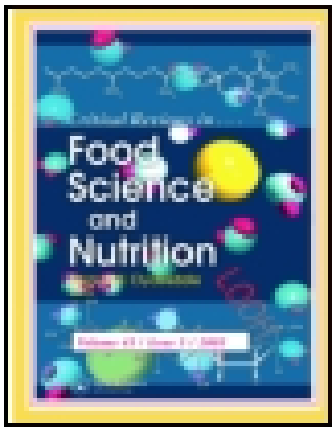

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Immunochemical Detection Methods for Gluten in Food Products: Where Do We Go from Here?

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Immunochemical detection methods for gluten in food products: Where do we go from here?

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Abstract

Accurate and reliable quantification methods for gluten in food are necessary to ensure proper product labelling and thus safeguard the gluten sensitive consumer against exposure. Immunochemical detection is the method of choice, as it is sensitive, rapid and relatively easy to use. Although a wide range of detection kits are commercially available, there are still many difficulties in gluten detection that have not yet been overcome. This review gives an overview of the currently commercially available immunochemical detection methods, and discusses the problems that still exist in gluten detection in food. The largest problems are encountered in the extraction of gluten from food matrices, the choice of epitopes targeted by the detection method, and the use of a standardized reference material. By comparing the available techniques with the unmet needs in gluten detection, the possible benefit of a new multiplex immunoassay is investigated. This detection method would allow for the detection and quantification of multiple harmful gluten peptides at once and would, therefore, be a logical advancement in gluten detection in food.

Keywords: Celiac disease, ELISA, multiplex immunoassay, epitopes

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Introduction

Celiac disease (CD) is one of the most common food related immune disorders, as it affects approximately 1% of the general population worldwide (Lionetti and Catassi, 2011; Reilly and Green, 2012). CD is an enteropathy that is triggered by the ingestion of gluten in genetically predisposed individuals. In these susceptible persons, consumption of gluten containing foods may result in an inflammation process damaging the mucosal villi, visible as villous atrophy and crypt hyperplasia. This damage can lead to a flattened mucosa, resulting in malabsorption of nutrients and deficiency-related complaints (Green and Cellier, 2007). The symptoms of CD are diverse and vary between patients. In classic manifestations of the disease, intestinal complaints such as abdominal pain and diarrhoea are often reported. However, CD can also manifest non-classic, with extra-intestinal subclinical signs; for instance osteoporosis and anaemia. An increasing number of CD patients shows no symptoms other than villous atrophy or a positive serology (Alaedini and Green, 2005). Especially in the latter cases, CD is difficult to diagnose and often remains undetected for a long period of time (Lionetti and Catassi, 2011). When left undiagnosed or untreated, CD can lead to serious complications such as enteropathy-associated T-cell lymphoma or intestinal adenocarcinoma (Green and Cellier, 2007). Although several new therapies are currently under investigation, the only treatment for CD to date is to completely eliminate gluten from the diet (McAllister and Kagnoff, 2012; Sollid and Khosla, 2011). Patients need to adhere to the gluten-free diet for their entire life, or risk relapse and progression of the disease.

To assist those with a gluten intolerance in making safe food choices, the European Commission adopted Commission Regulation 41/2009 on the labelling of gluten-free foodstuffs

(The Commission of the European Communities, 2009), which will be transferred under the new Commission Regulation 1169/2011 in 2014 (The European Parliament and the Council of the European Union, 2011). This regulation states that products that have been specially processed to reduce the gluten content can be labelled ógluten-freeô if the total gluten content is below 20 mg/kg, or óvery low glutenô if the total gluten content is between 20 and 100 mg/kg. The ógluten-freeô label may also be placed on products in which gluten containing cereals have been replaced by other ingredients, and the total gluten content does not exceed 20 mg/kg. To be able to ensure proper processing practices and the safety of gluten-free foodstuffs, the gluten content of these products must be determined accurately so that they can be labelled accordingly. Indeed, full compliance to the current and upcoming EC regulation is only possible if accurate and reliable detection methods for gluten in foodstuffs are available. Immunochemical analysis is most applied, as this method of detection is easy to use and provides rapid results. At this moment, more than 15 immunochemical methods for detecting gluten are marketed commercially. However, none of these methods seems to have overcome all difficulties in accurate gluten detection in food products (Diaz-Amigo and Popping, 2012; Haraszi et al., 2011). The largest problems are encountered in the full extraction of gluten proteins from food matrices, the choice on what epitopes should be targeted by the detection methods, and the production of a standardized reference material.

This review aims to evaluate the currently available immunochemical detection methods and the requirements and unmet needs in gluten detection during the production of foodstuffs. Based on these differences, the possible role of a new multiplex immunoassay for the detection of gluten in food is investigated.

Gluten in food

Gluten is a group of storage proteins that are found in wheat, rye, barley and possibly oats, and crossbreeds thereof. Gluten proteins can be divided based on their structural properties or based on their aggregation state, as shown in Table 1 (Osborne, 1907; Thatam et al., 2000). The High Molecular Weight (HMW) prolamins and Sulphur (S)-rich prolamins in wheat gluten are responsible for the viscoelastic properties and structure of dough. Products with wheat, rye or barley or flours thereof include bread, pasta, cookies and beer, amongst many others. Because of their physical properties, wheat gluten are often added to other foodstuffs as thickener, emulsifier or flow agent to improve product quality (Day et al., 2006). This is sometimes referred to as óhidden glutenô, as they can appear in foodstuffs that are expected to be gluten-free such as lunch meats, soups and sauces. For CD patients, this means that it is not easy to simply avoid gluten and that every label has to be checked to determine whether or not a product is safe to eat. However, labels can be confusing. Ingredients such as óflavouringsô, óstabilizersô or óhydrolysed vegetable proteinô, can indicate that the product contains gluten. Even when a food product label does not list any cereal related ingredients, there is the risk that the product might be contaminated with gluten during processing. Contamination during the cultivation of cereals can also occur, as is often the case with oats (Størsrud et al., 2003; Thompson, 2004). For this reason, product manufacturers often choose to label their products with a ómay contain glutenô label. For CD patients such a label holds little value and restricts their food choices unnecessarily.

Toxic and immunogenic gluten peptides

The immune response in CD that follows gluten ingestion is triggered by specific epitopes; amino acid sequences that are encountered within gluten peptides. Typically, relevant CD

epitopes in gluten are rich in proline residues, which makes them resistant to most digestive proteases (Hausch et al., 2002) and causes them to reach the intestinal tract intact. Although the exact molecular mechanism remains unknown until today, it is generally accepted that gluten epitopes that remain intact after gastro-intestinal digestion can trigger two different pathways (Figure 1): an innate response that targets the epithelium directly (Jabri et al., 2005; Londei et al., 2005), and an adaptive response in the lamina propria that is mediated by CD4+ T-cells and involves the secretion of auto-antibodies (Jabri et al., 2005; Qiao et al., 2012). According to the definitions used by Ciccocioppo *et al*, gluten peptides are considered toxic when they are able to induce damage to the mucosa when added in culture to a duodenal mucosal biopsy or when administered to the proximal and distal intestine *in vivo*. Immunogenic peptides are able to specifically stimulate HLA-DQ2 or HLA-DQ8 restricted T-cell lines and T-cell clones from the jejunal mucosa or peripheral blood of CD patients (Ciccocioppo et al., 2005b).

Epithelial damage and villous atrophy are mediated by the innate response, in which the intraepithelial lymphocytes (IELs) play an important role (Jabri and Sollid, 2006). When epithelial cells become stressed, they will express stress signals, most importantly MHC class I molecules and HLA-E molecules. These molecules are recognised by natural killer (NK) receptors present on the IELs. Upon binding, they mediate enterocyte destruction (Hüe et al., 2004). Furthermore, interleukin-15 (IL-15) is released, which causes upregulation of the NK receptors (Meresse et al., 2004) and maturation of antigen presenting cells (APCs). The causing factors of epithelial stress are still under debate. Toxic gluten peptides are assumed to be triggers, though recent research showed that the innate immune response was mediated by amylase trypsin inhibitors from wheat, rather than the wheat gluten peptides themselves (Junker et al.,

2012). These amylase trypsin inhibitors activated Toll-like receptor 4. Other possible causing factors of epithelial stress have been suggested as well, including viruses (Zanoni et al., 2006) and gut bacteria (Nadal et al., 2007).

Gluten peptides that remain intact after gastro-intestinal digestion can be transported across the epithelium mostly by transcellular pathways (Heyman et al., 2012). Once these peptides reach the lamina propria, an adaptive immune response can develop as well. Intestinal damage causes the release of the enzyme tissue transglutaminase (tTG), which plays an important role in the adaptive immune response against gluten. tTG increases the affinity of the gluten peptides for HLA-DQ2 and HLA-DQ8 molecules on APCs through glutamine residue deamidation, thereby boosting the inflammatory process and increasing the subsequent damage (Dieterich et al., 1997). The APCs present the gluten peptides to CD4+ T-cells, stimulating them to release pro-inflammatory cytokines including interferon-ɔ (IFN-ɔ). IFN-ɔ stimulates apoptosis in enterocytes via the IELs and the lamina propria mononuclear cells (LPMCs) (Ciccocioppo et al., 2005a; Ciccocioppo et al., 2001; Di Sabatino et al., 2001). Furthermore, the activated CD4+ T-cells activate B-cells. B-cells can take up tTG-gluten complexes through endocytosis, and then present gluten peptides on their surface (Qiao et al., 2012; Sollid et al., 1997). After recognition of these peptides by the CD4+ T-cells, the B-cells are activated and transform into plasma cells. The plasma cells then start secreting antibodies against the gluten peptides and tTG-gluten complexes. The inflammatory responses are triggered continuously as long as gluten peptides are present in the gut, and will become stronger with every ócycleô. Only adherence to a strict gluten-free diet will lead to those processes fading out. Not all gluten peptides are equally harmful to CD patients, as the number of epitopes present in these peptides may vary

strongly between different cereals (Vader et al., 2003) and between cereal species (van Herpen et al., 2006). The known number of different toxic gluten peptides is limited (Ciccocioppo et al., 2005b; Silano et al., 2009). Currently most medical research focuses on identifying the immunogenic peptides that trigger a T-cell response. An immunogenic gluten peptide can only trigger an adaptive immune response if it can be presented to CD4+ T-cells by HLA-DQ2 or HLA-DQ8 molecules. It is for the same reason that absence of the HLA-DQ2 and HLA-DQ8 genes is considered an exclusion criterion for CD (Rostom et al., 2004). Once a peptide is identified as immunogenic, it is considered relevant for those with CD, regardless whether or not it has also toxic properties. Sollid *et al* have recently listed the immunogenic gluten epitopes relevant for celiac disease (Sollid et al., 2012). Table 2 presents these epitopes, as well as their immunogenicity and the number of patients sensitive to the epitope. The potential strength of the immune response triggered by these epitopes varies. Some of the epitopes are immunodominant, which means they can start a strong T-cell response. As indicated by the number of responding patients, immunogenic gluten epitopes are not equally harmful for all CD patients, regardless of their immunogenicity. This implies that some gluten peptides, possibly even some immunogenic gluten epitopes could be tolerated by CD patients, depending on the patientsô sensitivity to the different immunogenic epitopes. Of course, in this scenario it becomes very important to accurately detect which immunogenic gluten epitopes are present in foodstuffs. Both HLA-DQ2 and HLA-DQ8 have preferences for particular amino acids on certain positions in the gluten peptide sequences (Figure 2), causing them to bind some peptides better than others. A better binding of the gluten peptide results in a higher stimulation of CD4+ T-cells and is therefore important for the immunogenicity of the peptide (Tollefsen et al., 2006). For HLA-DQ2, a large

hydrophobic residue at position P1 and negatively charged amino acids at positions P4, P6 and P7 are important for the recognition and binding of the peptide (Kapoerchan et al., 2010). HLA-DQ8 prefers negatively charged amino acids at positions P1 and P9 (Qiao et al., 2009). Gluten peptides that are not toxic and bind poorly or not at all to HLA-DQ2 or HLA-DQ8 molecules, could possibly be tolerated by CD patients. Various research groups are attempting to decrease the gluten exposure for CD patients by removing or reducing immunogenic gluten epitopes in wheat by selective breeding (Mitea et al., 2010), genetic deletions (van den Broeck et al., 2009b) and RNA interference (Gil-Humanes et al., 2010). The reasoning behind these approaches is the increased occurrence of harmful gluten epitopes in modern wheat varieties, as compared to old varieties as a result of modern wheat breeding practices (van den Broeck et al., 2010). Other strategies to decrease the gluten burden include oral enzyme therapeutics that reduce the amount of immunogenic gluten peptides that reach the intestine (Tye-Din et al., 2010a) and tTG inhibitors that target the deamidation process of the gluten epitopes, making them much less immunogenic (Dafik et al., 2012). Considering these developments, detecting the total gluten content in foodstuffs becomes less important, as this gives no information on whether or not the gluten in the product are safe to eat for CD patients. This specifically holds when fractions are used that are enriched in certain gluten proteins, such as gliadins. A gluten detection method that only detects and quantifies the amount of gluten epitopes is then more appropriate. As to date only limited data on toxic gluten epitopes is available and medical research currently focuses mostly on immunogenic gluten epitopes, detection and quantification should also be based on immunogenic gluten epitopes. Our knowledge on immunogenic CD epitopes is still increasing and this can be considered to be an on-going process. Therefore, methods for the detection of

gluten should preferably be flexible so they can be kept up to date with our most recent knowledge on relevant epitopes.

Immunochemical detection methods

Several methods for the detection of gluten proteins are available today, including immunochemical methods, mass spectrometry and DNA based methods. The advantages and disadvantages of these methods have been reviewed recently (Haraszi et al., 2011). Immunochemical analysis is the most applied detection method in food production, as it is sensitive enough to detect gluten in the mg/kg range. Furthermore, it is relatively easy to use and provides results relatively rapid, allowing food manufacturers to check their products on the site (Schubert-Ullrich et al., 2009). The most commonly used immunochemical analysis methods are the enzyme-linked immunosorbent assays (ELISAs) and the lateral-flow assays (LFAs), the latter often referred to as dipstick tests. ELISAs are used for quantitative measurements such as routine screenings of foodstuffs, whereas LFAs give only qualitative or semi-quantitative results and are often used to evaluate cleaning procedures. Several ELISA and LFA test kits for gluten detection are commercially available, as shown in Table 3. They all detect epitopes within the prolamin group and use a correction factor to calculate the total gluten content. Currently, a correction factor of 2 is applied, in accordance with the Codex Alimentarius.

In the early 1990s, Skerrit *et al* developed a gluten detection method using an antibody against the heat-stable ɤ-gliadins; 401/21 (Skerritt and Hill, 1991). This method was approved by the Association for Official Analytical Chemists (AOAC) for quantifying wheat gluten concentrations in food. The 401/21 antibody recognises HMW glutenins as well. This glutelin recognition, in combination with the low amount and large variation of ɤ-gliadins present

amongst cereal species (Wieser et al., 1994), leads to an error-prone detection of the total prolamin content. The 401/21 antibody responds weakly to hordeins, leading to an underestimation of the total gluten content in foods containing barley (Thompson and Méndez, 2008). Although new methods like the R5 method were developed with the purpose of overcoming this problem, the 401/21 method is still commercially available.

The R5 method was developed in 2003 by Valdés *et al* and later validated by Mendez *et al* (Méndez et al., 2005; Valdés et al., 2003). This method is the type I method according to the Codex Alimentarius Commission, thereby replacing the ɤ-gliadin ELISAs as the CODEX approved method (Joint Food and Agriculture Organization of the United Nations/World Health Organization Food Standards Program). The R5 antibody targets the epitope QQPFP which is present in Ŭ-/ɓ-, ɔ- and ɤ-gliadins, as well as in hordeins in barley and secalins in rye. However, as stated before, the occurrence of epitopes varies with the type of protein and cereal. Care must be taken in selecting a standard, as the R5 method overestimates hordein in barley-contaminated foods unless a hordein standard is used (Kanerva et al., 2006). A competitive format of the R5 method was developed to detect smaller gluten peptides in hydrolysed products (Haas-Lauterbach et al., 2012).

Morón *et al* developed the G12 method by raising antibodies against the toxic 33-mer gluten epitope, important in the innate response in celiac disease (Morón et al., 2008b). Both a sandwich and a competitive ELISA format are available. The G12 antibody reacts with prolamins from wheat, rye, barley and some oat varieties that contain certain toxic gluten fragments. However, its sensitivity for oat is almost fiftyfold lower than for rye, and over a hundredfold lower than for wheat and barley (Morón et al., 2008a). As a result, the sensitivity for

oat is too low in the legally relevant concentration range. Furthermore, this cross-reactivity inhibits the detection of wheat, rye and barley contaminations in oat.

Koning and co-workers developed an ELISA method for detecting the T-cell stimulatory epitope RPQQPYP in Ŭ-gliadins from wheat (Spaenij-Dekking et al., 2004). The method also detects homologous epitopes in rye, barley and oats. As only Ŭ-gliadins are targeted, the results from this method are expressed as Ŭ20-gliadin contents.

Unmet needs in gluten detection

There are several problems with gluten detection that all currently available detection methods run into. First, there are differences in what researchers believe that should be detected by gluten detection methods. The G12 method focusses on a highly toxic gluten epitope, whereas the Glia-Ŭ20 ELISA detects one of the most immunogenic epitopes. Both state that it is more sensible to detect those gluten peptides that are most harmful to CD patients as this is most informative to those patients, yet they do not focus on the same peptides. The R5 method focuses on a sequence present in both toxic and immunogenic epitopes. All methods aim to quantify the total gluten content present in food, as required by the Commission Regulation 41/2009 to be able to qualify products as gluten-free. However, all methods use antibodies directed against a specific prolamin epitope. It is therefore possible that they miss other epitopes that add up in calculating the total gluten content and therefore lead to false negative results.

A second problem in gluten detection comes from the fragmentation of the gluten peptides. During certain types of food processing such as hydrolysis, gluten proteins are broken down into fragments. A part of these fragments does not contain the two necessary binding sites for a sandwich ELISA or LFA to detect them, causing a large underestimation of the total gluten

content of the product if a two-sided detection method is used. To overcome this error, one-sided, so called competitive ELISA formats have been developed recently. The competitive format is not compatible with all extraction methods, however, as the chemicals in extraction solutions can interfere with the specific binding of the antibody in these test kits (Mena et al., 2012). Therefore, most competitive assays rely on an ethanol solution for extraction and miss out on the use of reducing agents that are required to improve the extraction of gluten proteins from the processed food samples.

Third, extracting gluten from food matrices is a huge hurdle that is still not fully overcome. Gluten proteins are near-insoluble in water, but prolamins are solvable in ethanol and glutelins in dilute acid and alkali. Due to the difference in solubility characteristics of these different gluten protein groups (Table 4), full extraction remains troublesome. The currently available ELISAs therefore aim to extract and detect the prolamin group and apply a correction factor to calculate the total gluten content. However, the extraction procedure for prolamins often includes a step to reduce disulfide bonds which are formed during food processing, to improve prolamin extraction from food matrices. This step also improves the solubility of the glutelins in ethanol (Wieser, 2007). Antibodies that can also detect glutelins, such as the 401/21 antibody, therefore give a higher signal than antibodies that can only detect prolamins. By applying the correction factor to this higher signal, the total gluten content of the product is further overestimated. Wieser and Koehler have questioned the validity of using this one single correction factor before, because the ratio between prolamins and glutenins can significantly differ per type of cereal (Wieser and Koehler, 2009). Their adjusted correction factors range from 1.07 in einkorn to 1.71 in barley, meaning that the currently applied correction factor of 2

overestimates the total gluten content. Also, gluten is a group of proteins with a varying composition. Environmental factors have a big influence on gluten composition and total protein content in cereals, even within the same variety (Hamer and van Vliet, 2000). With this in mind, the application of one single correction factor for all different gluten compositions, in all types of cereals, seems to simplify too much. Especially when the focus is shifting from detecting gluten as a whole to detecting harmful gluten epitopes, it becomes essential that all epitopes ï at least the most relevant ones- are fully extracted from the food matrices so they can be detected and quantified without the use of a correction factor. A two-step extraction protocol for a highly complete extraction of gliadins and glutenins from wheat flour has been published, that could be used in gluten epitope quantification (van den Broeck et al., 2009a). However, it is not mentioned whether or not this protocol is also useful to extract hordeins, secalins, avenins and glutelins from other cereal flours or for heat treated products. Other food matrices may have additional extraction difficulties, as is the case with heated foodstuffs. Due to exposure to high temperatures gluten proteins can form strong aggregates. These changes lead to differences in solubility and thus extractability, which cause an underestimation of the amount of gluten detected (Bugyi et al., 2013; Hong et al., 2012). It is important to realise that as long as the gluten extraction methods for food matrices are flawed, gluten detection methods only provide a relative indication of the true gluten content in food.

A final problem with gluten detection is the lack of a standardized reference material. There is currently no certified reference material for gluten. Therefore, all available commercial ELISA kits contain their own set of standards for quantification of the gluten content. This can be gliadin, ógluten, or in case of the Ğ20 method, the Ğ20 gliadin peptide. Schwalb *et al* recently

compared different reference materials and found differences in both the crude protein content and the distribution of prolamin and glutelin fractions (Schwalb et al., 2011). Calibration of the same ELISA kits with different reference materials would thus lead to different results in gluten quantification. The Prolamin Work Group (PWG) has produced and characterized a gliadin reference material for gluten analysis with ELISAs (van Eckert et al., 2006). Although the material was not approved by the Institute for Reference Material and Measurements of the European Commission (IRMM), it is still used as it is the best characterized gliadin material to date. However, by using a gliadin standard as reference material, the quantification of prolamins from cereals other than wheat becomes less accurate. Also, detection methods using antibodies that recognise both prolamins and glutelins cannot be calibrated by a reference material containing gliadin alone, unless the prolamin/glutelin ratio of the samples is known. Another issue is that when native proteins, for instance gliadin, are used as reference material, the effect of food processing on the gluten epitopes is not taken into account (Doña et al., 2008). This includes deamidation processes. For that reason, the possibilities of using a reference material with gliadin present in a food matrix is being investigated (Bugyi et al., 2013).

All these factors together can cause a significant difference between the amount of gluten present, and the amount of gluten detected. An overestimation of the total gluten content might lead to an unnecessary restriction in foodstuffs for CD patients, while an underestimation might result in products begin labelled ógluten-freeô incorrectly and therefore putting CD patients at risk of gluten exposure.

New immunochemical detection methods

ELISA and LFA methods are aimed against prolamin peptides and, therefore, only detect and quantify the corresponding gluten protein group accurately. Techniques that allow for the simultaneous detection of multiple different peptides are available, and are making their way into the field of food allergen detection. These multiplex immunoassays combine antibodies against various peptides in a single detection method, thereby reducing time and sample size needed to analyse foodstuffs on multiple allergens. Several techniques are available to detect and quantify allergens, including biosensors using resonance-enhanced absorption (REA) or surface plasmon resonance (SPR), and flow cytometric assays such as the Luminex xMAP Technology. Biosensors make use of a chip on which antibodies against the target molecules are immobilized. They can also be used as a competitive detection method, in which case the antigens are immobilized on the chip so they can compete for antibody binding with the target molecules in samples. The REA technique uses antibodies labelled with golden particles. These antibodies are dispersed above a highly reflective mirror. When antigens bind to these antibodies and they are illuminated with white light, they will give a strong colour signal which can be measured spectroscopically (Mayer et al., 2001). SPR does not require the labelling of antibodies. Instead, this technique uses chips with a gold surface that reflects light. When the antibodies bind antigens, the refractive index value of the light changes. These changes can be measured by a variety of SPR sensors to quantify the amount of bound antigen (Homola et al., 2005). The Luminex xMAP Technology is a two-sided method that uses colour encoded beads instead of a chip. These beads are coated with antibodies that bind the antigens. In case of a competitive format, the beads are coated with the antigens. Two lasers detect both the colour of the bead for identification of the bead ï and therefore the detected target compound, and the amount of target

present in the sample (Vignali, 2000). An immunochip biosensor for the detection of the egg white allergens ovalbumin and ovomucoid, based on REA was developed by Maier *et al* (Maier et al., 2008). Although their method could detect both allergens in food matrices, some food matrices such as pasta gave false negative results. The authors stressed the importance of improving the extraction procedures to obtain more reliable results. Rebe Raz *et al* developed an on-chip direct iSPR-based method which detects several major allergens, including peanut, egg and several types of nuts (Rebe Raz et al., 2010). This group only encountered a minor effect of food matrices on the method performance, as they used an existing extraction method also used for ELISAs. The sensitivity of their method was comparable to that of an ELISA, though the process was much less time consuming. Haasnoot *et al* developed a Luminex-based immunoassay to detect soy, pea and soluble wheat proteins in milk powder (Haasnoot and Du Pré, 2007). The authors were able to quantify the three plant proteins accurately, provided that the treatment of the samples was known. This way, suitable calibration standards could be prepared under the same conditions.

There are some interesting possibilities in gluten detection with a multiplex immunoassay. Gluten peptides from both the prolamin and the glutelin groups have proven to be harmful for CD patients. A more reliable estimate of the total gluten content of a product could be determined by combining antibodies against prolamin and glutelin peptides in a single detection method. If cereal-specific prolamin antibodies could be raised and combined in a multiplex immunoassay, this would allow for more accurate gluten detection in products containing rye and barley. Full detection and quantification of all gluten proteins would be very beneficial for these products, as the ratio between prolamins and glutelins can differ greatly from

the ratio found in wheat (Wieser and Koehler, 2009). Application of the criticized single correction factor to calculate the total gluten content could be avoided in this manner. Another interesting possibility in gluten detection with the use of a multiplex immunoassay is to narrow down the focus even further to the harmful gluten epitopes. If antibodies were raised against the most relevant gluten epitopes, the detection of these specific epitopes could prove more relevant than detecting the total gluten content. A multiplex immunoassay can be updated by adding antibodies against more epitopes, and therefore can keep up with our increasing knowledge on harmful gluten epitopes. Also, by combining antibodies against the most relevant epitopes in a single detection method, the possibility of a false negative result decreases. Van den Broeck *et al* have investigated the possibilities of breeding a wheat variety with reduced CD-epitopes, based on small varieties in amino acid sequences between different gluten peptides (van den Broeck et al., 2011). If such a wheat variety could be bred, quantifying the total gluten content of food products containing this variety would be less appropriate. However, a detection method that can detect the presence of the harmful epitopes in these products would be very welcome.

The development of a multiplex immunoassay for the detection of gluten still involves some obstacles, comparable to those described above for the ELISA kits. The largest problems are to be expected in antibody specificity, extraction procedures and availability of a reference material. For the multiplex immunoassay, suitable antibodies for both prolamin and the glutelin peptides have to be developed, as well as cereal-specific antibodies. The cereal-specific antibodies should be raised against a sequence that is always present in these cereals, and preferably against an immunogenic epitope. Monoclonal antibodies are preferred, as these are more specific than polyclonal antibodies. This reduces the possibility of cross-reactions with

gluten-free cereals such as maize and rice. A high specificity will be required especially for the cereal-specific antibodies, as cross-reactions between gluten-containing cereals are very common. The used extraction procedure should be capable of extracting both prolamin and glutelin peptides from food matrices. As both groups have different solubility properties, this might mean that an extra extraction step will have to be implemented to extract all relevant peptides, as compared to the extraction method used by the ELISA detection kits that only need to extract the prolamin peptides. As with all immunochemical detection methods, a final difficulty will be to select an appropriate reference material. So far, creating a standard reference material for gliadin alone has proven to be challenging. For the multiplex immunoassay, reference materials for both gliadin and glutenin would have to be created, as well as references for the prolamins and glutelins in rye and barley. If the developed cereal-specific antibodies do not cross-react with each other, a mixture of a set amount of wheat, rye and barley peptides might be a feasible option.

In case of using the multiplex immunoassay to detect harmful epitopes, additional problems present themselves. Highly specific antibodies are vulnerable to miss gluten epitopes if these are modified by chemical treatments, for instance during the extraction procedure. During the development of the multiplex immunoassay, the intended extraction procedure should therefore be studied even more intensively. In this case, a reference material consisting of the targeted gluten epitopes distributed in a food matrix might be the most accurate representation. However, such a material will be difficult to standardize.

Conclusion

Although major developments in detection of gluten have been achieved over the last two decades, there is still a strong need for further improvements. Immunochemical detection is the most applied method in food production, as it is sensitive, rapid in providing results and relatively easy to use. To facilitate the detection of smaller gluten fragments, competitive ELISAs have been developed in addition to sandwich formats. However, none of the commercially available immunochemical detection methods are able to detect each of the two harmful gluten fractions; prolamins and glutelins. Problems concerning extraction methods still have not been fully overcome and, so far, no certified reference material is available. Developing a multiplex immunoassay to detect prolamin and glutelin peptides simultaneously seems to be the logical advancement in gluten detection. By also including cereal-specific antibodies, detection of the gluten content of products containing rye and barley would be greatly improved. This way, the use of the criticized single correction factor could be avoided, resulting in a more accurate detection and quantification of the total gluten content in food products. Furthermore, a multiplex immunoassay would be very useful in the direction we are currently heading; detecting those epitopes that are harmful to CD patients. A multiplex immunoassay could combine antibodies against multiple harmful epitopes in one detection method. It would give an opportunity to search for gluten-containing products that are safe to eat for CD patients. This would be greatly beneficial for the alternative CD treatments that are being investigated; treatments that focus on avoiding harmful gluten epitopes instead of gluten altogether. If the obstacles for developing a multiplex immunoassay can be overcome, this detection method would help providing consumers with more accurate food labels. This would further improve both food safety and the variety of choice in food products for CD patients everywhere.

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Table 1: Classification of gluten proteins based on structural properties and aggregation state

	Structure *(Tatham et al)*			**Aggregation state** *(Osborne)*	
	S-rich prolamins	*S-poor prolamins*	*HMW prolamins*	*Prolamins*	*Glutelins*
Wheat	Ŭ-gliadins ɔ-gliadins B-type LMW glutenins C-type LMW glutenins	ɤ -gliadins D-type LMW glutenins	HMW glutenins	Gliadins	Glutenins
Rye	ɔ-secalins	ɤ -secalins	HMW secalins	Secalins	Glutelins
Barley	ɔ-hordeins B hordeins	C hordeins	D hordeins	Hordeins	Glutelins
Oat	avenins*			Avenins	Glutelins

*Existence of subgroups uncertain.

S: Sulphur, HMW: High Molecular Weight, LMW: Low Molecular Weight.

Table 2[1]: Immunogenic gluten epitopes relevant in celiac disease

Name	Sequence (9)	Immunogenicity	Responding patients	Reference
DQ2.2-glut-L1	PFS**E**Q**E**QPV	+*	6/26	(Bodd et al., 2012)
DQ2.5-glia-α1a	PFPQP**E**LPY	+*	17/17	(Arentz-Hansen et al., 2000)
DQ2.5-glia-α1b	PYPQP**E**LPY	+*	9/15	(Arentz-Hansen et al., 2002; Mamone et al., 2012)
DQ2.5-glia-α2	PQP**E**LPYPQ	+*	13/17	(Arentz-Hansen et al., 2000)
DQ2.5-glia-α3	FRP**E**QPYPQ	+	4/20	(Vader et al., 2002)
DQ2.5-glia-ω1	PQQSFP**E**QQ	+*	7/20	(Sjöström et al., 1998; Vader et al., 2002)
DQ2.5-glia-ω2	IQP**E**QPAQL	+	5/20	(Qiao et al., 2005; Vader et al., 2002)
DQ2.5-glia-ω3	QQP**E**QPYPQ	+	9/13	(Arentz-Hansen et al., 2002; Qiao et al., 2005)
DQ2.5-glia-ω4a	SQP**E**Q**E**FPQ	+/-	7/13	(Arentz-Hansen et al., 2002; Qiao et al., 2005)

DQ2.5-glia-ɔ4b	PQP**E**Q**E**FPQ	+	8/13	(Qiao et al., 2005)
DQ2.5-glia-ɔ4c	QQP**E**QPFPQ	+	3/13	(Arentz-Hansen et al., 2002; Qiao et al., 2005)
DQ2.5-glia-ɔ4d	PQP**E**QPFCQ	ND	ND	Qiao, unpublished
DQ2.5-glia-ɔ5	QQPFP**E**QPQ	+	9/13	(Arentz-Hansen et al., 2002; Qiao et al., 2005)
DQ2.5-glia-ɤ1	PFPQP**E**QPF	+*	5/14	(Camarca et al., 2009; Tye-Din et al., 2010b)
DQ2.5-glia-ɤ2	PQP**E**QPFPW	+*	ND	(Tye-Din et al., 2010b)
DQ2.5-glut-L1	PFS**E**Q**E**QPL	+/-	3/20	(Vader et al., 2002)
DQ2.5-glut-L2	FSQQQ**E**SPF	+/-	4/20	(Vader et al., 2002)
DQ2.5-hor-1	PFPQP**E**QPF	+	4/8	(Vader et al., 2003)
DQ2.5-hor-2	PQP**E**QPFPQ	+/-	4/8	(Vader et al., 2003)
DQ2.5-hor-3	PIP**E**QPQPY	+*	ND	(Tye-Din et al., 2010b)
DQ2.5-sec-1	PFPQP**E**QPF	+*	3/8	(Vader et al., 2003)
DQ2.5-sec-2	PQP**E**QPFPQ	+*	3/8	(Vader et al., 2003)
DQ2.5-ave-	PYPEQ**E**EPF	-	3/8	(Vader et al., 2003)

1a				
DQ2.5-ave-1b	PYPEQ**E**QPF	+	2/8	(Vader et al., 2003)
DQ8-glia-ω1	**E**GSFQPSQ**E**	+*	2/2	(van de Wal et al., 1998)
DQ8-glia-ω1a	**E**QPQQPFPQ	+	8/9	(Tollefsen et al., 2006)
DQ8-glia-ω1b	**E**QPQQPYP**E**	+	1/2	(Tollefsen et al., 2006)
DQ8-glut-H1	QGYYPTSPQ	+	2/8	(van de Wal et al., 1999)
DQ8.5-glia-ω1	**E**GSFQPSQ**E**	+*	3/3	(Kooy-Winkelaar et al., 2011)
DQ8.5-glia-ω1	PQQSFP**E**Q**E**	+*	1/3	(Kooy-Winkelaar et al., 2011)
DQ8.5-glut-H1	QGYYPTSPQ	+	1/3	(Kooy-Winkelaar et al., 2011)

[1]Adapted from Sollid *et al* (Sollid et al., 2012). *: immunodominant; ND: no data. Glutamate residues formed by tTG deamidation are underlined.

Table 3: Commercially available immunochemical gluten detection kits

Test format	Antibody	Main epitope	Type of test	Test manufacturers*
ELISA	401/21	-	Sandwich	Diagnostic Innovations; ELISA Systems; ELISA Technologies; Neogen
	R5	QQPFP	Sandwich/ Competitive	BioControl Systems; Ingenasa; Neogen; R-Biopharm
	G12	QPQLPY	Sandwich/ Competitive	Biomedal Diagnostics; Romer Labs
	Glia-Ŭ20	RPQQPYP	Competitive	Europroxima
	pAb, not specified	-	Sandwich/ Competitive	Astori Lab; Diagnostic Automation; Immunolab GmbH; Incura; Morinaga Institute of Biological Science; Neogen
LFA	401/21	-		Diagnostic Innovations; ELISA Technologies; Neogen
	R5	QQPFP		R-Biopharm
	G12	QPQLPY		Incura; Romer Labs

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Table 1: Solubility of cereal proteins*

Cereal	Protein fraction	Ethanol	Dilute acid and alkali	Ratio
Wheat	Gluten	Gliadins (45-47%)	Glutenins (26-27%)	1.7-1.8
Rye	Gluten	Secalins (45-50%)	Glutelins (16-21%)	2.1-3.1
Barley	Gluten	Hordeins (31-36%)	Glutelins (36-39%)	0.8-1.0

*Percentages of the total protein content after Schwalb *et al* (Schwalb et al., 2012).

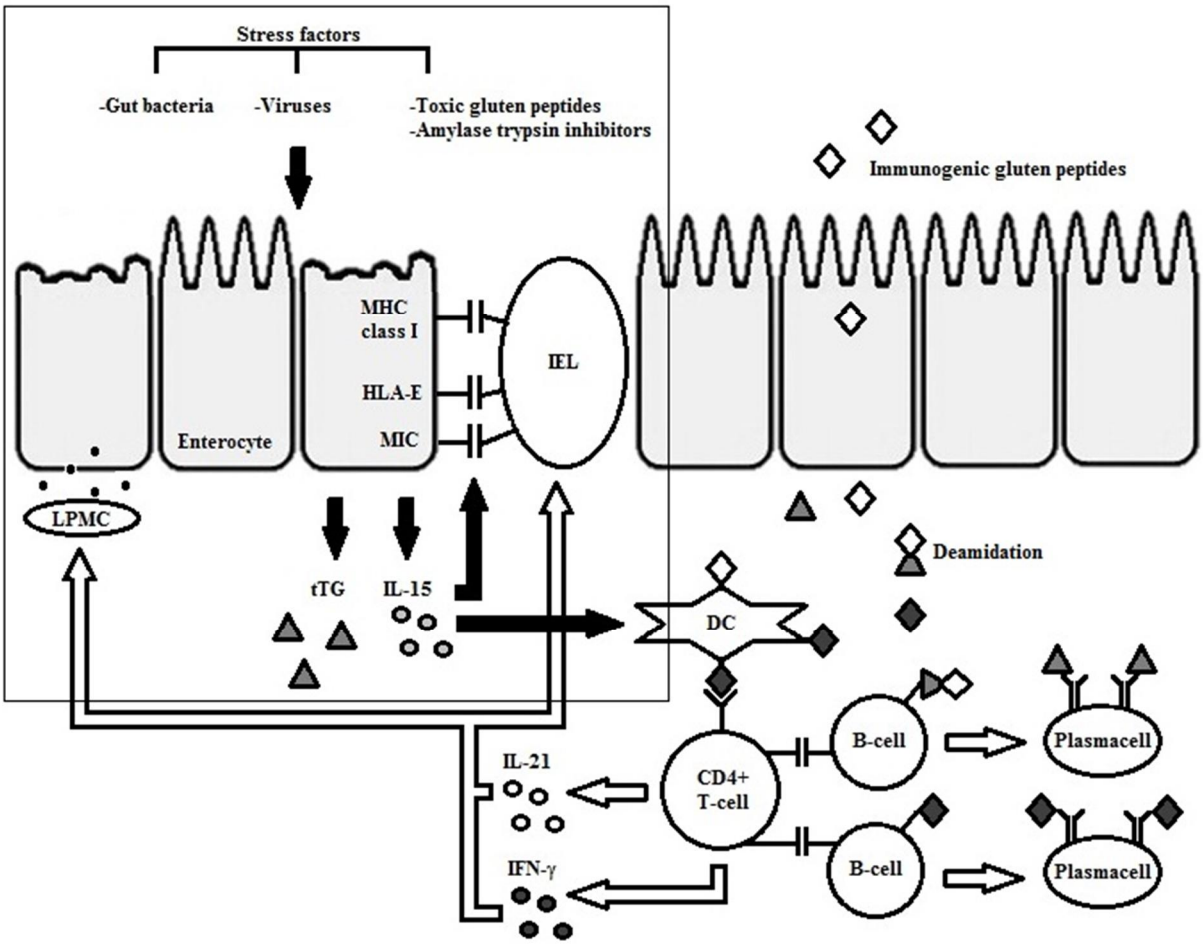
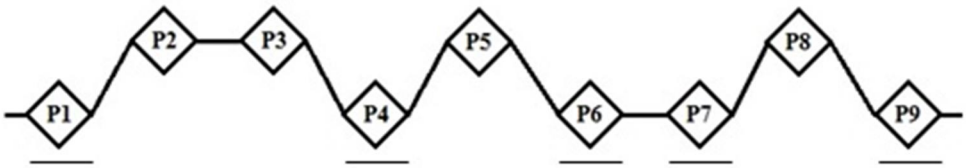


Figure 1: Pathways triggered by gluten peptides in celiac disease. The innate response is triggered by stress factors and includes activation of the intraepithelial lymphocytes (IELs), shown in the box. The adaptive response is triggered by immunogenic gluten peptides and includes the production of antibodies against gluten and tissue transglutaminase (tTG). DC: dendritic cell; LPMC: lamina propria mononuclear cell.



	P1	P4	P6	P7	P9
HLA-DQ2	P F, I, L E	E, D Q A	E P A	Q F, L, Y, W, A E, D	Q F, L, Y, W, A
HLA-DQ8	E, D Q F, L, M	A, I, V, M D	D A, I, Y, V R	D A, I, Y, V R	D P, G Q, N

Figure 2: Preferred positions (P) for amino acids in gluten peptides of HLA-DQ2 and HLA-DQ8. Data obtained from (Stepniak et al., 2008; van de Wal et al., 1996) and (Godkin et al., 1997)