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Classics in a new perspective: gluten as a special food safety and analytical challenge

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1. SUMMARY

In the last couple of decades, the nutritional role and perception of gluten became controversial. In one hand, gluten proteins play a central role in determining the baking quality of wheat and other cereals. On the other hand, hypersensitivity reactions triggered by gluten in susceptible individuals have become subjects of growing interest. Of these gluten-related disorders, with an estimated global prevalence of 1%, the most important one is celiac disease (CD), which is an autoimmune disorder accompanied by villous atrophy. CD can manifest in a wide range of symptoms, its only treatment option is a lifelong gluten-free (GF) diet. To support compliance to this diet, current EU legislation maximizes the gluten-content of products sold with a GF label in 20 mg/kg. It necessitates accurate quantification of gluten in this low concentration range. The method-of-choice for this purpose is the immunoanalytical-based ELISA (enzyme-linked immunosorbent assay). However, validation of different ELISA methods and the comparability of their results and, consequently, the reliability of the data they provide is problematic. The major goal of this paper is to introduce the analytical and protein chemistry issues behind this problem and the efforts to improve the conditions of the methodology. We are also including the special role of oats in the GF diet in an attempt to provide the widest possible overview of the food safety and analytical challenges represented by gluten.

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2. Introduction

Wheat and other cereals, such as rye, barley and oats, have long been staple foods in the human diet due to their significant contribution to our daily energy, protein and fiber intake and their high contents of certain vitamins and bioactive phytochemicals **[1]**. Gluten is a collective term referring to a protein fraction of wheat, rye, barley (and their crossbred varieties, such as triticale) that plays a very important role in the baking quality of these cereals **[2]**. However, these very same proteins (in certain cases alongside other protein types) are able to trigger different hypersensitivity reactions in susceptible people. Of these, the most important one is celiac disease (CD), with a global prevalence of 1%. CD is a chronic autoimmune disorder that appears in genetically predisposed individuals upon dietary gluten exposure. Due to the inflammation of the small intestine and the atrophy of the intestinal villi, CD can manifest in a range of symptoms, like malabsorption-induced nutritional deficiencies, anemia, gastrointestinal and other atypical phenomena (e.g. neurological disorders, infertility, etc.). Currently CD is incurable; its only treatment is a lifelong gluten-free (GF) diet **[3, 4]**.

To support CD patients in complying with their diet, it is mandatory for food producers to indicate the presence of gluten in their products, or the absence thereof if the product was specifically manufactured to meet the special needs of the celiac customer. According to current EU regulations, in line with the recommendation of Codex Alimentarius, products with a gluten content below 20 mg/kg can be labelled GF **[5, 6, 7]**. The law defines gluten as “a protein fraction from wheat, rye, barley, oats or their crossbred varieties and derivatives thereof, to which some persons are intolerant and which is insoluble in water and 0,5 M sodium chloride solution” **[6]**. Nevertheless, this definition does not do justice to the utter complexity of gluten. This complexity originates from the high number of protein subunits that gluten consists of and the genetic and environmental variability. The two major components of gluten are the alcohol-soluble prolamins (what gliadins, rye secalins, barley hordeins and oat avenins) and the alcohol-unsoluble glutelins (in wheat: glutenins). While gliadins are monomeric proteins, glutelins are large, aggregated biopolymers **[2, 8, 9]**. Celiac-toxic epitopes with various immunogenicity have been identified in both protein groups, with certain gliadin epitopes showing the highest levels of toxicity. In vitro and in silico methods indicate a high number of potentially toxic epitopes, but in patients, more than 90% of immune reactions are caused by a few so-called immunodominant epitopes. Besides the sheer number of epitopes, the estimation of toxicity is further hindered by the fact that the number of these epitopes may vary within and across cereal species **[10, 11, 12]**.

Monitoring the compliance of food products sold as GF with the 20 mg/kg threshold requires such analytical methods that are able to quantify gluten accurately and reliably in this low concentration range. There are several suitable methodologies including PCR (polymerase chain reaction) or liquid chromatography coupled to mass spectrometry. However, the method-of-choice in routine analysis is the ELISA (enzyme-linked immunosorbent assay) which is based on the formation and detection of a specific antigen-antibody complex. The advantages of the method are its specificity, sensitivity, ease of use and relatively low cost. While gluten quantification has no reference methods, Codex Alimentarius recommends ELISA as the method to be used for this purpose **[5, 13]**.

Because of the lack of reference methods, more and more ELISA assays appeared and the advent of the methodology brought along a number of studies drawing attention to the fact that different ELISA methods may provide different results when analyzing the same sample, which became an important reliability issue **[14, 15, 16]**.

3. Specific problems of gluten analysis

The variability of gluten ELISA results is caused by several intertwined factors that affect method development and validation, as depicted in ***Figure 1***.

The core of the problem is in part the pathomechanism of CD and the complexity of gluten proteins. As described in the Introduction, celiac disease can be triggered by a high number of epitopes located on a number of protein subunits. The amount of these epitopes expressed in different cereal species and cultivars strongly depends on genetic and environmental factors. This is one of the reasons of the lack of reference methods and reference materials, which makes method development and validation very difficult. These issues together lead to the variability of methods, which finally peaks in the observed variation of the analytical results. Major elements of this methodological variability are the applied antibodies, the sample preparation steps and the calibrating materials. Beside these, we must also keep in mind the effects of complex matrices and food processing procedures on protein structure and solubility, which may further increase measurement uncertainty through modifying the extractability and immunoaffinity of proteins. Overcoming these obstacles requires an urgent harmonization of gluten analytical methods of which a key element is the development of suitable reference materials (RMs) **[17, 18, 19, 20, 21]**.

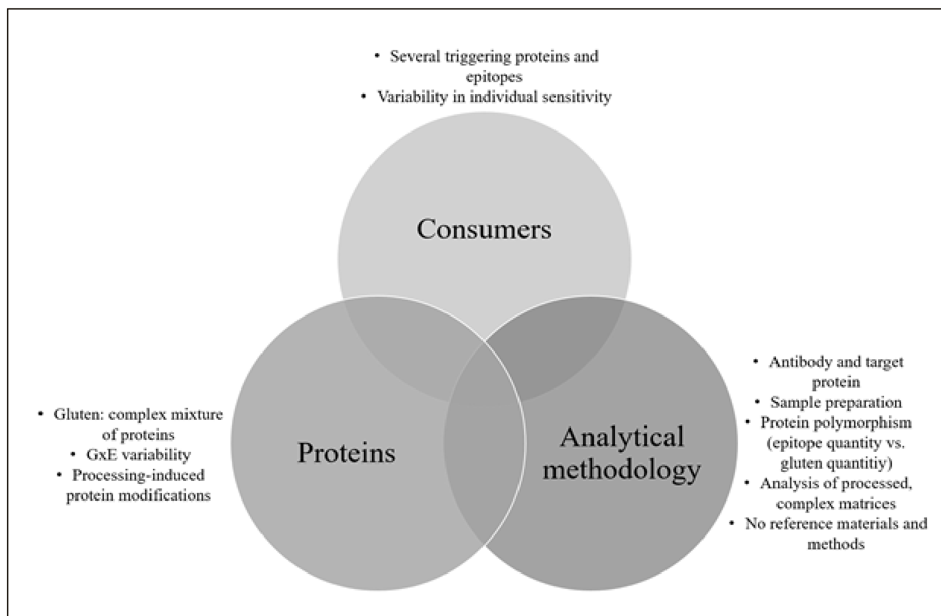

Figure 1. Factors affecting gluten quantification (edited by the authors)

4. Reference material development efforts for the quantification of gluten

The problem of gluten reference materials has been in the focus of the researchers of the field for more than 20 years, but a universally accepted, certified reference material is still out of reach **[17, 22]**. An important milestone of the reference material development was the creation of the so-called PWG (Prolamin Working Group)-gliadin. PWG gliadin was isolated from a mixture of the 28 most common European wheat varieties. It is a high-purity, very well characterized material, and is commonly used as an ELISA calibrating material to this day. However, it could not obtain the status of a certified RM due to uncertainties about the reproducibility of its production and its long-term availability **[17, 23]**.

Our research group joined the RM development efforts in 2008 as a member of the Allergen Working Group of the EU FP6 MoniQA Network of Excellence (FOOD-CT-2006-036337). In the first phase of our work, we developed such RM candidates that contained a known amount of gluten in a processed food matrix ***(Figure 2)***. As a gluten source, we used the above-mentioned PWG gliadin **[24, 25]**. This RM candidate was subsequently used in a number of experiments that aimed to identify the factors that act as sources of error in ELISA and thus contribute to analytical uncertainty **[14, 19, 26, 27, 28]**.

Figure 2. Gluten reference material candidate model product

As the questions of the disadvantages of PWG gliadin emerged, the Reference Material Working Group, coordinated by our research group under the auspices of the legal successor of the MoniQA project, the MoniQA Association (https://www.moniqa.org), contemplated that the development of a brand new reference material is necessary. The Working Group decided to start the work from scratch and to rethink this problem from the basics. For this, two questions had to be answered. The first one was which wheat variety or varieties are the most promising to represent a global sample population considering genetic and environmental variability. The other one was the question of the format of gluten in the new RM: should it be a flour, a gluten isolate of a gliadin isolate. To answer these questions the Working Group collected 23 commercial wheat varieties from a number of countries and continents. The samples were thoroughly characterized with a complex analytical methodology (including chemical composition, two gluten ELISAs and separation techniques). Based on the results a set of selection criteria including both qualitative and quantitative elements was created that we used to choose five such wheat cultivars that we deemed suitable to be used in a reference material **[29, 30]**. Then, flours, gluten and gliadin isolates made from the five varieties and their mixture were further analyzed in terms of protein composition with no significant differences found. As a conclusion of this work, we chose the flour of the mixture of the five varieties as a new RM. Its application is supported by its easy preparation that is also feasible in pilot plant conditions without changes in quality and that it contains not only gliadins but every other protein types as well **[31, 32]**. This is particularly advantageous because a common criticism towards gluten ELISAs is that they usually provide their results in gliadin units that are recalculated to gluten using a multiplying factor of two. This approach comes from the theory that the prolamin to glutelin ratio in gluten is 1:1. However, a growing body of evidence suggests that this ratio can be very different, which may also cause inaccuracy when calculating the results **[33]**. This explains that the latest method developments are moving to the direction of using several antibodies simultaneously to be able to detect not only prolamins but glutelins as well **[34]**. The flour reference material that is now available for analytical applications through the MoniQA Association fits this approach well.

Therefore, in case of wheat, a considerable progress occurred in the RM development. However, not only wheat but rye and barley also triggers celiac disease. While there is a lot less information about these cereals in this context, the available studies indicate that gluten antibodies show different affinity towards rye and barley prolamins. This may lead to under- or overestimation of the gluten content of samples with rye or barley contamination that makes it necessary to create new reference materials developed specifically for rye and barley **[35, 36]**.

By recognizing this demand, our international research group is now working on the repetition of the experiments described for wheat, this time for rye and barley. So far, we have collected more than 120 barley and more than 50 rye samples and analyzed their chemical composition, gluten content as per ELISA and protein content and composition determined by separation techniques. We used the results for setting up new selection criteria. The selected seven rye and eight barley cultivars are currently being analyzed [publication underway]. The expected outcome of this work is the development of new rye and barley RMs that independently or in combination with each other and the wheat material could help to improve the conditions of gluten analysis.

5. A short but important detour: oats and the gluten-free diet

The previous sections covered the analytical aspects related to wheat, rye and barley. In the celiac context we must also involve oats and its controversial role in the gluten-free diet. An improper GF diet may be accompanied by nutritional problems such as the reduced intake of fibers, vitamins and minerals, an increase in saturated fatty acids and a higher glycemic load **[37]**. These disadvantages could be counterbalanced by the ingestion of oats due to their high fiber and antioxidant, and relatively high unsaturated fatty acid content **[38]**.

Oats are generally considered safe for celiac patients because they contain significantly less prolamins than wheat, and contrary to the analogous proteins of wheat, oat avenins are less resistant to digestive enzymes **[39]**. The vast majority of clinical studies dealing with the capacity of oats to trigger CD also conclude that oat consumption in moderate amounts (20-25 g/day for children, 50-70 g/day for adults) is safe for celiac patients in remission **[40, 41]**. However, some other studies found that in certain cases, oats can pose a risk for celiac consumers and, while only a low amount, but some oat avenin epitopes were found to be able to induce CD. It is also important to note, that genetic and environmental variability is also present for oats, which can affect the presence of potentially toxic epitopes. Thus, it becomes necessary to screen the presence of toxic epitopes in oat varieties **[42, 43, 44, 45]**.

This controversy also appears in international law. While Australia and New Zealand explicitly rejects the inclusion of oats in the GF diet [46], in the EU, the US and Canada it is permitted to introduce the so-called "pure oats" specifically produced for CD patients in the diet **[6, 47, 48]**. The issue of pure oats production is of paramount importance as different studies found that 13-88% of commercial oat products are contaminated with gluten to various extents. Contamination may occur at any step of the production chain **[49, 50]**.

Pure oat production must be handled with exceptional care and is built on two pillars. In one hand, it must be ensured that seeds do not contain toxic epitopes, which requires pre-screening of oat varieties and the development of a suitable analytical methodology **[51]**. In the past couple of years, our research group got involved in these tasks **[52, 53]**. On the other hand, gluten contamination must be avoided in the entire production process. This requires serious efforts and compliance to special protocols that aim for the complete elimination of the risk of gluten contamination (e.g. confirming seed purity, safety lanes between land plots, the application of dedicated machinery and tools, segregated storage and processing, etc.) ***(Figure 3)*** **[54]**. The detection of the presence of unwanted gluten in oats is yet another analytical challenge. While ELISAs using the R5 antibody are suitable tools for this purpose, because they do not cross-react with oats but they do recognize wheat, rye and barley (with the limitations described earlier), in case of oats a specific difficulty is that only a few contaminating grains can pose a health risk. To mitigate this risk, sampling protocols developed specifically for oats have been established **[55, 56]**.

In conclusion, the role of oats in the gluten-free diet keeps being a matter of debate to this day. While the nutritional benefits of oat consumption are beyond doubt, the safety of oats must be further assessed in clinical trials. Another important task is the improvement of pure oats production protocols and the related analytical methodologies.

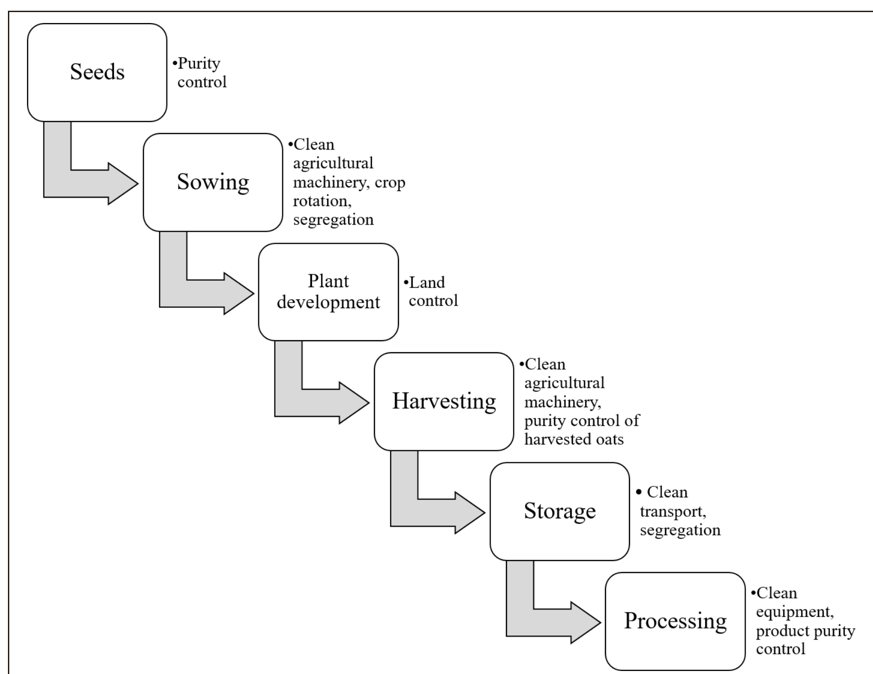

Figure 3. Major steps of a pure oats production protocol ***[57]***

6. Summary

Gluten proteins have long been an integral part of our diet through the consumption of cereals, they are very important in determining the quality of a range of staple foods, thus they deserve to be in the center of cereal science for a long time. In the last couple of decades, they also came to the forefront due to their connection to diseases such as celiac disease, non-celiac gluten sensitivity or wheat allergy. In this paper, we focused on disorders treated by a gluten-free diet, primarily celiac disease, to demonstrate the special food safety and analytical challenges represented by gluten.

While we have at our disposal a relatively large repertoire of analytical methods, it is very important to be aware of their possibilities and limitations, especially in case of the routinely used ELISA. Some of these limitations can be at least partially eliminated or improved with new findings of protein chemistry, immunology and clinical studies, which corroborates the need of the continuation of the research efforts presented in this article. However, others will always be present due to the innate characteristics of the methodology, which makes it necessary to create new analytical solutions, of which a good example is the quick evolution of proteomics **[58]**.

Consequently, the handling of gluten as a food safety problem requires a multidisciplinary approach. It needs the close cooperation of clinical research, lawmakers, food producers, food analytics and a range of other areas. Our research group integrated in this system through the improvement of the conditions of gluten analysis with the goal of making gluten quantification more reliable and as a result of that, to contribute to the safety and better quality of life of people living with celiac disease.

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