-turns. These turns form a tighter helix than an \( \alpha \)-helix and thus enable proteins to be packed more efficiently into a small space. This is convenient for a plant to store vital amino acids, but makes it difficult for enzymes to hydrolyze the tight structures of prolamins. As a consequence, these proteins are poorly degraded by gastric and pancreatic digestive proteases in gastrointestinal tract. Some partially hydrolyzed peptides may enter into the intestinal epithelium and have access to the propia lamina by a mechanism than remain unknown, causing damage in celiac disease patients. The glutamine residues of these peptides are deamidated by a tisular transglutaminase (tTG) turning them into glutamic
acid, increasing the immunoestimulatory potential of the peptides as the negative charge enhance the joining of the peptide to the DQ2 or DQ8 receptors of antigen presenting cells causing a response from intestinal CD4+ T cells and damage in mucosal villi. However, this disease not only affects the gut, but also it is a systemic disease that may cause injury to the skin, liver, joints, brain, heart, and other organs.

Gluten proteins can be classified in different ways regarding its characteristics and species. Wheat, barley and rye contain celiac-active prolamins, whereas maize, rice, millet and sorghum do not. Oats contains low amounts of the prolamin type avenin. Wheat, rye, barley and some oat cultivars have been established to trigger celiac disease, whereas maize, rice and buckwheat were found not to be harmful.

Prolamins can be divided into groups based on their sulfur content, size or sequence homologies. Shewry and Tatham divided prolamins based on their sulfur content into S-poor, S-rich and High molecular weight (HMW) prolamins. Whereas Wieser divided prolamins into three groups based on their size: HMW (80000-120000 Da), medium molecular weight (MMW) (52000-80000 Da) and low molecular weight (LMW) (30000-52000 Da) groups. The HMW group consists of HMW glutenin subunits of wheat, HMW secalins, and D-hordeins. The MMW group consists of omega-type gliadins and secalins and C-hordeins. The LMW group consists of alpha/beta gliadins and gamma-gliadins, gamma secalins (monomeric gamma-40 and polymeric gamma-75), gamma-hordeins, LMW glutenins and B-hordeins. The storage proteins of oats are different from those of wheat, barley and rye. Avenins are monomeric and polymeric proteins, and can be divided into groups based their molecular weights. The molecular weights of alpha-avenins are about 12000-18000 Da and those of gamma-avenins about 22000-35000 Da. The HMW are similar to LMW-GS (Low molecular weight glutenin subunits) from wheat (Table 1).
Table 1. Characterization of storage protein types of wheat, barley rye and oats.

| Group        | Wheat                  | Barley      | Rye                     | Oats          | Type       |
|--------------|------------------------|-------------|-------------------------|---------------|------------|
| Prolamins    | Alpha/Beta gliadins    | Gamma hordeins | Gamma 40k-secalins       | Alpha avenins | Monomeric  |
|              | Gamma gliadins         |             | Omega secalins          |               |            |
|              | Omega gliadins         | C hordeins  |                         |               |            |
|              | B Hordeins             |             |                         |               |            |
| Glutelins    | HMW glutenins          | D hordeins  | Gamma 75k-secalins      | LMW avenins   | Polymeric  |
|              | LMW glutenins          |             | HMW secalins            |               |            |

The calculation of gluten content is usually performed based on the assumption of a 1:1 ratio between gliadins and glutenins, as traditionally, gluten proteins have been divided into roughly equal fractions according to their solubility in alcohol–water solutions: the soluble gliadins and the insoluble glutenins. Nevertheless, some studies have reported slight differences in the ratio between gliadins and glutenins, suggesting a factor around 65:35 mainly for barley and rye and depending on the variety and specie of the cereal\(^9\).

CD goes in remission when the patients are put on a gluten-exclusion diet, and patients relapse when gluten is reintroduced into the diet\(^{10,11}\). Complying with a gluten-free diet (GFD) is difficult and affects the patients’ quality of life, but a strict diet is critical to reduce morbidity and mortality\(^12\). However, this generates numerous social and economic repercussions. It is not easy to maintain a diet with zero gluten content because gluten contamination of food is commonplace. Even products specifically targeted at dietary treatment of CD may contain tiny amounts of gluten proteins, either because of the cross-contamination of originally gluten-free cereals during the milling,
storage, and manipulation, or because of the presence of wheat starch as a major ingredient. Therefore, standardized methods of analysis are needed to quantitatively determine the gluten content of food and provide the basis for enforcing regulations regarding use of the term “gluten-free” in food labelling.

2. Analytical Tools for Gluten Analysis

2.1. Factors Affecting Gluten Analysis

2.1.1. Modifications of Proteins During Manufacturing of Foods

Proteins in foods are modified during manufacturing by different processes to improve their functionally and increase their usage in different applications in the food industry. These modifications include mainly deamidation, transamidation and degradation by different types of hydrolysis. All of these modifications can also happen naturally due to enzymes in cereal seeds that are released when cells are broken down during processing. Hydrolysis may decrease the toxicity of gluten\textsuperscript{13} but this fragmentation of peptides can make more difficult the analysis of gluten in these foods\textsuperscript{14}. Deamidation of gluten proteins decreases the affinity and recognition of antibodies to gluten proteins and peptides, which may lead to underestimation when immunoassays are used to quantify gluten content of foods\textsuperscript{15}. During the processing of some foods, proteins are treated at high temperatures in a dry state at a neutral pH, forming isopeptide bonds between lysine and 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. The so-called cocktail solution combines reducing and disaggregating agents to extract gluten proteins completely as this ensures that protein aggregates are disrupted\textsuperscript{16}. However, it is not compatible with all the techniques used for gluten analysis because beta-mercaptoethanol interferes with the specific binding of the antibodies. In order to solve this problem, another extraction
solution called UPEX (universal prolamin and glutelin extractant solution) leads to a complete extraction and it is compatible with all gluten analysis procedures\textsuperscript{14}. 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\textsuperscript{17} 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 (patent WO 2011/07039 A2).

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). The UGES procedure gave high extraction efficiency from both simple and complex matrices even if they had been heat-processed.

### 2.2.2. Interference of Ingredients

There are certain foods in which ingredients may interfere with the results yielding lower or higher values than real gluten content. For instance, in the case of chocolate and other foods containing tannins, when a spiked sample with a known value of gluten is analyzed, the observed recovery is lower than expected. Tannins are plant polyphenols that bind and precipitate proteins (such as gliadins) and yield large tannic acid-gliadin complexes, therefore interfering in the determination of the gluten content in food. As well as gliadins, other proteins such as gelatin are susceptible to bind polyphenols. In order to solve this problem a modified extraction protocol combining the UPEX solution with fish gelatin and polyvinylpyrrolidone (PVP) must be applied. This modified protocol should be applied routinely or at least when analyzing foods containing unknown ingredients\textsuperscript{14}.

In addition, other proteins may interfere in the analysis resulting in an overestimation of gluten content. This phenomenon has been observed when gluten is analyzed in soy based foods such as soy drinks after extraction with 60% ethanol. Nevertheless, when UPEX solution is used for extracting gluten
proteins, the interference components do not remain in solution and there is no overestimation. As that interferences were not observed in the main ingredient in soy drinks (soybeans) it was suggested that processing soybeans to produce soy drinks might cause changes in the solubility of these proteins leading them to remain in suspension in 60% ethanol but nor in UPEX/60% ethanol\textsuperscript{14}.

2.1.3. Standards for Gluten Analysis

Another critical point in gluten analysis is the use of a correct standard representative of gluten proteins to be analyzed in any kind of foods. The Working Group on Prolamin Analysis and Toxicity (PWG) gliadin standard is the most used internationally standard for gluten analysis. The PWG standard preparation was developed as part of a multi-centre project whose aim was to produce an international reference standard which would enable validation of quantitative results obtained using different methods. This standard is obtained from a mixture of 28 wheat cultivars representative of the European wheat-producing countries\textsuperscript{18}. A conventional protocol for prolamin extraction was followed, with some modifications made for the purpose of obtaining a large quantity with few contaminants. Characterization was then begun by the most wide-ranging methodology available (RP-HPLC, polyacrylamide gel electrophoresis, capillary electrophoresis, MALDI-TOF MS, immunoassays). Its stability and solubility were also evaluated. In this manner, a highly stable and completely soluble reagent was obtained which has been extensively characterized and can be used as reference material\textsuperscript{19}.

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 hydrolyzed standard to quantify peptides of partially hydrolyzed gluten in fermented wheat, rye, and barley products\textsuperscript{19}. Commercial foods usually have only partial hydrolysis of proteins and, when the proteins are exhaustively hydrolyzed, the toxicity for celiac patients of the peptides generated usually disappears. Comparison of the intact PWG gliadin standard
with a partially enzymatically digested gliadin standard demonstrated that the resulting curves were 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 prepare a reproducible hydrolyzed standard\(^\text{14}\).

However, other strategies based on the use of immunotoxic peptides of gluten as standard are being developed for the analysis of samples using hydrolyzed gluten. This standard presented a high degree of repeatability, reproducibility and stability and the results obtained were correlated with the potential relative immunotoxicity of gluten\(^\text{20,21}\).

### 2.2. Immunological Techniques

The most used methods for gluten analysis in foods are based on immunological analysis. Immunological methods are based on the antibodies raised against the different prolamin fractions or specific sequences found in gluten proteins. The requirement for the assays is that they should measure the harmful proteins and peptides, regardless of the type of food or manufacturing process\(^\text{22}\).

There are many immunoanalytical-based commercial kits available for the quantification of gliadin/gluten/wheat proteins, including rapid test kits (lateral flow device assay format).

#### 2.2.1. Enzyme-Linked Immunosorbent Assays (ELISAs)

Since the mid-1980s, multiple immunochemical gluten analysis methods have been developed\(^\text{23}\). The earlier methods were reviewed by Howdle and Losowsky\(^\text{24}\). Two ELISAs formats, sandwich and competitive, are the recommended methods for gluten analysis in gluten-free foods. The sandwich method is based on two antibodies. The first is called coating antibody and the second detecting antibody. The coating antibody is bound to the bottom of the microplate wells and the detecting antibody is used to recognize the antigens attached to the coating antibody. An enzyme is linked to the
detecting antibody. Commonly used enzymes include horseradish peroxidase (HRP) and alkaline phosphatase (AP). The purpose of the enzyme is to induce a color reaction involving a chromogen, which can be measured by spectrophotometric methods. The coating and detecting antibody can be the same antibody or they can be different. For this type of analysis, the sample protein must have at least two epitopes recognized for the two antibodies. Therefore, the sandwich technique is not suitable for hydrolyzed proteins.

The competitive method is based on the competition between sample proteins and standard proteins. Only one antibody is used in this assay, which makes it suitable for also detecting small, hydrolyzed proteins and peptides. However, the robustness of the method may not be as good as that obtained with the sandwich format, since nonspecific binding is more likely when only one binding site is needed for detection. The enzyme in competitive systems can be conjugated with the antibody or with the standard peptide/protein. In the competitive assay format, the intensity of the color reaction is inversely proportional to the amount of antigen in the sample.

Many ELISA assays, both sandwich and competitive, are commercially available (Table 2). However, the results obtained with such kits are often noncomparable, since they target different gluten components and differ in antibody specificity, extraction conditions and matrix effects.\(^\text{25-27}\)

Other immunological ELISA systems based on different antibodies have also been developed. McKillop et al.\(^\text{28}\) and Troncone et al.\(^\text{29}\) developed ELISAs based on polyclonal rabbit antisera against gliadin with very low detection limits. The assay of McKillop was not tested with heated samples and that of Troncone reacted with proteins from rice and maize that are not harmful for celiac patients. In 1988 Friis\(^\text{30}\) also developed an ELISA using a polyclonal rabbit antibody; however, this antibody additionally recognized proteins from buckwheat. Other antibodies were raised against different epitopes of prolamins as proposed Freedman et al.\(^\text{31}\) and Chirdo et al.\(^\text{32,33}\).

Ellis et al.\(^\text{34}\) developed an ELISA based on the PN3 antibody, for the toxic 19-mer peptides. Subsequently, a competitive ELISA was developed with the
same antibody\textsuperscript{36}. The competitive assay based on the PN3 detected equally harmful peptides from wheat, barley, rye and oats. Neither of these above-mentioned methods are commercially available.

### Table 2. Enzyme-linked immunosorbent assays for gluten detection.

| Name of antibody | Type of antibody | Antibody raised against | Main recognition epitope | ELISA | LOD\textsuperscript{**} | Reference |
|------------------|------------------|--------------------------|--------------------------|-------|--------------------------|-----------|
| -                | pAb              | $\alpha$-Gliadin Gliadin| -                        | Sandwich Competitive | 1-20 ng/ml\textsuperscript{***} | (23)      |
| -                | pAb              | Gliadin                  | -                        | Sandwich | 22 ng/ml               | (28)      |
| -                | pAb              | Gliadin                  | -                        | Sandwich | 5 ng/ml                | (29)      |
| -                | mAb              | Gliadin                  | -                        | Sandwich | 15 ng/ml               | (31)      |
| -                | pAb              | Gliadin                  | -                        | Competitive | 13 ng/ml             | (30)      |
| 401/21           | mAb              | $\omega$-Gliadin         | -                        | Sandwich | 100-150 ng/ml         | (38)      |
| -                | pAb              | Gliadin                  | -                        | Competitive | 1 ng/ml              | (33)      |
| 13B4 12A1        | mAb              | Gliadin                  | -                        | Sandwich (12A1) Competitive (13B4) Competitive (12A1) | 1 ng/ml 20 ng/ml 5 ng/ml | (34)      |
| R5               | mAb              | Secalin                  | QQPFP                    | Sandwich Competitive | 1.5 ng/ml 0.36 ng/ml | (46) (14) |
| PN3              | mAb              | 19-mer                   | QQQPFP                   | Sandwich Competitive | 4 ng/ml 128 ng/ml | (34) (36) |
| Glia$\alpha$-2/9 Glia$\gamma$-1 | mAb | $\alpha$-Gliadin $\gamma$-Gliadin | LQPFPQPQ QQRPFQ | Competitive | 12 ng/ml | (42) |
| 8D4 7C6          | 2 x mAb 1 x pAb | Gliadin                  | QQSFPQQ QQTQPQPQ QPFRPQ | Sandwich | 5 ng/ml | (40) (41) |
| G12 A1           | mAb              | 33-mer                   | QPQLPY QLPYPQ           | Sandwich Competitive | 0.6 ng/ml 0.4 ng/ml | (50) (52) |
| -                | pAb              | Gliadin                  | -                        | Sandwich | 0.3 $\mu$g/ml         | Morinaga Institute of Biological Science Inc., Crystal Chem Inc.) |
| -                | -                | -                        | -                        | Sandwich | 5 $\mu$g/ml           | Neogen    |

\textsuperscript{*}polyclonal antibody (pAb), monoclonal antibody (mAb); \textsuperscript{**}limit of detection (LOD); \textsuperscript{***}limit of quantification.
2.2.1.1. ω-Gliadin ELISA and Others

Skerrit and Hill\textsuperscript{37,38} developed a sandwich format that was approved as an official method by AOAC (Association of Official Agricultural Chemist) and it was used for many years in gluten analysis. This method is based on an antibody that recognizes the heat-stable ω-gliadin fraction. This is an advantage, since that fraction remains unchanged during the processing of food. However, the disadvantage of the method is that the different relative content of the ω-fraction among cereal species causes considerable variation in the quantitative result\textsuperscript{2,39}. In addition, fertilization may have strong effect on the protein composition of the grain. These changes in protein composition affect the immunological analysis results, especially when using the ω-specific antibody. Other disadvantage is that this method has only a weak response to barley hordeins. This method is no longer in general use; however it can still be obtained from different companies.

ImmunoTech (Pardubice, Czech Republic), developed a gliadin ELISA kit based on two monoclonal antibodies against two different epitopes of gliadin and one polyclonal antibody\textsuperscript{40,41}. It recognizes wheat, rye and spelt with the same efficiency, but barley with an efficiency of only about 20-30%.

An ELISA method for detecting α-gliadins was developed by Koning and co-workers. Initially, the research group developed several antibodies against T-cell stimulatory epitopes. The antibodies were raised against synthetic peptides that represented T-cell stimulatory epitopes in α-gliadin, γ-gliadin, LMW glutenin and HMW glutenin\textsuperscript{42,43}. The antibodies were very specific to the epitopes they were raised against and were able to detect homologous epitopes in other cereals (barley, oats, wheat, rye and triticale). However, as the method was further developed, only the α-gliadin antibody was selected for inclusion in the final ELISA. Because of this, the results of the method are expressed as α-gliadin contents.

The Morinaga Wheat Protein ELISA method has been validated in an interlaboratory study supported by The Japanese Ministry of Health, Labor and Welfare and is based in the use of a polyclonal antibody to wheat gliadin that detects multiple epitopes. The antibody also cross-reacts with hordeins.
and secalins with a lesser degree than with wheat and therefore this assay underestimates both barley and rye protein content in contaminated foods.

2.2.1.2. R5 ELISA

The sandwich R5 ELISA is the most common enzyme immunoassay format used in detection of gluten proteins. It is highly sensitive and specific for gluten proteins and it is especially useful for the quantification of antigens when their concentration is low, when they are contained in samples with a large amount of other non-gluten proteins, or both. This assay is based on the R5 antibody, using two antibodies (R5 antibody and the R5 conjugated antibody) that bind to different sites on the antigen. The R5 antibody recognizes potential toxic-celiac epitopes which occur repeatedly in prolamins, mainly QQPFP, QQQFP, PQPFP, LQPFP, QQPYP, QLPYP, that are contained in toxic-celiac peptides such as Gliadin 33 mer peptide, Gliadin 26 mer peptide and Gliadin 25 mer peptide. This ELISA has a limit of quantification of 1.56 ppm of gliadins and, combined with what is known as the cocktail extraction solution, it is internationally accepted by the Codex Alimentarius Commission as the method for determining gluten content in gluten-free foods. In hydrolyzed foods, the quantification of gluten by the sandwich R5 ELISA is not accurate enough as two intact epitopes are required to quantify the gluten content.

The competitive R5 ELISA, based on the R5 monoclonal antibody, leads to a precise quantification of both intact and fragmented gluten because in this technique only one antibody is used and therefore only one epitope is required for complete determination of gluten. In addition, the competitive system is cheaper and faster than the sandwich system ELISA. The Codex Alimentarius Commission states that a modification of the R5 assay (competitive ELISA) has to be applied for the detection of hydrolyzed gluten. The cocktail extraction solution is not compatible with this competitive technique, but the combination of the competitive assay with the UPEX solution described above leads to accurate and 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 (LOQ of 0.30 ppm of gliadins). 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\textsuperscript{25}.

\section*{2.2.1.3. G12 and A1 ELISA}

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 recognize the specific intramolecular regions responsible for such toxicity. Nevertheless, there are many such regions and even today not all have been identified.

Recent advances in the celiac field strongly recommend updating the concept of “gluten detection” to “potential relative immunotoxicity of gluten” for the safety of celiac consumers of food. Two monoclonal antibodies, A1 and G12, were raised against the immunodominant peptide 33-mer\textsuperscript{48}. The 33-mer peptide from α-2 gliadin is a principal contributor to gluten immunotoxicity\textsuperscript{49}. The reactivity of these antibodies was correlated with the potential immunotoxicity of the dietary grains from which the proteins were extracted\textsuperscript{50,51}.

A sandwich ELISA using the monoclonal G12 and A1 antibodies gave very promising results for gluten analysis across a range of samples\textsuperscript{52,53}. This method had a detection limit for wheat, barley, and rye prolamins of 0.6 ng/mL. Furthermore, the reactivity 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\textsuperscript{20,50,52}.

A competitive ELISA method was also developed for the detection of toxic gluten peptides in hydrolyzed 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\textsuperscript{50,51}.  

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2.2.2. Western Blot

Single and two-dimensional gel electrophoresis (both SDS-PAGE and A-PAGE) have been used by different authors in order to characterize wheat, barley, rye and oat proteins from cereal grains of different species\textsuperscript{7,54,55}. Nevertheless, these techniques have not enough sensibility for detecting gluten in gluten-free foods. The western blot techniques lead to a qualitative or semi-quantitative analysis of these proteins and therefore are very useful for the confirmation of gluten content in foods avoiding false positives or negative results. Proteins separated in one-dimensional SDS-PAGE are electrotransferred onto a polyvinylidene difluoride membrane where proteins are adsorbed. Afterwards, a specific antibody is added, such as the R5 antibody\textsuperscript{16}, G12 antibody\textsuperscript{51,53} or anti cells T gliadin alpha-20 antibody\textsuperscript{56}.

2.2.3. Lateral Flow Devices (LFDs) and Dipsticks

LFDs are used to qualitatively or semi-quantitatively determine whether gluten is present in a food product. LFDs and dipsticks for rapid and sensitive qualitative detection of gluten are available\textsuperscript{57}. LFDs are usually what we think of as “dipstick” tests. Most employ sandwich type methodologies. They utilize a line of fixed antibody on a surface strip and a second antibody conjugated with colored “nano” size particles. When a liquid sample extract is applied to the strip, the conjugate and the sample start to migrate across the surface of the strip together. If the sample extract has the protein or compound present (gluten) and the conjugate can recognize its epitope (binding site), under the right conditions they will bind together. Now that they are “hooked” together as they come in contact with the line of antibodies that are fixed to the strip, these antibodies will also bind to the protein forming a sandwich complex, “sandwiching” the protein (gluten) between the two antibodies. As the conjugate complex starts to accumulate on the surface of the strip the “nano” particles start to become visible.
2.2.4. Biosensors

A number of biosensors for detecting gliadin contamination in gluten-free foods have been developed but are not yet commercially available. Two electrochemical biosensors have been described\textsuperscript{58,59}. One uses an antibody raised against the immunodominant epitope of gliadin with a LOD of 5.5 µg/L. The second is based on the adsorption of anti-gliadin Fab fragments on gold surfaces. The LOD for gliadin was evaluated by impedance (LOD=0.42 mg/L) and amperometry (LOD=3.29 µg/L).

A quartz crystal microbalance biosensor incorporating gold nanoparticles was able to detect gliadin with a LOD of 8 µg/Kg\textsuperscript{60}. Another biosensor used anti-gliadin antibody-conjugated immunomagnetic beads and fluorescence-dye-loaded immunoliposomal nanovesicles (IMLNs) to form sandwich\textsuperscript{61}, the LOD for gliadin was 0.6 mg/L.

Recently, Amaya-González et al.\textsuperscript{62} have described an electrochemical competitive enzyme–linked assay on magnetic particles, which allows the measurement of as low 0.5 ppb of gliadin standard.

2.3. Non-Immunological Techniques

The quantitative analysis of prolamins is mainly based on immunological methods, but mass spectrometric and chromatographic techniques have also been used\textsuperscript{63,64}. In addition, in non-processed foods, the PCR techniques have an interesting role confirming the presence of gluten by a DNA pathway. The use of complementary and alternative non-immunological systems to confirm the results of the immunological methods are essential for validation of methods and avoiding false negative or positive results.

2.3.1. Proteomic Techniques

Applying proteomics to analysis of gluten in foods is of great interest to complement other techniques and to achieve the maximum accuracy in the results. There are several studies that use proteomics techniques for characterization of gluten proteins in grains to confirm and increase flour
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quality\textsuperscript{65-69}. Nevertheless, in gluten-free foods the wide dynamic range of gluten proteins (low amount compared with the other major proteins) represents a major problem in analyzing them\textsuperscript{70}.

Mass spectrometry (MS) methods have a high sensibility and they are widely used nowadays for identification, characterization and quantification of proteins and peptides. Depending on the different method of ionization, 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 celiac disease and to observe the different patterns of gliadins, hordeins, secalins, and avenins in grains depending on the type of cultivar and variety studied\textsuperscript{71}. Afterwards, the technique was optimized for gluten analysis in foods\textsuperscript{72}.

Even though MALDI-TOF gluten analysis is very useful, identifying gluten based on the analysis of intact proteins is not enough, due to extensive sequence similarities among gluten proteins; the results for hydrolyzed 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 beneficial. MS/MS is a process in which an ion formed in an ion source is mass-selected in the first phase, reacted and fragmented, and then the charged products from the reaction are analyzed in the second phase. The classical workflow approach consists of separating protein mixtures by electrophoresis, digest the sample by the enzyme trypsin breaking down proteins into peptides, and, finally, identifying those using MS. Most scientists engaged in proteomics separate proteins by electrophoresis\textsuperscript{73}. Nevertheless, more advanced shotgun proteomics approaches overcome the protein separation stage by digesting the entire protein mixture into peptides and separating them with one or two liquid chromatography (LC) steps. In addition to the classical methods of 2-DE and DIGE, MS-based quantification methods have gained increasing popularity. There are two broad groups of quantitative methods in MS-based proteomics: relative and absolute quantitative proteomics. In addition, quantitative proteomics can be classified into two major approaches: differential stable isotope labeling and label-free techniques (Figure 1).
There are several difficulties associated with proteomic analysis of prolams and glutelins as they are a complex mixture of proteins. In addition, there are a limited number of sequences of wheat, barley, and rye that are loaded and registered in public databases, especially for barley and rye, whose sequences are less registered than in the case of wheat, making more difficult the identification of proteins and peptides. Appropriate sample preparation procedures are also essential for correct sample analysis. Enrichment strategies are essential for successful protein identification, because the dynamic ranges of the proteins are very different and the proteins of interest are present in a substantially smaller quantity than other major proteins. The fact that gluten is formed by a high number of different proteins is another rate-limiting step in proteomic workflows. Enzymatic digestion of proteins by endoproteases is a key step in protein identification by MS. Trypsin, the most widely-used enzyme for this purpose, cleaves C-terminal to
lysine and arginine, but in the case of gluten proteins these cleavage points are not appropriate for generating peptides easily quantifiable by these techniques. Therefore it is necessary to use different enzymes with different cleavage points to obtain other, more appropriate gluten fragments and obtain a complete characterization, but the digestion by these enzymes is less reproducible.

The analysis of hydrolyzed peptides, such as in beers, is also of great interest to test the possible remaining toxic peptides for people with celiac disease, but the comprehensive annotation of the beer proteome is challenged both by the high concentration range of the protein entities and by a severe degree of processing-induced modifications. Other authors have characterized by proteomics techniques the content of prolamins in beer finding different peptides considering different types of beer.

The application of analysis of proteotypic peptides in gluten analysis is very promising. The first step in developing a method for gluten detection by identification of proteotypic peptides by MS is the selection of the best proteotypic peptides to be monitored that represent the most important prolamin and glutelin proteins, including those with proven immunogenicity and toxicity. The peptides must be unique to gluten proteins and must ionize efficiently and chromatograph in a stable, reproducible manner. Prior to MS analysis, proteins need to be broken up into peptides by enzymatic digestion. The common peptides for wheat, barley, and rye would be the best potential representative peptides for gluten analysis in all kind of foods when the origin of the contamination is not clear.

### 2.3.2. 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 and they are very useful to achieve the characterization of different cultivars and selection of genotypes coding for gluten proteins with the best bread-making quality. Nevertheless, there are few studies regarding the application of this technique to the analysis of gluten in foods. One of the
first studies used a PCR combined with agarose gels to detect wheat contaminations in oats\textsuperscript{84}. Afterwards, a quantitative PCR system combined with agarose gels was developed to detect simultaneously contamination of wheat, barley and rye in gluten-free food\textsuperscript{85}. Nevertheless, using agarose gels has some disadvantages and the most efforts for the detection and quantification of wheat, barley and/or rye DNA, have been in Q-PCR\textsuperscript{86-88}.

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 which presents a quantification limit of 20 pg DNA/mg. Comparing this Q-PCR system with the prolamin levels determined with the most commercially available R5 ELISA it was demonstrated that with the exception of some hydrolyzed and highly processed food samples (such as beers, syrups, malt extracts, breakfast cereals...), the rest of the food with prolamin levels above the R5 ELISA quantification limit (1.5 mg/kg) gave positive signals with the Q-PCR system. Therefore, this Q-PCR system 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 celiacs but also for individuals with wheat allergy\textsuperscript{89}.

Other authors have developed a DNA-based allergen-multiplex ligation-dependent probe amplification method that includes the determination of gluten in foods which might constitute a complementary method to the traditional protein-based methods\textsuperscript{90}. 

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3. Analytical Tools for the Selection of Oat Varieties with No Toxicity in Celiac Disease

Cultivated oats are hexaploid cereals belonging to the genus *Avena* L., which is found worldwide in almost all agricultural environments. Recently, oats have been receiving increasing interest as human food, mainly because this cereal could be suitable for consumptions by celiac patients. Several varieties of oats are available and all of them present very interesting nutritional and other healthy properties.

The presence of oats in a GFD is still a subject of controversial. Oats differ from other cereals in their prolamin content. The percentage of proline and glutamine (amino acids abundant in toxic regions) in avenin is lower than in other toxic cereals. Some clinical researchers state that patients with celiac disease tolerate oats without signs of intestinal inflammation. In contrast, other studies confirmed the toxicity of oats in certain types of patients with celiac disease. Arentz-Hansen et al. described the intestinal deterioration suffered by some patients with celiac disease following the consumption of oats while on a GFD. Avenin 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 patients with celiac disease who consumed 50 g/day of oats over 12 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 patients with celiac disease.

Comparison of the different studies are complicated by the different study designs, the different conditions used in the testing, the number of subjects included in each study and the reporting of the purity control of the oat material used in the clinical trials. Another relevant factor in different designs is the absence of information on the oat variety used. Silano et al. investigated the immunogenic effect of avenins from four oat cultivars using peripheral lymphocytes from patients with CD. All the varieties of oats tested (Lampton, Astra, Ava, and Nave) by these investigators were immunogenic with differences in their capacity to induce a response. However, other study...
confirmed that *Avena genziana* and *Avena potenza* do not display *in vitro* activities related to CD pathogenesis\(^5\).

The utility of the G12 antibody to identify potentially toxic oat varieties for celiac patients has been reported\(^6\) (Patent No.: WO2013098441 A1). This finding allowed classification of oat varieties into three groups based in their degree of affinity for the G12 antibody: a highly recognized group, one of moderate recognition, and one with no reactivity\(^6\). These results were confirmed by MALDI-TOF, SDS-PAGE and western blot by showing that the number, relative intensity of the peaks and protein profile obtained for the nine oat varieties differ from one another. The potentially immunotoxicity of the different types of oats was determined by T cell proliferation and interferon \(\gamma\) release. The reactivity that T-cells isolated from celiac patients exhibited with three oat varieties (one from each of the classified groups) correlated directly with the moAb G12 reactivity. The diversity observed in the reactivity to the different oat cultivars suggests variations in the avenin composition, and therefore in the amount of immunotoxic epitopes similar to the 33-mer present in these varieties. This gives a rational explanation for why only some oats trigger an immunological response.

In comparison with wheat gliadins, the avenins have been little studied, and the number of full avenin genes present at the moment in the databases is limited and from few genotypes, so that the variability of avenin genes in oats is not well represented. It has recent been known that, like wheat, oat grains have both monomeric and polymeric avenins\(^7\). A direct correlation between the immunogenicity of the different varieties of oats and the presence of the specific peptides with a higher/lower potential immunotoxicity has been found, that could explain why certain varieties of oats are toxic for celiac patients and other not\(^7\). The incorporation of some varieties of oats in food products not only may improve the nutritional quality but also may provide a treatment for various illnesses and would be welcomed by patients with celiac disease (Patent No.: WO2013098441 A1).
4. Policies and Regulation

The presence of high number of gluten components, the variation in the extraction efficiency, and the lack of reference materials representative of all kind of foods, are some issues that hinder the implementation of equivalent laws at national level and the comparison of data across the different methods\textsuperscript{97}.

In January 2009, the European Commission published a new European Regulation concerning the composition and labeling of foodstuffs suitable for people intolerant to gluten. “Gluten-free” food were defined as dietary foods consisting of or made only from one or more ingredients which do not contain wheat, rye, barley, or oats, and the gluten content does not exceed 20 mg/kg in them as sold to the final consumer\textsuperscript{98}. In addition, foods specially processed to reduce the gluten content to a level above 20 up to 100 mg/Kg were defined as food consisting of one or more ingredients from wheat, rye, barley oats or their crossbred varieties which have been specially processed to reduce the gluten content to that levels. Based on this, labeling, advertising and presentation of the products shall bear the terms, “gluten-free” (not exceeding 20 mg/Kg) and “very low gluten” (not exceeding 100 mg/Kg). This Regulation shall apply as from 1 January 2012. Regarding to oats content in food, according to the Codex Alimentarius for food for special dietary use for persons intolerant to gluten, CODEX STAN118-1979 (revised 2008, \texttt{http://www.codexalimentarius.net/web/more_info.jsp?id_sta=291}), oats can be tolerated by most but not all people who are intolerant to gluten. Moreover, the Commission Regulation (EC) No 41/2009 (\texttt{http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2009:016:0003:0005:EN:PDF}) concerning the composition and labeling of foodstuffs suitable for people intolerant to gluten, also states that the possible adverse effect of oats is an issue of ongoing study and investigation by the scientific community. In addition, a major concern is the contamination of oats with wheat, rye or barley that can occur during grain harvesting, transport, storage and processing, that should be taken into consideration with regard to labeling of those products.
Nowadays, the method for determination of the gluten content in gluten-free foods accepted internationally by the Codex Alimentarius Commission is the sandwich ELISA based on the R5 antibody. As mentioned above, the principal limitation of the sandwich R5 ELISA is that it is essential that at least two epitopes of the sequences recognized by the monoclonal antibody R5 be present simultaneously in a protein or peptide. However, in hydrolyzed foods (such as baby foods, syrups and beers), gluten proteins are fragmented during food processing and converted into peptides in which only one toxic peptide may appear. In this case, the quantification of gluten by sandwich R5 ELISA would be incorrect, yielding less than the real gluten content. The Codex Alimentarius Commission states that “for the detection of hydrolyzed gluten a modification of the R5 assay (competitive ELISA) has to be applied”.

In 2013 the Protein & Enzymes Technical Committee of AACC International initiated a collaborative study of a method for gluten quantitation in selected foods using a G12 antibody sandwich ELISA system. Recently, this method has been approved as AACC International Method (AACC 38-52.01; Newsletter 2014; 5 (1): 1-5). In March 2014, at the AOACI mid-year meeting, the G12 Sandwich ELISA has been adopted as an AOACI 1st Action Method.

In August 2013, gluten-free labeling regulations for gluten-free foods were established for the first time by the Food and Drug Administration (FDA) of the United States. These gluten limits are based on Codex standards and define the term “gluten-free” for voluntary use in the labeling of foods when any presence of gluten is less than 20 ppm. In general, foods may be labeled “gluten-free” if the food either is inherently gluten free; or does not contain an ingredient that is: 1) a gluten-containing grain (any type of wheat, rye, barley), or crossbreeds of these grains; 2) derived from a gluten-containing grain that has not been processed to remove gluten; or 3) derived from a gluten-containing grain that has been processed to remove gluten (e.g., wheat starch), if the use of that ingredient results in the presence of 20 parts per million (ppm) or more gluten in the food. The final rule applies to all FDA-regulated foods, including dietary supplements. The rule excludes those foods
whose labeling is regulated by the U.S. Department of Agriculture (USDA) and the Alcohol and Tobacco Tax and Trade Bureau (TTB). Generally, USDA regulates the labeling of meats, poultry, and certain egg products (FDA regulates the labeling of shell eggs). TTB regulates the labeling of most alcoholic beverages, including all distilled spirits, wines that contain 7 percent or more alcohol by volume, and malted beverages that are made with both malted barley and hops. All foods imported into the United States must meet also these requirements to make a gluten-free claim. Manufacturers who elect to analyze their foods for gluten can select the test methods most appropriate for them, considering the type of foods they manufacture, and FDA recommends the use of scientifically valid methods to obtain reliable and consistent results (http://www.fda.gov/food/guidanceregulation/guidancedocumentsregulatoryinformation/allergens).

In Australia and New Zealand claims in relation to gluten content of food are prohibited unless the expressly permitted cases. A claim to the effect that a food is gluten free must not be made in relation to a food unless the food contains no detectable gluten; and no oats or their products; or cereals containing gluten that have been malted, or their products. In addition, a claim to the effect that a food has low gluten content must not be made in relation to a food unless the food contains no more than 20 mg gluten per 100 g of the food (Standard 1.2.8 Federal Register of Legislative Instruments F2012C00218).
5. Conclusions

CD is a common autoimmune disorder that has genetic, environmental, and immunological components. The ingestion of gluten proteins contained in wheat, barley, rye, and in some cases oats, leads to characteristic inflammation, villous atrophy, and crypt hyperplasia in the CD patient’s upper small intestine. Safety of gluten free foods can be only ensured by providing reliable methods of gluten detection and quantitation. The high variety of gluten components and other ingredients contained in foods after manufacturing make extraction efficiency and detection very difficult.

Methods for gluten analysis are available for the control of “gluten-free” products. Different immunological and non-immunological techniques are being applied to increase the sensitivity and provide supplementary information on gluten protein identification, taking into account that methods for gluten analysis must be sensitive enough to quantify low levels of gluten in foods to fit in the food regulations.

The quantitative analysis of gluten is mainly performed by ELISA methods. Proteomics techniques are promising tools for quantification of gluten, whereas DNA-based methods are useful tools to detect eventual contaminations. The limit values of 20 and 100 mg/Kg of gluten in “gluten-free” and “very low gluten” foods, respectively, help managing the diet of most celiac patients efficiently.
References

1. Breiteneder H, Radauer C. *A classification of plant food allergens.* J Allergy Clin Immunol. 2004; 113(5): 821-30
   http://dx.doi.org/10.1016/j.jaci.2004.01.779
   PMid:15131562

2. Shewry PR, Tatham AS. *The characteristics, structures and evolutionary relationships of prolamins.* In: Shewry PR, Casey R (Eds.). Seed Proteins. The Netherlands: Kluwer Academic Publishers. 1999; 11-34.
   http://dx.doi.org/10.1007/978-94-011-4431-5_2

3. Osborne TB. *The proteins of the wheat kernel.* Publication no. 84 Carnegie Institution of Washington, Press of Judd & Detweiler, Inc, Washington DC, USA. 1907.
   http://dx.doi.org/10.5962/bhl.title.22763

4. Stern M, Ciclitira PJ, van Eckert R, Feighery C, Janssen FW, Mendez E et al. *Analysis and clinical effects of gluten in coeliac disease.* European journal of gastroenterology & hepatology. 2001; 13(6): 741-7.
   http://dx.doi.org/10.1097/00042737-200106000-00023

5. Shewry PR, Tatham AS. *The prolamin storage proteins of cereal seeds: structure and evolution.* Biochem J. 1990; 267(1): 1-12.
   PMid:2183790 PMcid:PMC1131235

6. Wieser H. *Comparative investigations of gluten proteins from different wheat species I. Qualitative and quantitative composition of gluten protein types.* Eur Food Res Technol. 2000; 211(4): 262-8.
   http://dx.doi.org/10.1007/s002170000165

7. Real A, Comino I, de Lorenzo L, Merchan F, Gil-Humanes J, Gimenez MJ et al. *Molecular and immunological characterization of gluten proteins isolated from oat cultivars that differ in toxicity for celiac disease.* PloS one. 2012; 7(12): e48365.
   http://dx.doi.org/10.1371/journal.pone.0048365
   PMid:23284616 PMcid:PMC3524229

8. Chesnut RS, Shotwell MA, Boyer SK, Larkins BA. *Analysis of avenin proteins and the expression of their mRNAs in developing oat seeds.* Plant Cell. 1989; 1(9): 913-24.
   http://dx.doi.org/10.1105/tpc.1.9.913
   PMid:2535531 PMcid:PMC159827

9. Wieser H. *Chemistry of gluten proteins.* Food Microbiol. Review. 2007; 24(2): 115-9.
   http://dx.doi.org/10.1016/j.fm.2006.07.004
   PMid:17008153
10. Sollid LM. *Coeliac disease: dissecting a complex inflammatory disorder*. Nat Rev Immunol. 2002; 2(9): 647-55. 
http://dx.doi.org/10.1038/nri885
PMid:12209133

11. Bernardo D, Pena AS. *Developing strategies to improve the quality of life of patients with gluten intolerance in patients with and without coeliac disease*. Eur J Intern Med. Editorial. 2012; 23(1): 6-8.

12. Corrao G, Corazza GR, Bagnardi V, Brusco G, Ciacci C, Cottone M et al. *Mortality in patients with coeliac disease and their relatives: a cohort study*. Lancet. 2001; 358(9279): 356-61. 
http://dx.doi.org/10.1016/S0140-6736(01)05554-4

13. Greco L, Gobbetti M, Auricchio R, Di Mase R, Landolfo F, Paparo F et al. *Safety for patients with celiac disease of baked goods made of wheat flour hydrolyzed during food processing*. Clinical gastroenterology and hepatology: the official clinical practice journal of the American Gastroenterological Association. 2011; 9(1): 24-9. 
http://dx.doi.org/10.1016/j.cgh.2010.09.025
PMid:20951830

14. Mena MC, Lombardia M, Hernando A, Mendez E, Albar JP. *Comprehensive analysis of gluten in processed foods using a new extraction method and a competitive ELISA based on the R5 antibody*. Talanta. 2012; 91: 33-40. 
http://dx.doi.org/10.1016/j.talanta.2011.12.073
PMid:22365676

15. Kanerva P, Brinck O, Salovaara H, Loponen J. *Deamidation of gluten proteins and peptides decreases the antibody affinity in gluten analysis assays*. J Cereal Sci. 2011; 53(3): 335-9. 
http://dx.doi.org/10.1016/j.jcs.2011.02.003

16. Garcia E, Llorente M, Hernando A, Kieffer R, Wieser H, Mendez E. *Development of a general procedure for complete extraction of gliadins for heat processed and unheated foods*. European journal of gastroenterology & hepatology. 2005; 17(5): 529-39. 
http://dx.doi.org/10.1097/00042737-200505000-00010

17. Han Z, Wu F, Deng G, Qian G, Yu M, Jia Y. *Structural and expresional analysis of the B-hordein genes in Tibetan hull-less barley*. Genetica. 2010; 138(2): 227-39. 
http://dx.doi.org/10.1007/s10709-009-9415-6
PMid:19856114

18. van Eckert R, Berghofer E, Ciclitira PJ, Chirdo F, Denery-Papini S, Ellis HJ et al. *Towards a new gliadin reference material–isolation and characterisation*. J Cereal Sci. 2006; 43: 331-41. 
http://dx.doi.org/10.1016/j.jcs.2005.12.009
19. Gessendorfer B, Koehler P, Wieser H. Preparation and characterization of enzymatically hydrolyzed prolamins from wheat, rye, and barley as references for the immunochemical quantitation of partially hydrolyzed gluten. Analytical and bioanalytical chemistry. 2009; 395(6): 1721-8. 
   http://dx.doi.org/10.1007/s00216-009-3080-6  
   PMid:19763549

20. Comino I, Real A, Gil-Humanes J, Piston F, de Lorenzo L, Moreno ML et al. Significant differences in coeliac immunotoxicity of barley varieties. Mol Nutr Food Res. 2012; 56(11): 1697-707. 
   http://dx.doi.org/10.1002/mnfr.201200358  
   PMid:22968973

21. Comino I, Real A, Moreno ML, Montes R, Cebolla A, Sousa C. 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. 2013; 93(4): 933-43. 
   http://dx.doi.org/10.1002/jsfa.5830  
   PMid:22886585

22. Denery-Papini S, Nicolas Y, Popineau Y. Efficiency and Limitations of Immunochemical Assays for the Testing of Gluten-free Foods. Journal of Cereal Science. 1999; 30(2): 121-31. 
   http://dx.doi.org/10.1006/jcrs.1999.0268

23. Windemann H, Fritschy F, Baumgartner E. Enzyme-linked immunosorbent assay for wheat alpha-gliadin and whole gliadin. Biochimica et biophysica acta. 1982; 709: 110-21. 
   http://dx.doi.org/10.1016/0167-4838(82)90428-9

24. Howdle PD, Losowsky MS. Review of methods for measuring gliadins in food. Gut. Review. 1990; 31(6): 712-3.

25. Immer U, Haas-Lauterbach S. 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. Journal of AOAC International. 2012; 95(4): 1118-24. 
   http://dx.doi.org/10.5740/jaoacint.CS2012_01  
   PMid:22970580

26. van Eckert R, Bond J, Rawson P, Klein CL, Stern M, Jordan TW. Reactivity of gluten detecting monoclonal antibodies to a gliadin reference material. Journal of Cereal Science. 2010; 51(2): 198-204. 
   http://dx.doi.org/10.1016/j.jcs.2009.11.012

27. Diaz-Amigo C, Popping B. Accuracy of ELISA detection methods for gluten and reference materials: a realistic assessment. Journal of agricultural and food chemistry. 2013; 61(24): 5681-8. 
   http://dx.doi.org/10.1021/jf3046736  
   PMid:23713744
28. McKillop DF, Gosling JP, Stevens FM, Fottrell PF. Enzyme immunoassay of gliadin in food. Biochem Soc Trans. 1985; 13: 486-7.

29. Troncone R, Vitale M, Donatiello A, Farris E, Rossi G, Auricchio S. A sandwich enzyme immunoassay for wheat gliadin. 1986: 0022-1759.

30. Friis SU. Enzyme-linked immunosorbent assay for quantitation of cereal proteins toxic in coeliac disease. Clin Chim Acta. 1988; 178(3): 261-70.

http://dx.doi.org/10.1016/0009-8981(88)90234-3

31. Freedman AR, Galfre G, Gal E, Ellis HJ, Ciclitira PJ. Monoclonal antibody ELISA to quantitate wheat gliadin contamination of gluten-free foods. J Immunol Methods. 1987; 98(1): 123-7.

http://dx.doi.org/10.1016/0022-1759(87)90445-5

32. Chirdo FG, Añón MC, Fossati CA. Development of high-sensitive enzyme immunoassays for gliadin quantification using the streptavidin-biotin amplification system. Food and Agricultural Immunology. 1998; 10(2): 143-55.

http://dx.doi.org/10.1080/09540109809354977

33. Chirdo FG, Añón MC, Fossati CA. Optimization of a competitive ELISA with polyclonal antibodies for quantification of prolamins in foods. Food and Agricultural Immunology. 1995; 7(4): 333-43.

http://dx.doi.org/10.1080/09540109509354893

34. Ellis HJ, Rosen-Bronson S, O'Reilly N, Ciclitira PJ. Measurement of gluten using a monoclonal antibody to a coeliac toxic peptide of A gliadin. Gut. 1998; 43(2): 190-5.

http://dx.doi.org/10.1136/gut.43.2.190
PMid:10189843 PMCID:PMC1727224

35. Sturgess R, Day P, Ellis HJ, Lundin KE, Gjertsen HA, Kontakou M et al. Wheat peptide challenge in coeliac disease. Lancet. 1994; 343(8900): 758-61.

http://dx.doi.org/10.1016/S0140-6736(94)91837-6

36. Bermudo Redondo MC, Griffin PB, Garzon Ransanz M, Ellis HJ, Ciclitira PJ, O'Sullivan CK. Monoclonal antibody-based competitive assay for the sensitive detection of coeliac disease toxic prolamins. Analytica Chimica Acta. 2005; 551(1-2): 105-14.

http://dx.doi.org/10.1016/j.aca.2005.07.023

37. Skerritt JH, Hill AS. Enzyme immunoassay for determination of gluten in foods: collaborative study. J Assoc Off Anal Chem. 1991; 74(2): 257-64.
PMid:2050607

38. Skerritt JH, Hill AS. Monoclonal antibody sandwich enzyme immunoassays for determination of gluten in foods. Journal of agricultural and food chemistry. 1990; 38(8): 1771-8.
http://dx.doi.org/10.1021/jf00098a029

39. Thompson T, Mendez E. Commercial assays to assess gluten content of gluten-free foods: why they are not created equal. J Am Diet Assoc. Review. 2008; 108(10): 1682-7.
40. Gabrovská D, Rysov J, Filov V, Plicka J, Cuhra P, Kubk M et al. *Gluten Determination by Gliadin Enzyme-Linked Immunosorbent Assay Kit: Interlaboratory Study*. Journal of AOAC International. 2006; 89(1): 154-60. PMid:16512241

41. Sánchez D, Tučková L, Burkhard M, Plicka J, Mothes T, Hoffmanová I et al. *Specificity Analysis of Anti-gliadin Mouse Monoclonal Antibodies Used for Detection of Gliadin in Food for Gluten-free Diet*. Journal of agricultural and food chemistry. 2007; 55(7): 2627-32. http://dx.doi.org/10.1021/jf0630421 PMid:17335223

42. Spaenij-Dekking EH, Kooy-Winkelaar EM, Nieuwenhuizen WF, Drijfhout JW, Koning F. *A novel and sensitive method for the detection of T cell stimulatory epitopes of alpha/beta- and gamma-gliadin*. Gut. 2004; 53(9): 1267-73. http://dx.doi.org/10.1136/gut.2003.037952 PMid:15306583 PMCid:PMC1774189

43. Mitea C, Havenaar R, Drijfhout JW, Edens L, Dekking L, Koning F. *Efficient degradation of gluten by a prolyl endoprotease in a gastrointestinal model: implications for coeliac disease*. Gut. 2008; 57(1): 25-32. http://dx.doi.org/10.1136/gut.2006.111609 PMid:17494108

44. Sharma GM. *Immunoreactivity and detection of wheat proteins by commercial ELISA kits*. Journal of AOAC International. 2012; 95(2): 364-71. http://dx.doi.org/10.5740/jaoacint.SGE_Sharma PMid:22649920

45. Kahlenberg F, Sanchez D, Lachmann I, Tuckova L, Tlaskalova H, Méndez E et al. *Monoclonal antibody R5 for detection of putatively coeliac-toxic gliadin peptides*. Eur Food Res Technol. 2006; 222(1-2): 78-82. http://dx.doi.org/10.1007/s00217-005-0100-4

46. Valdes I, Garcia E, Llorente M, Mendez E. *Innovative approach to low-level gluten determination in foods using a novel sandwich enzyme-linked immunosorbent assay protocol*. Eur J Gastroenterol Hepatol. 2003; 15(5): 465-74. http://dx.doi.org/10.1097/01.meg.0000059119.41030.df PMid:12702901

47. Codex Alimentarius Commission. *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*. 2006; ALINORM 06/29/23.

48. Morón B, Cebolla Á, Manyani H, Álvarez-Maqueda M, Megías M, Thomas MC et al. *Sensitive detection of cereal fractions that are toxic to celiac disease patients by using monoclonal antibodies to a main immunogenic wheat peptide*. The American journal of clinical nutrition. 2008; 87(2): 405-14. PMid:18258632
49. Shan L, Molberg O, Parrot I, Hausch F, Filiz F, Gray GM et al. Structural basis for gluten intolerance in celiac sprue. Science. 2002; 297(5590): 2275-9.
http://dx.doi.org/10.1126/science.1074129
PMid:12351792

50. Moron B, Bethune MT, Comino I, Manyani H, Ferragud M, Lopez MC et al. Toward the assessment of food toxicity for celiac patients: characterization of monoclonal antibodies to a main immunogenic gluten peptide. PloS one. 2008; 3(5): e2294.
http://dx.doi.org/10.1371/journal.pone.0002294
PMid:18509534 PMcid:PMC2386552

51. Ehren J, Moron B, Martin E, Bethune MT, Gray GM, Khosla C. A food-grade enzyme preparation with modest gluten detoxification properties. PloS one. 2009; 4(7): e6313.
http://dx.doi.org/10.1371/journal.pone.0006313
PMid:19621078 PMcid:PMC2708912

52. Moron B, Cebolla A, Manyani H, Alvarez-Maqueda M, Megias M, Thomas MC et al. Sensitive detection of cereal fractions that are toxic to celiac disease patients by using monoclonal antibodies to a main immunogenic wheat peptide. The American journal of clinical nutrition. 2008; 87(2): 405-14.
PMid:18258632

53. Mena MC, Hernando A, Lombardía M, Albar J. The application of proteomics in gluten analysis: Identification and characterization of prolamins and glutelins through mass spectrometry. Proceeding of the 23rd Meeting Working Group on Prolamin Analysis and Toxicity. 2009; 23-8.

54. Salmanowicz BP, Nowak J. Diversity of monomeric prolamins in triticale cultivars determined by capillary zone electrophoresis. Journal of agricultural and food chemistry. 2009; 57(6): 2119-25.
http://dx.doi.org/10.1021/jf803326z
PMid:19228059

55. Shuaib A, Hussain MS. The past and future of neuroprotection in cerebral ischaemic stroke. European neurology. 2008; 59(1-2): 4-14.
http://dx.doi.org/10.1159/000109254
PMid:17917451

56. van den Broeck HC, America AH, Smulders MJ, Bosch D, Hamer RJ, Gilissen LJ et al. A modified extraction protocol enables detection and quantification of celiac disease-related gluten proteins from wheat. J Chromatogr B Analyt Technol Biomed Life Sci. 2009; 877(10): 975-82.
http://dx.doi.org/10.1016/j.jchromb.2009.02.035
PMid:19288254
57. Immer U, Haas Lauterbach S. *Gluten detection in molecular biological and immunological techniques and applications for food chemist*. In: Propping B, Diaz-Amigo C, Hoenicke K (Eds.). Molecular Biological and Immunological Techniques and Applications for Food Chemists. John Wiley & Sons, Inc. 2010: 359-75.  
http://dx.doi.org/10.1002/9780470637685.ch19

58. Nassef HM, Bermudo Redondo MC, Ciclitira PJ, Ellis HJ, Fragoso A, O'Sullivan CK. *Electrochemical immunosensor for detection of celiac disease toxic gliadin in foodstuff*. Anal Chem. 2008; 80(23): 9265-71.  
http://dx.doi.org/10.1021/ac801620j  
PMid:19551990

59. Nassef HM, Civit L, Fragoso A, O'Sullivan CK. *Amperometric immunosensor for detection of celiac disease toxic gliadin based on Fab fragments*. Anal Chem. 2009; 81(13): 5299-307.  
http://dx.doi.org/10.1021/ac9005342  
PMid:19469538

60. Chu PT, Lin CS, Chen WJ, Chen CF, Wen HW. *Detection of gliadin in foods using a quartz crystal microbalance biosensor that incorporates gold nanoparticles*. Journal of agricultural and food chemistry. 2012; 60(26): 6483-92.  
http://dx.doi.org/10.1021/jf2047866  
PMid:22694361

61. Chu PT, Wen HW. *Sensitive detection and quantification of gliadin contamination in gluten-free food with immunomagnetic beads based liposomal fluorescence immunoassay*. Analytica Chimica Acta. 2013; 787: 246-53.  
http://dx.doi.org/10.1016/j.aca.2013.05.014  
PMid:23830446

62. Amaya-Gonzalez S, de-Los-Santos-Alvarez N, Miranda-Ordieres AJ, Lobo-Castanon MJ. *Aptamer binding to celiac disease-triggering hydrophobic proteins: a sensitive gluten detection approach*. Anal Chem. 2014; 86(5): 2733-9.  
http://dx.doi.org/10.1021/ac404151n  
PMid:24502317

63. Sealey-Voyksner JA, Khosla C, Voyksner RD, Jorgenson JW. *Novel aspects of quantitation of immunogenic wheat gluten peptides by liquid chromatography-mass spectrometry/mass spectrometry*. J Chromatogr A. 2010; 1217(25): 4167-83.  
http://dx.doi.org/10.1016/j.chroma.2010.01.067  
PMid:20181349

64. Wieser H. *Investigations on the extractability of gluten proteins from wheat bread in comparison with flour*. Z Lebensm Unters Forsch A. 1998; 207: 128-32.  
http://dx.doi.org/10.1007/s002170050306

559
65. Hurkman WJ, Tanaka CK, Vensel WH, Thilmony R, Altenbach SB. Comparative proteomic analysis of the effect of temperature and fertilizer on gliadin and glutenin accumulation in the developing endosperm and flour from Triticum aestivum L. cv. Butte 86. Proteome science. 2013; 11(1): 8.
http://dx.doi.org/10.1186/1477-5956-11-8
PMid:23432757 PMCID:PMC3599944

66. Pompa M, Giuliani MM, Palermo C, Agriesti F, Centonze D, Flagella Z. Comparative Analysis of Gluten Proteins in Three Durum Wheat Cultivars by a Proteomic Approach. Journal of agricultural and food chemistry. 2013. 61(11): 2606-17.
http://dx.doi.org/10.1021/jf304566d
PMid:23414385

67. Guo G, Lv D, Yan X, Subburaj S, Ge P, Li X et al. Proteome characterization of developing grains in bread wheat cultivars (Triticum aestivum L.). BMC plant biology. 2012; 12: 147.
http://dx.doi.org/10.1186/1471-2229-12-147
PMid:22900893 PMCID:PMC3480910

68. Altenbach SB, Vensel WH, Dupont FM. Analysis of expressed sequence tags from a single wheat cultivar facilitates interpretation of tandem mass spectrometry data and discrimination of gamma gliadin proteins that may play different functional roles in flour. BMC plant biology. 2010; 10: 7.
http://dx.doi.org/10.1186/1471-2229-10-7
PMid:20064259 PMCID:PMC2827424

69. Mamone G, De Caro S, Di Luccia A, Addeo F, Ferranti P. Proteomic-based analytical approach for the characterization of glutenin subunits in durum wheat. Journal of mass spectrometry: JMS. 2009; 44(12): 1709-23.
http://dx.doi.org/10.1002/jms.1680

70. Mamone G, Picariello G, Addeo F, Ferranti P. Proteomic analysis in allergy and intolerance to wheat products. Expert review of proteomics. 2011; 8(1): 95-115.
http://dx.doi.org/10.1586/epr.10.98
PMid:21329430

71. Camafeita E, Alfonso P, Mothes T, Mendez E. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometric micro-analysis: the first non-immunological alternative attempt to quantify gluten gliadins in food samples. Journal of mass spectrometry: JMS. 1997; 32(9): 940-7.
http://dx.doi.org/10.1002/(SICI)1096-9888(199709)32:9<940::AID-JMS550>3.0.CO;2-2

72. Hernando A, Valdes I, Mendez E. 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: JMS. 2003; 38(8): 862-71.
http://dx.doi.org/10.1002/jms.502
PMid:12938107
73. Granvogl B, Ploscher M, Eichacker LA. Sample preparation by in-gel digestion for mass spectrometry-based proteomics. Anal Bioanal Chem. Review. 2007; 389(4): 991-1002.  
http://dx.doi.org/10.1007/s00216-007-1451-4  
PMid:17639354

74. Gao M, Deng C, Zhang X. Magnetic nanoparticles-based digestion and enrichment methods in proteomics analysis. Expert Rev Proteomics. 2011; 8(3): 379-90.  
http://dx.doi.org/10.1586/epr.11.25  
PMid:21679118

75. Vaezzadeh AR, Deshusses JM, Waridel P, Francois P, Zimmermann-Ivol CG, Lescuyer P et al. Accelerated digestion for high-throughput proteomics analysis of whole bacterial proteomes. J Microbiol Methods. 2010; 80(1): 56-62.  
http://dx.doi.org/10.1016/j.mimet.2009.10.019  
PMid:19913576

76. Zhang K, Wu S, Tang X, Kaiser NK, Bruce JE. A bifunctional monolithic column for combined protein preconcentration and digestion for high throughput proteomics research. J Chromatogr B Analyt Technol Biomed Life Sci. 2007; 849(1-2): 223-30.  
http://dx.doi.org/10.1016/j.jchromb.2006.11.029  
PMid:17150420

77. Salplachta J, Marchetti M, Chmelik J, Allmaier G. A new approach in proteomics of wheat gluten: combining chymotrypsin cleavage and matrix-assisted laser desorption/ionization quadrupole ion trap reflectron tandem mass spectrometry. Rapid Commun Mass Spectrom. 2005; 19(18): 2725-8.  
http://dx.doi.org/10.1002/rcm.2092  
PMid:16124027

78. Picariello G, Mamone G, Nitride C, Addeo F, Camarca A, Vocca I et al. Shotgun proteome analysis of beer and the immunogenic potential of beer polypeptides. Journal of proteomics. 2012; 75(18): 5872-82.  
http://dx.doi.org/10.1016/j.jprot.2012.07.038  
PMid:22868252

79. Colgrave ML, Goswami H, Howitt CA, Tanner GJ. What is in a beer? Proteomic characterization and relative quantification of hordein (gluten) in beer. Journal of proteome research. 2012; 11(1): 386-96.  
http://dx.doi.org/10.1021/pr2008434  
PMid:21999962

80. Salmanowicz BP, Dylewicz M. Identification and characterization of high-molecular-weight glutenin genes in Polish triticale cultivars by PCR-based DNA markers. Journal of applied genetics. 2007; 48(4): 347-57.  
http://dx.doi.org/10.1007/BF03195231  
PMid:17998591
81. Huang XQ, Cloutier S. *Molecular characterization and genomic organization of low molecular weight glutenin subunit genes at the Glu-3 loci in hexaploid wheat* (*Triticum aestivum* L.). TAG Theoretical and applied genetics Theoretische und angewandte Genetik. 2008; 116(7): 953-66.  
http://dx.doi.org/10.1007/s00122-008-0727-1  
PMid:18305921

82. Liu SW, Gao X, Lu BR, Xia GM. *Characterization of the genes coding for the high molecular weight glutenin subunits in Lophopyrum elongatum*. Hereditas. 2008; 145(1): 48-57.  
http://dx.doi.org/10.1111/j.0018-0661.2008.2029.x  
PMid:18439233

83. Zhang X, Liu D, Jiang W, Guo X, Yang W, Sun J et al. *PCR-based isolation and identification of full-length low-molecular-weight glutenin subunit genes in bread wheat* (*Triticum aestivum* L.). TAG Theoretical and applied genetics Theoretische und angewandte Genetik. 2011; 123(8): 1293-305.  
http://dx.doi.org/10.1007/s00122-011-1667-8  
PMid:21830110

84. Köppel E, Stadler M, Lüthy J, Hübner P. *Detection of wheat contamination in oats by polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA)*. Zeitschrift für Lebensmitteluntersuchung und -Forschung A. 1998; 206(6): 399-403.  
http://dx.doi.org/10.1007/s002170050281

85. Dahinden I, von Büren M, Lüthy J. *A quantitative competitive PCR system to detect contamination of wheat, barley or rye in gluten-free food for coeliac patients*. Eur Food Res Technol. 2001; 212(2): 228-33.  
http://dx.doi.org/10.1007/s002170000252

86. Hernandez M, Esteve T, Pla M. *Real-time polymerase chain reaction based assays for quantitative detection of barley, rice, sunflower, and wheat*. Journal of agricultural and food chemistry. 2005; 53(18): 7003-9.  
http://dx.doi.org/10.1021/jf050797j  
PMid:16131102

87. Zeltner D, Glomb M, Maede D. *Real-time PCR systems for the detection of the gluten-containing cereals wheat, spelt, kamut, rye, barley and oat*. European Food Research and Technology A. 2009; 228(3): 321-30.  
http://dx.doi.org/10.1007/s00217-008-0937-4

88. Rønning SB, Berdal KG, Andersen CB, Holst-Jensen A. *Novel Reference Gene, PKABA1, Used in a Duplex Real-Time Polymerase Chain Reaction for Detection and Quantitation of Wheat- and Barley-Derived DNA*. Journal of agricultural and food chemistry. 2006; 54(3): 682-7.  
http://dx.doi.org/10.1021/jf052328n  
PMid:16448168
89. Mujico JR, Lombardía M, Mena MC, Méndez E, Albar JP. A highly sensitive real-time PCR system for quantification of wheat contamination in gluten-free food for celiac patients. Food Chemistry. 2011; 128(3): 795-801. http://dx.doi.org/10.1016/j.foodchem.2011.03.061

90. Mustorp SL, Dromtorp SM, Holck AL. Multiplex, quantitative, ligation-dependent probe amplification for determination of allergens in food. Journal of agricultural and food chemistry. 2011; 59(10): 5231-9. http://dx.doi.org/10.1021/jf200545j PMid:21452891

91. Suttie JM, Reynolds SG. Fodder oats: A world overview, 2004. Food and Agriculture Organization of the United Nations (FAO). Accessed on 16 October 2012. http://www.fao.org/docrep/008/y5765e/y5765e00.htm

92. Pulido OM, Gillespie Z, Zarkadas M, Dubois S, Vavasour E, Rashid M et al. Introduction of oats in the diet of individuals with celiac disease: a systematic review. Adv Food Nutr Res. Review. 2009; 57: 235-85.

93. Arentz-Hansen H, Fleckenstein B, Molberg O, Scott H, Koning F, Jung G et al. The molecular basis for oat intolerance in patients with celiac disease. PLoS Med. 2004; 1(1): e1. http://dx.doi.org/10.1371/journal.pmed.0010001 PMid:15526039 PMcid:PMC523824

94. Silano M, Di Benedetto R, Maialetti F, De Vincenzi A, Calcaterra R, Cornell HJ et al. Avenins from different cultivars of oats elicit response by coeliac peripheral lymphocytes. Scandinavian journal of gastroenterology. 2007; 42(11): 1302-5. PMid:17852883

95. Maglio M, Mazzarella G, Barone MV, Gianfrani C, Pogna N, Gazza L et al. Immunogenicity of two oat varieties, in relation to their safety for celiac patients. Scandinavian journal of gastroenterology. 2011; 46(10): 1194-205. http://dx.doi.org/10.3109/00365521.2011.603159 PMid:21843037

96. Comino I, Real A, de Lorenzo L, Cornell H, Lopez-Casado MA, Barro F et al. Diversity in oat potential immunogenicity: basis for the selection of oat varieties with no toxicity in coeliac disease. Gut. 2011; 60(7): 915-22. http://dx.doi.org/10.1136/gut.2010.225268 PMid:21317420 PMcid:PMC3112367

97. Diaz-Amigo C, Popping B. Gluten and gluten-free: issues and considerations of labeling regulations, detection methods, and assay validation. Journal of AOAC International. 2012; 95(2): 337-48. http://dx.doi.org/10.5740/jaoacint.SGE_Diaz-Amigo PMid:22649917
98. Commision Regulation (EC) No 41/2009 of 20 January 2009 concerning the composition and labelling of foodstuffs suitable for people intolerant to gluten. 2009.

99. Codex Alimentarius International Food Standards. Accessed on 28 August 2013. http://www.codexalimentarius.net/web/more_info.jsp?id_sta=291