White Paper : The Current State of Scientific Knowledge about Gluten

April 27, 2018

Published by:

Allergen Control Group Inc.

420 Main Street East, Unit 553

Milton, Ontario

Canada L9T 5G3

www.glutenfreecert.com

info@glutenfreecert.com

The Canadian Celiac Association would like to thank Agriculture and Agri-Food Canada for funding this project as well as the Allergen Control Group Inc. and EnvironeX Group which led and contributed to this document.

Table of Contents

Introduction 2

1. Gluten—A Complex Group of Cereal Proteins 4

1.1 Definition 4

1.2 Protein Classification in Gluten-containing Cereals 4

1.3 Gluten-containing Cereals in the Food Industry 6

1.4 Gluten-free Replacements 6

1.5 Effects of Processing on Gluten Proteins 6

2. Gluten-related Disorders 8

2.1 Celiac Disease 8

2.2 Dermatitis Herpetiformis 9

2.3 Gluten Ataxia 9

2.4 Wheat Allergy 10

2.5 Non-celiac Gluten Sensitivity 10

2.6 Epidemiology 10

3. Regulations 11

3.1 Overview of Worldwide Regulations 11

3.2 The 20 ppm Limit Establishment 15

3.3 Effects of Environmental Conditions and Cross-contamination 16

3.4 The Specific Case of Gluten-free Oats 17

4. Analytical Techniques for the Detection and Quantification of Gluten 18

4.1 Immunochemical Techniques 19

4.2 Genomics-based Methods 24

4.3 Proteomics Approach 25

4.4 Non-specific Surface Testing Method 26

4.5 Emerging Technologies in Gluten Detection 27

Conclusion 28

Glossary 30

References 33

APPENDIX 1 - Comparison of commercially available ELISA test kits i

Introduction

Over the past decade, demand for gluten-free products has skyrocketed. More and more products labelled gluten-free have appeared on store shelves. The many people who suffer from celiac disease or other gluten-related disorders benefit from this wider range of products because, for them, a strict gluten-free diet is essential to avoid aggravating their condition. However, these products must be sufficiently safe and reliable to be truly gluten-free and meet regulatory specifications. A wide range of testing methods for detecting and quantifying gluten are also on the market. It is often difficult for manufacturers of gluten-free products to select a reliable testing method that meets their needs.

This white paper is a source of detailed information on the current state of scientific knowledge about gluten. It is more specifically intended for all people working in the gluten-free products industry who want to learn more about the topic. The vocabulary used is sometimes technical, so there is a glossary at the end of the document.

The first section of the document covers the current scientific knowledge on the chemistry of gluten, a highly complex group of proteins. This section explains the challenges of accurately detecting and quantifying it. Solutions for replacing cereals containing gluten will also be covered in this section as well as the effect of transformation processes on gluten proteins.

The next section covers the main gluten-related health disorders. Gluten proteins may provoke different types of physiological reactions and a wide range of symptoms in susceptible people. Gluten-related diseases and conditions include celiac disease, dermatitis herpetiformis, gluten ataxia, wheat allergy, non-celiac gluten sensitivity and others. Celiac disease or gluten enteropathy (1) is the best-known gluten-related health disorder. It is estimated that approximately 1% of the population is affected by celiac disease. Currently, the only treatment for this disease is to maintain a strictly gluten-free diet.

Then, an overview of the various regulations worldwide is included, more specifically regulations in Canada, the US, the European Union and New Zealand/Australia. It is important to know that these regulations are primarily based on the standards of the Codex Alimentarius, which defines gluten-free food as special dietary food composed of ingredients that do not contain wheat, rye, barley, oats and their cross-bred varieties, and in which the gluten content does not exceed 20 ppm. However, according to the Codex Alimentarius Commission, allowance of gluten-free oats, i.e., oats that have not been contaminated by wheat, rye or barley, needs to be determined nationally.

The final section of this white paper presents the various testing techniques for detecting and quantifying gluten available on the market. Several tests have been developed, for example immunoassays, methods based on DNA detection and genomic approaches that use mass

spectrometry. The selection of an appropriate testing method depends on several factors such as the matrix, processing steps, time, costs, knowledge and the accuracy of results (quantitative results, qualitative results, identification of the source of contamination). This section will help readers understand the important concepts related to selecting the optimal testing methodology depending on the type of matrix and/or the procedure.

1. Gluten—A Complex Group of Cereal Proteins

This section summarizes current scientific knowledge on gluten chemistry. A good understanding of the various concepts in this section makes it possible to link the several issues related to testing techniques for detecting and quantifying gluten.

1.1 Definition

Gluten is defined by the **Codex Alimentarius** as the "protein fraction from wheat, rye, barley, oats or their cross-bred varieties and derivatives thereof, to which some persons are intolerant and that is insoluble in water and 0.5M NaCl" (2).

Gluten includes the different species of wheat (e.g. durum, spelt, farro, Khorasan wheat, einkorn, emmer, etc.), rye and barley as well as the cross-bred hybrids of these cereals (e.g. triticale, which is a cross between wheat and rye).

1.2 Protein Classification in Gluten-containing Cereals

Gluten is a complex mixture of storage proteins commonly divided into two different subgroups: **prolamins** and **glutelins** (3). **Prolamins** are the protein fraction that can be extracted using 40–70% ethanol, while **glutelins** are the protein fraction soluble in alkali or acid solutions. Depending on the source, **prolamins** and **glutelins** are found in different forms in gluten (Table 1). Other protein fractions are also found in cereals, as illustrated in Figure 1. For example, **albumin**, which is water-soluble, and **globulins**, which are salt-soluble (4).

Table 1 : Protein fractions of gluten according to the variety

Variety	Prolamins	Glutelins
Wheat	α/β-gliadins γ-gliadins ω-gliadins	LMW[1] glutenin HMW[2] glutenin
Rye	γ-40 secalins ω-secalins	γ-75 secalins HMW[2] secalins
Barley	C-hordeins γ-hordeins	B-hordeins D- hordeins
Oats	α/β-avenins γ-avenins	LMW[1] avenins

1. LMW: low molecular weight 2. HMW: high molecular weight

Figure 1 provides a general portrait of the protein composition of the various cereals containing gluten. It shows that the composition of the different protein fractions varies greatly between cereals but also between varieties of the same type. In general, **prolamins**

and **glutelins** are estimated to occur in about the same ratio in gluten based on the typical composition of wheat. Therefore, a multiplication factor of two is often used to calculate the total gluten content in the ELISA methods. However, this ratio varies depending on varieties, genetics, growing conditions and environmental influences (4).

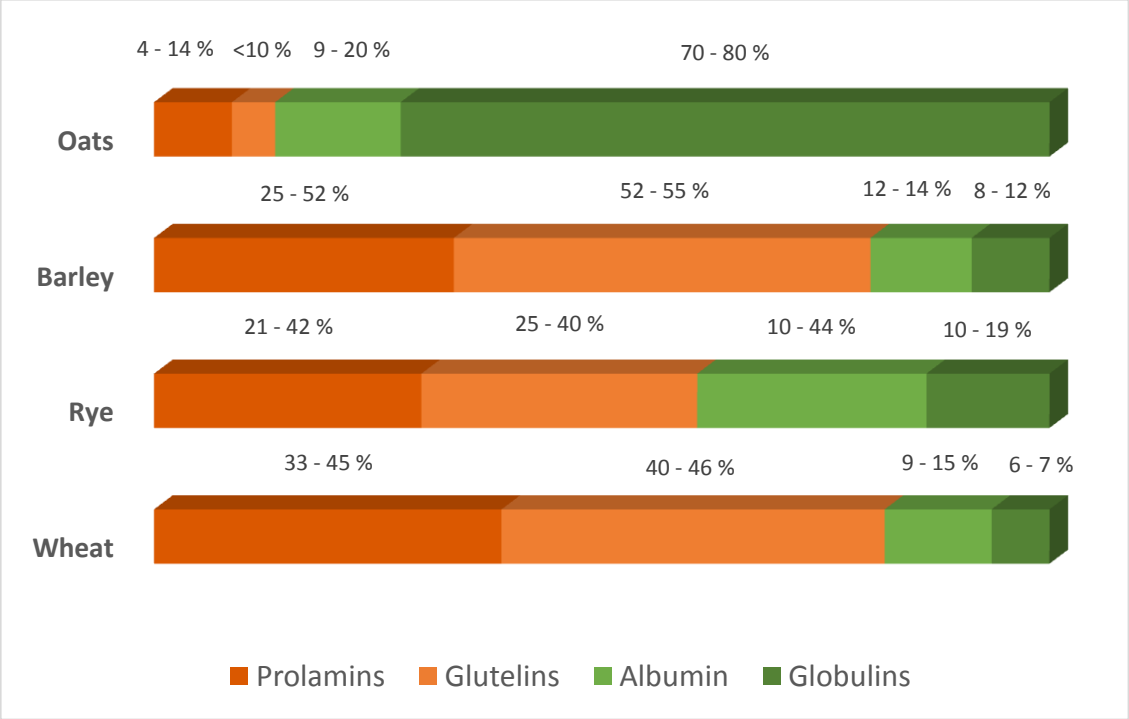

Figure 1. Protein composition in cereals containing gluten

Gluten protein fractions are not all harmful for people with celiac disease. The **peptides** that are responsible for triggering celiac disease are **prolamins** and **glutelins.** In Figure 1, these groups of proteins are represented by the colour orange. The protein fraction that participates in triggering celiac disease therefore varies greatly depending on the source. However, it is important to note that **albumin** and **globulins** (in green), can be harmful for people with wheat allergy, because the triggering mechanisms for these two diseases are different.

Gluten proteins can also be distinguished based on their sulphur content, structural properties or size (5). For example, monomeric **prolamins** contain intramolecular disulphide bonds, weak hydrogen bonds and are easily soluble in aqueous alcohols (6). However, polymeric **glutelins** also contain intermolecular bonds, which allow them to form a network when cooked or baked (7). The use of reducing agents, acid or **enzymes** is thus necessary to break disulphide bonds before their solubilization in alcohol solutions during the **extraction** procedure. This is why cocktail solutions are sometimes used.

Lastly, the variations observed in the protein composition of the various sources of gluten make their detection and quantification challenging. For more details on this topic, please see section 4, which covers testing methods.

1.3 Gluten-containing Cereals in the Food Industry

Gluten proteins are widely recognized for their technological properties. Wheat gluten can form a viscoelastic dough crucial in the bread-making process, whereas **prolamins** of other cereal species lack this property (7). This unique characteristic can be attributed to the polymeric fraction of wheat, which allows gluten to form a complex network. Wheat proteins are also used as thickeners, emulsifiers, polishing agents and flavour enhancers in multiple foodstuffs such as sauces and soups (5). Furthermore, it is used as a filler in meat and fish products, desserts, drugs, carriers of spice, flavouring extracts and concentrates. Wheat flour is often used as a substrate of **enzymes**, bacterial cultures and yeast. Hydrolyzed and fermented **peptides** from rye, barley and wheat are also found in many manufactured products such as beer, distilled alcohol, vinegar, baby food, soy sauce and glucose syrup. (8)

1.4 Gluten-free Replacements

Amaranth, buckwheat, corn, millet, quinoa, rice, sorghum, soybeans, teff and other gluten-free replacements do not contain gluten proteins. Their proteins differ in their **amino acid** composition, as they contain less **prolamin** and **glutelin** proteins. This makes these replacements harmless to people with celiac disease or those with gluten intolerance and suitable to use in gluten-free formulations in combination with starchy ingredients such as potato or tapioca starch.

1.5 Effects of Processing on Gluten Proteins

Gluten proteins are often modified to increase their use in different applications, which can also affect protein conformation without reducing celiac effect (5). For example, baking and cooking make gluten proteins more difficult to extract, as glutenin proteins form new disulphide bonds and aggregates. These changes result in lower gluten protein solubility and therefore lower detection rates if the extraction protocol is not modified.

Some ingredients such as wheat starch, wheat starch hydrolysates (e.g. glucose syrup) (9), sugar alcohols (e.g. sorbitol, maltitol) and wheat maltodextrin can be made gluten-free using certain special processes (10). Similarly, alcohol and vinegar distillation usually enables the elimination of any residual harmful proteins (11). During distillation, liquid is heated and volatile components like alcohol and flavours are separated from non-volatile materials like proteins and sugars. The residual products are usually safe for people with celiac disease, but an inadequate distillation process could have an impact on the purity of the final product.

There is also the increased potential of cross-contamination in a gluten-rich environment. Total protein content of the distilled products should be tested to verify its purity.

Fermented products (e.g. beer) and hydrolyzed products (e.g. hydrolyzed wheat proteins, flavor enhancers) may not be suitable for people with celiac disease. During the **fermentation** and **hydrolyzation** processes, proteins are broken into smaller fragments, which makes them difficult to detect and quantify. Other processes such as **deamination** and **transamination** can also affect the conformation of proteins and make them difficult to detect (5). Special attention should therefore be given to the testing method and the interpretation of the test results for these types of products (see section 4).

2. Gluten-related Disorders

Cereal containing gluten can trigger different types of immune responses in susceptible individuals. This section covers the best-known gluten-related disorders including celiac disease, wheat allergy, non-celiac gluten sensitivity, dermatitis herpetiformis and gluten ataxia.

2.1 Celiac Disease

Celiac disease is a chronic small intestinal immune-mediated enteropathy induced by the ingestion of gluten proteins in genetically predisposed individuals (12). The immune reaction causes typical lesions characterized by a flattening of the villi in the small intestine that leads to a generalized inflammation and malabsorption of nutrients.

Typical signs and symptoms of celiac disease vary greatly from one person to another both in extent, time and severity. Additionally, the symptoms of celiac disease are often similar to symptoms of many other disorders, making diagnosis difficult. Infants and children usually display symptoms of diarrhea and abnormal stretching of the abdomen. They may also show symptoms of malnutrition such as short stature, anemia, dental defects, failure to thrive or developmental delay. In adults, gastrointestinal complaints are common and include abdominal pain, flatulence and diarrhea. Weight loss is common, but symptoms of weight gain and constipation are not unheard of. Further symptoms vary and can include mouth ulcers, extreme fatigue, bone pain and others. Only certain individuals with celiac disease suffer typical gastrointestinal symptoms, while others may display no visible symptoms. These people will present with positive serology results and typical intestinal damage, but they will not show any symptoms of the disease for a long period of time. However, long-term malnutrition may lead to more severe disorders such as bone diseases (e.g. osteoporosis), growth delay in children, infertility and predisposition to other diseases (e.g. type 1 diabetes, thyroiditis, Sjogren's syndrome and cancers) (13). Celiac disease is linked to gluten sensitivity, and biopsy (sampling and testing) is usually performed to confirm diagnosis.

W. K. Dicke, a Dutch pediatrician, first established the link between the disease and the consumption of wheat gliadin in the 1950s. He suggested treating patients with a lifelong gluten-free diet (12). Soon after, barley, rye and oats were also suggested to be harmful due to their close relationship to wheat (5).

Since that time major progress has been made to explain the pathogenesis of celiac disease. It is now recognized that two genes (HLA-DQ2 and HLA-DQ8) are mainly responsible for the immune response to gluten **peptides** (13). Due to their high proline (P) and glutamine (Q) content, gluten proteins are particularly resistant to digestive **enzymes**. In people affected by

celiac disease, tissue transglutaminase-2 **antibodies** (tTG2) convert the glutamine residue in gluten **peptides** into glutamic acid, which triggers a chain reaction leading to the atrophy of the mucosal villi (5). However, this response cannot fully explain tissue damage, since about 30% of the general population carries these genes without developing the disease (14). Environmental factors, abnormalities in the immune system and genetic syndromes may also influence the incidence of celiac disease (15).

The immunological reactions to celiac disease are most likely due to **antibody**-binding **epitopes** with a short toxic **amino acid** sequence (16). Many **peptides** were found to be active in celiac disease (17), and their presence in gluten proteins was studied. The 33-mer, a **peptide** that contains 33 **amino acids** (LQLQPFPQPQLPYPQPQLPYP-QPQLPYPQPQPF), is considered the most important contributor to celiac disease (5). Many studies have tested its harmfulness in people with celiac disease. However, this **peptide** does not explain the overall pathogenesis of the disease, and hundreds of other **peptides** from wheat, rye and barley proteins may be involved in celiac disease (4). Most peptides seem to contain a large amount of proline (P) and glutamine (Q) residues, which give them their specific ability to protect **epitopes** from digestive **enzymes** and improve the effectiveness of binding sites with **antibodies**. Recently, glutenin fractions of wheat were also found to be harmful for people with celiac disease (18).

2.2 Dermatitis Herpetiformis

Dermatitis herpetiformis is an autoimmune skin disease related to the exposure of gluten in genetically susceptible individuals. Gluten triggers the production of immunoglobulin A (IgA) **antibodies**, which leads to an immune reaction in the bloodstream (19). The manifestation on the skin is characterized by itchy lesions, papules and vesicles mainly located on the surface of the elbows, knees, buttocks and back of the neck (15). People affected by this disease also present with lesions on their small intestinal villi but do not usually present with digestive symptoms. Patients need to adhere to a strict gluten-free diet (20) to prevent the appearance of skin lesions. A skin biopsy (sample and test) is generally performed to confirm the diagnosis.

2.3 Gluten Ataxia

Gluten ataxia is a rare and serious auto-immune disease triggered by the ingestion of gluten in genetically susceptible individuals (21). Gluten ataxia is similar to celiac disease but instead of affecting the gut, it affects the brain and central nervous system. More specifically, **antibodies** produced in response to gluten ingestion attack the cerebellum, eventually causing irreversible effects such as difficulties with balance, loss of coordination, fatigue, and speaking and swallowing difficulties (22). To date, this neurological disorder is not well

understood by experts, and diagnosis can be difficult as affected people do not present with gastrointestinal symptoms (23). However, they must adhere to a strict gluten-free diet.

2.4 Wheat Allergy

Wheat allergy is an immunoglobulin E (IgE)-mediated immune disorder triggered by the ingestion of wheat proteins. The reaction is not only due to gluten proteins, but also **albumin** and **globulins**. When wheat is ingested, the body's immune system becomes sensitized and overreacts by producing IgE **antibodies** against wheat proteins, which causes the release of inflammatory molecules (24). The reaction leads to a range of symptoms that may rapidly progress from mild (e.g. rashes, hives, itchy skin, swelling, cramps, diarrhea, vomiting) to severe (e.g. breathing problems, drop in blood pressure, loss of consciousness) (24). Allergic reactions to food usually occur within a few minutes or hours of ingestion and can be fatal. The effects of wheat allergy are completely different from the pathology of celiac and non-celiac gluten sensitivity reactions.

2.5 Non-celiac Gluten Sensitivity

Non-celiac gluten sensitivity (NCGS) is a non-autoimmune reaction to gluten. People suffering from this condition do not present with villous atrophy like people with celiac disease but can experience gastrointestinal symptoms when they consume food containing gluten (25). The symptoms usually disappear when gluten is removed from the diet and reappear when gluten is reintroduced (26). Typical symptoms include abdominal pain, bloating, diarrhea and constipation, and affected people can also be pertubed by various symptoms such as acid reflux, fatigue, skin rashes, muscle pain, headaches and depression. There is currently no detection test to diagnose non-celiac gluten sensitivity.

2.6 Epidemiology

Celiac disease affects approximately 1% of the total population of Western countries including Canada, the United States and northern European countries (13). It is also found in other populations but is rare in Russia and northern Asia (5). Its prevalence appears to be on the rise, but it is difficult to estimate how many people may have a silent or latent form of celiac disease (27). Dermatitis herpetiformis affects approximately 0.001% of the general population (20). Non-celiac gluten sensitivity prevalence is difficult to establish, as it is often self-diagnosed in the absence of biomarkers (25). However, its prevalence has been estimated to be at least 6% (28). In Canada, food allergies of all types affect around 6% of children and 3% to 4% of adults (29). A similar prevalence was found in the United States (13), although the occurrence of wheat and barley allergies is only approximately 0.1% (10).

3. Regulations

The following section summarizes the legislation in force around the world, particularly in Canada, the United States, the European Union, New Zealand and Australia. Regulations can change rapidly, and it is important to refer to the latest version of the legislation applicable in the country where the gluten-free products in question are sold.

3.1 Overview of Worldwide Regulations

At the international level, food standards, guidelines and codes of practice are collected in the Codex Alimentarius. Many countries throughout the world use these standards to establish their own standards and regulations. The Codex Committee on Nutrition and Foods for Special Dietary Uses (CCNFSDU) has been responsible for developing labelling standards for gluten-free products for several years. Gluten-free foods are currently defined in CODEX STAN 118-1979 as follows: "dietary foods consisting of or made only from one or more ingredients which do not contain wheat (i.e. all *Triticum* species, such as durum wheat, spelt and khorasan wheat, which is also marketed under different trademarks such as KAMUT), rye, barley, oats or their cross-bred varieties, and the gluten level does not exceed 20 mg/kg in total, based on food as sold or distributed to the consumer" and/or "dietary foods consisting of one or more ingredients from wheat (i.e. all *Triticum* species, such as durum wheat, spelt, and khorasan wheat, which is also marketed under different trademarks such as KAMUT), rye, barley, oats or their cross-bred varieties, which have been specially processed to remove gluten, and the gluten level does not exceed 20 mg/kg in total, based on food as sold or distributed to the consumer". (2)

Using these guidelines, many countries have established their own standards and regulations for voluntary gluten-free food labelling in addition to the ingredient declaration on food packaging. Most of the countries (e.g., Canada, European Union, New Zealand/Australia) decided to include wheat, barley, rye, oats and their cross-bred varieties in their gluten definition. Table 2 summarizes authorized claims and conditions.

It should be noted that many privately owned, voluntary certification standards are loosely based on these regulations where the gluten-free products are sold. However, many of these are consumer-driven standards and certification processes that are generally more restrictive than government regulations in an effort to provide the perception of the higher level of protection that consumers desire.

Table 2: Authorized Gluten-free Claims and Conditions

Type of product	Canada	USA	European Union	Australia/ New Zealand
Foods that inherently do not contain gluten (e.g. fresh vegetables, milk, eggs)	Gluten-free claims generally not authorized (check with CFIA and Health Canada)	**Gluten-free** if < 20 ppm	**Gluten-free** if < 20 ppm	**Gluten-free** if no detectable gluten
Foods with gluten-containing grains that have been processed to remove gluten[1] (e.g. wheat starch, glucose syrup, maltodextrin)	**Gluten-free** if ≤ 20 ppm (check with CFIA and Health Canada)	**Gluten-free** if < 20 ppm	**Gluten-free** if ≤ 20 ppm **Very low gluten** if ≤ 100 ppm	**Low gluten** if < 200 ppm
Gluten-free foods that might contain gluten as a result of cross-contamination	**Gluten-free** if ≤ 20 ppm	**Gluten-free** if < 20 ppm	**Gluten-free** if ≤ 20 ppm	**Gluten-free** if no detectable gluten **Low gluten** if < 200 ppm
Oats	**Gluten-free** if ≤ 20 ppm[2]	**Gluten-free** if < 20 ppm[3]	**Gluten-free** if ≤ 20 ppm[2] **Very low gluten** if ≤ 100 ppm	Claims not authorized

1. Processing steps should be validated to demonstrate their effectiveness in removing gluten.
2. Gluten-free claims are allowed only for specially produced gluten-free oats if they can be verified as gluten-free.
3. Oats are treated as a gluten-free grain but may contain gluten as a result of cross-contamination.

3.1.1 Canadian regulation

In Canada, gluten-free foods are regulated under Division 24 of the *Food and Drug Regulations*, "Foods for Special Dietary Use", which includes foods that have been specially processed or formulated to meet the particular dietary requirements of a person in whom a physical or physiological condition exists as a result of a disease, disorder or injury; or for whom a particular effect, including but not limited to weight loss, is to be obtained by a controlled intake of foods. Section B.24.018 of the *Food and Drug Regulations* states that:

> *It is prohibited to label, package, sell or advertise a food in a manner likely to create an impression that it is a gluten-free food if the food contains any gluten protein or modified gluten protein, including any gluten protein fraction, referred to in the definition "gluten" in subsection B.01.010.1(1).* (30) .

Gluten is defined as any gluten protein from the grain of any of the following cereals or the grain of a hybridized strain created from at least one of the following cereals: barley, oats, rye, triticale, wheat or any modified gluten protein, including any gluten protein fraction that is derived from any of these cereals.

To meet Canadian regulations, foods labelled “gluten-free” must contain no more than 20 ppm of gluten as a result of cross-contamination and no intentionally added gluten sources. However, products made from a gluten-containing grain ingredient can also bear a gluten-free claim if the manufacturer can demonstrate that the food was processed to reduce gluten to a level of less than 20 ppm (e.g., wheat starch, wheat maltodextrin, wheat glucose syrup). (31).

The “gluten-free” claims for specially produced oats that contain no more than 20 ppm of gluten from wheat, rye, barley, or their hybrid strains have also been allowed since May 2015. The goal is helping people with celiac disease gain access to a wider selection of nutritious products.

Fermented and hydrolyzed foods or foods containing fermented and hydrolyzed ingredients are also authorized to bear “gluten-free” claims as long as they are clearly labelled. However, beers are not authorized to carry “gluten-free” claims as they must meet the standards of composition in the *Food and Drug Regulations*, which means that they are always made from barley and/or wheat. However, Health Canada and the CFIA do not object to the use of the following precautionary statement: “This product is fermented from grains containing gluten and [processed or treated or crafted] to remove gluten. The gluten content of this product cannot be verified, and this product may contain gluten”. In this case, the manufacturer must be prepared to provide evidence to substantiate their claim, including a detailed description of the method used to remove gluten from the product, appropriate gluten assay results for the finished product, and the name and the manufacturer of the assay.

It is important to note that most certification schemes (e.g., GFCP) do not allow fermented products to which a gluten-containing ingredient has intentionally been added, nor the precautionary statements permitted under regulations. In contrast, similar products made from cereals that do not contain gluten may use a “gluten-free” claim if they meet all other requirements.

Products that inherently do not contain gluten (e.g., fresh vegetables, milk, and eggs) are not authorized to bear a “gluten-free” claim because they do not meet the intent of Division 24 of the *Food and Drug Regulations* on Foods for Special Dietary Use.

3.1.2 US regulation

In the United States, the FDA’s Final Rule concerning gluten-free food labelling (21 CFR Part 101.91) has been in force since August 2014 (32). Gluten-free foods were defined by the FDA as food and dietary supplements that does not contain any of the following:

1) *An ingredient that is a gluten-containing grain (e.g., spelt wheat);*
    - *An ingredient that is derived from a gluten-containing grain and that has not been processed to remove gluten (e.g., wheat flour); or*

- *An ingredient that is derived from a gluten-containing grain and 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 (i.e., 20 milligrams (mg) or more gluten per kilogram (kg) of food);*

- Inherently does not contain gluten;

or

2) *Any unavoidable presence of gluten in the food bearing the claim in its labeling is below 20 ppm gluten (i.e., below 20 mg of gluten per kg of food).*
3) *that do not contain the following:*
   - *an ingredient that is a gluten-containing grain (e.g., spelt wheat);*
   - *an ingredient that is derived from a gluten-containing grain and that has not been processed to remove gluten (e.g., wheat flour);*
   - *an ingredient that is derived from a gluten-containing grain and that has been processed to remove gluten (e.g., wheat starch), if the use of that ingredient results in the presence of 20 ppm or more gluten in the food;*

   or
4) *that does not inherently contain gluten and any unavoidable presence of gluten in the food bearing the claim in its labelling is below 20 ppm gluten.*

Other claims such as “no gluten,” “free of gluten” and/or “without gluten” are also authorized on food packaging and must meet the intent of the regulation.

Unlike Canada, voluntary gluten-free food labelling in the United States applies to any food meeting the requirements, including inherently gluten-free foods and oats. However, since these products could be cross-contaminated by gluten, they must also respect the 20 ppm threshold.

American gluten-free labelling regulations also exclude drugs, cosmetics and products that are regulated by the U.S. Department of Agriculture (USDA) and the Alcohol and Tobacco Tax and Trade Bureau (TTB), which includes meats, poultry, certain egg products and most alcoholic beverages.

Most fermented beverages are regulated by the TTB under the *Federal Alcohol Administration Act* (FAA Act). Malt beverages are defined as beverages made with both malted barley and hops and cannot be labelled as gluten-free. However, the TTB may accept the use of one of the following specific statements: “Product fermented from grains containing gluten and [processed or treated or crafted] to remove gluten. The gluten content of this product cannot be verified, and this product may contain gluten” or “This product was distilled from grains containing gluten, which removed some or all of the gluten. The gluten content of this product cannot be verified, and this product may contain gluten”. However, most private certification schemes (e.g. GFCP) do not allow any intentional addition of gluten even with a disclaimer permitted under regulations. Other alcoholic beverages produced

without any ingredients that contains gluten (e.g. wine fermented from grapes, vodka) may use a gluten-free claim.

Lastly, beers that do not meet the definition of a malt beverage under the *FAA Act* are subject to the same labelling requirements as foods administered by the FDA. On November 18, 2015, the FDA published a proposed rule in the Federal Register to establish requirements for fermented, hydrolyzed and distilled foods or ingredients that are labelled as gluten-free. Under the proposed rule, these products were permitted to bear a gluten-free claim if the manufacturer keeps records showing that the ingredients are gluten-free before fermentation and that the risk of cross-contamination is under control. Records to demonstrate conformity should be kept.

3.1.3 European Union Regulation

As of July 20, 2016, Regulation (EU) No. 828/2014 on *the requirements for the provision of information to consumers on the absence or reduced presence of gluten in food* came into force in the European Union (33). The labelling requirements apply to all prepackaged and non-prepackaged foods (including meals served in cafes, restaurants, schools and hospitals).

European foodstuffs can bear a gluten-free claim if they contain no more than 20 ppm of gluten. Additionally, food containing one or more ingredients made from wheat, rye, barley, oats or their cross-bred varieties that have been specially processed to reduce the gluten content to a level of no more than 100 ppm of gluten can bear a “very low gluten” claim. These statements may be accompanied by the following affirmations: “suitable for people intolerant to gluten,” “suitable for coeliacs,” “specifically formulated for people intolerant to gluten” or “specifically formulated for coeliacs,” under certain conditions. Other statements used to provide information to consumers on the absence or reduced presence of gluten in food are no longer authorized.

3.1.4 Australia New Zealand Regulation

The Australia New Zealand Food Standards Code (Standard 1.2.7) defines gluten-free foods as foods that have no detectable gluten, no oats and no cereals containing gluten that have been malted (34). The threshold is based on the best available testing method, which is currently represented by 3 ppm. The “low gluten” label can also be used if food contains a detectable gluten content of less than 200 ppm.

3.2 The 20 ppm Limit Establishment

It is recognized that people affected by celiac disease can tolerate variable trace amounts of gluten in food without causing adverse health effects (13) (31). However, the amount of gluten that is harmful to people with celiac disease is still debated (35). The FDA addressed this question by establishing an internal interdisciplinary group, the Threshold Working Group (15). According to their report, 20 ppm represents the lowest level at which analytical

methods could, at the time of publication (2013), reliably and consistently detect gluten in most food matrices. In addition, a review of available human challenge studies was conducted and concluded that most individuals with celiac disease can tolerate variable trace amounts and concentrations of gluten in foods (including levels that are less than 20 ppm of gluten) without causing adverse health effects (36). A Canadian study has also determined that at a level not exceeding 20 ppm of gluten, exposure to gluten would remain below 10 mg per day. This represents the maximum threshold of gluten that is unlikely to cause intestinal mucosa damage and not pose a health risk for most Canadians with celiac disease (37). This is the level generally considered acceptable in most countries with gluten labelling requirements and by most non-government organizations that represent consumers with a medical need to seek out a gluten-free diet.

As it is extremely difficult to manufacture foodstuffs completely free of gluten even when correctly managed, a 20 ppm limit also enables individuals to purchase a variety of safe food products appropriate for their needs. Setting a much lower threshold could restrict the availability of products labelled gluten-free and would not be beneficial to people affected by celiac disease. It could be argued that this is the case in Australia and New Zealand, where gluten-free means undetectable.

3.3 Effects of Environmental Conditions and Cross-contamination

When cereals, grains and other crops inherently gluten-free are grown, harvested, stored and transported in the same regions as wheat, barley and rye, it may result in cross-contamination. Crop rotation and shared agricultural fields, harvesting equipment, transportation vehicles and storage silos are examples of cross-contamination risks (35). Contamination can also occur during the processing stages, when production equipment is shared (e.g. milling and packaging equipment) and when good manufacturing practices are not followed (38). Errors due to substitution with gluten-containing ingredients, employee traffic flow and dust can also contribute to contamination if not correctly managed.

Consequently, gluten-free product consumers and manufacturers should be aware that the gluten content of some products may not be accurately reflected in the ingredient list. Some products may bear warning statements. However, the use of precautionary labelling is voluntary. Consumers cannot therefore rely on voluntary allergen-advisory labelling to make decisions about which products they should buy. Moreover, several studies have concluded that even when labelled “gluten-free,” some food products contain more than 20 ppm of gluten (e.g. breakfast cereals, flours) (35) (39). Food safety program management is critical to prevent the presence of gluten even in dedicated gluten-free facilities. Cross-contamination is a serious compliance issue. Gluten contamination of food products, if found, is subject to a risk assessment and depending on the findings, products may be recalled.

3.4 The Specific Case of Gluten-free Oats

Numerous studies have investigated the impact of oats intake by people with celiac disease. As illustrated in figure 1, oat protein is recognized to be structurally different from other gluten-containing cereals, as non-celiac driver **globulins** are found in about 70–80% of the total protein content (5). Moreover, oats contain less proline (P) and glutamine (Q) **amino acids** in its protein sequences (3). This could explain why evidence suggests that the majority of people affected by celiac disease can consume oats that are uncontaminated with wheat, rye and barley in moderate amounts without any adverse effect on their health (40) (41) (42). However, some people seem to be sensitive to oats (i.e. avenins), and several publications have shown that certain oats varieties could be harmful for some individuals with celiac disease (43) (44) (45).

According to the **Codex Alimentarius**, the allowance of oats in food may be determined at the national level (2). The United States allows unlimited use of oats in gluten-free products. In Canada, gluten-free claims were authorized in May 2015 for specially produced oats with a maximum acceptable level of no more than 20 ppm of gluten (46) in order to help people with celiac disease access a greater range of nutritious products (5) (38). However, they recommend that people with celiac disease consult a health practitioner before and during the introduction of gluten-free oats into their diet. The same approach is used in the European Union.

A b concluded that the general commercial oats supply is highly contaminated, since approximately 88% of the oats samples tested contained gluten sources above 20 ppm (38). Similar results were obtained in an American study (47). This means that to consistently achieve the food safety outcome of < 20 ppm of gluten, special steps must be taken to ensure that the oats themselves are gluten-free and meet regulatory and consumer requirements.

There is great debate within the celiac community and food industry as to how gluten-free oats production can be achieved. Regulators are more concerned about the outcome, whereas the celiac community and food industry want a proactive and preventive approach. Failures by industry are costly in terms of reduced consumer confidence and potential food recalls. Although regulators generally support the concept of preventative measures, they do not mandate these measures for gluten-free oats.

4. Analytical Techniques for the Detection and Quantification of Gluten

Testing of gluten-free products, although not currently a regulatory requirement, makes it possible to increase the level of trust in the processes in place to guarantee the gluten-free status of a product. Manufacturers are thus encouraged to test raw materials, final products and products being processed based on risk assessment to detect the presence of gluten. This section explores the various gluten testing techniques available on the market to provide an understanding of their specifications in order to determine the optimal testing methodology.

The **Codex Alimentarius** recommends that the quantification and detection of gluten in food should be based on sensitive and specific methods such as an immunological method with a detection limit of 10 ppm or below. The method should be validated and calibrated against certified reference material. In addition, the method should use an **antibody** that reacts with the cereal protein fractions that are toxic for people intolerant to gluten and that does not cross-react with other food components that could either under-report or over-report gluten (2).

Most international organizations and regulatory agencies recommend the use of testing methods that are fully validated and certified by the Association of Analytical Communities International (AOAC INTERNATIONAL). Harmonized guidelines were recently published for validating gluten ELISA methodologies to help test manufacturers develop a confident validation protocol for submission to AOAC INTERNATIONAL or other regulatory bodies (48). AOAC INTERNATIONAL has two programs by which methods are evaluated and approved: the AOAC Official Methods of Analysis Program (OMA) and AOAC-RI Performance Tested Methods Program (PTM). The PTM program is an independent review that verifies if methods perform according to the manufacturer's documented claims. It includes one independent laboratory study and an expert peer review of data. Certification is granted for certain matrices only. The OMA program is a much more rigorous program that includes the PTM program and a collaborative study. In addition, to achieve the OMA Final Action status, the method should be used in the industry for two years without any issue. Some tests have also obtained approvals from the American Association of Cereal Chemists International (AACC International).

Although an immunological method is recommended for gluten analysis, the use of other methods is not excluded. A wide variety of other analytical approaches has been developed and is currently in use, including DNA-based methods and proteomic methods. Manufacturers must select the test method most appropriate for their needs according to the type of food they make and the type of contamination suspected. In many cases, selecting the appropriate test method might be difficult. Some methods provide quantitative

test results while others are considered qualitative. Some techniques produce results in less than 15 minutes while others require many hours.

Each method uses a similar analytical process. The first critical step involves sampling and sample preparation. As only a very small amount of product is usually tested, it is important to pick a representative sample of the whole product. A representative sample can be obtained by using a recognized sampling plan and statistical methods. Effective sampling plans can be formulated by taking into consideration the lot size, homogeneity and nature of the sample. A non-representative sample is unlikely to provide analytical results that truly reflect the production process with a high level of confidence. Sample preparation steps can include drying, milling, grinding to a powder, blending and mixing. The proteins are then extracted and the samples analyzed using the various techniques. Finally, the results are interpreted by taking into consideration the limitations of the method.

4.1 Immunochemical Techniques

Immunochemical techniques are the most commonly used methods for analyzing gluten in food. Enzyme-linked immunosorbent assays (ELISA) are based on **antibodies** raised against specific **amino acid** sequences (called **epitopes**), and the reaction can be visualized by a quantifiable colour reaction (49). The immunoassays are specific, sensitive and easy to use, especially for routine analysis. They require relatively inexpensive equipment and give rapid results for a wide variety of foods (50). It is possible to carry out numerous analyses at the same time in only a few minutes to a few hours. However, certain food matrices might be difficult to analyze due to matrix interferences and cross-reactions (see section 4.1.4). ELISA test kits are available in two formats: sandwich and competitive.

Using the sandwich format ELISA test kit, a coating **antibody** is bound to the bottom of the microplate wells to detect the **antigen** (i.e. gluten protein). The detecting **antibody** then binds to the **antigen** attached to the coating **antibody** and a linked **enzyme** induces a colour reaction (5). The **antigen** concentration is calculated by comparing the absorbance measurement to the standard calibration curve. This method is reliable for assessing the gluten content of foods when gluten proteins are intact or relatively intact. However, it is not suitable for measuring the gluten content of highly hydrolyzed or fermented proteins because the protein in such products might not be large enough for the **antibodies** to attach to two different binding sites. Poor antibody binding could lead to underestimation of the gluten content (51). In the latter case, competitive format methods must be employed.

When using competitive format ELISA test kits, a known quantity of the standard protein is bound to a coated plate. The sample is mixed with the **antibodies** linked with **enzymes** and the mixture is added to the ELISA plate. A competition for the binding sites occurs during incubation. After washing, the enzymatic substrate is added and a colour reaction occurs,

which can be compared to the calibration curve. In this test, the intensity of the colour reaction is inversely proportional to the amount of **antigen** in the sample. Competitive ELISA methods use only one **antibody** and are thus suitable for detection of small hydrolyzed proteins (5). However, this test might not be as accurate as the sandwich method for intact proteins since only one binding site is required, which could affect the specificity of the test result (3).

Qualitative ELISA test kits such as lateral flow devices are also available on the market. In this test, a line of **antibodies** is fixed to a surface strip. When the gluten **extraction** solution is absorbed by the strip, the sample and **antibodies** migrate together across the surface of the strip. If the sample contains gluten, the **antibodies** will recognize specific **epitopes** and bind to them, as in the sandwich test. The conjugated complex will accumulate on the surface of the strip and the reaction will become visible if a specific concentration is reached (51). A control line appears on the strip if the test works correctly. These simplified versions of the ELISA method offer a direct reading of the results in less than 15 minutes without using any laboratory equipment. They are thus cheaper and require less expertise. However, samples are analyzed individually and the results are sometimes difficult to read.

Many types of ELISA test kits from several companies are available on the market. The main characteristics of the available test kits are presented in Appendix 1. When compared to each other, test kits do not necessarily give similar results because **antibodies** target different **peptide** sequences, different reference standards are used to calibrate the assay and the **extraction** protocols differ (49) (4). In addition, an accurate measurement cannot be performed, as ELISA methods cannot distinguish the source of cereals, and a standard multiplication factor of two is often used to calculate the total gluten content (49). This prediction is based on an assumption that gliadins provide approximately 50% of the gluten proteins in wheat, but this differs among cereal varieties and cultivars, as stated in section 1.2 (4). To overcome this problem, recent studies have suggested developing detection methods based on the recognition of both gliadins and glutenins in combination with an adapted reference material (52).

4.1.1 Antibodies

The various commercial test kits available on the market rely on different types of **antibodies**, mainly the Skerritt, R5 and G12 **antibodies**. Each **antibody** reacts with distinct sites from different **peptides** to a different degree. The various protein fractions of gluten were previously detailed in Table 1 in the first section of this document. Please refer to it for more information.

4.1.1.1 Skerritt Antibody

The Skerritt **antibody** (401.21) was developed by Skerritt and Hill in the late 1980s (5). This monoclonal **antibody** mainly recognizes HMW-glutenins, presumably LMW-glutenins and ω-gliadins and to a small degree α- and γ-gliadins (52). More specifically, it recognizes the PQPQPFPQE and PQQPPFPEE (50) **epitopes**. This method can be used with both raw and processed foods, as the ω-gliadin fractions are heat-stable. However, test results can differ considerably because the amount of ω-gliadin fluctuates significantly between cereal species and varieties (53). This **antibody** was found to give a weak response to barley **prolamins**, underestimate gliadins from wheat and overestimate **prolamins** from triticale and rye (4). Moreover, the Skerritt antibody was also shown to have a stronger affinity to glutenins than gliadins, which could lead to a miscalculation of the total gluten content depending on the standard material used (54).

A sandwich test based on the Skerritt antibody was the first commercially available ELISA method for gluten detection and was adopted as the AOAC Official Method 991.19 in 1995. It was used for many years in gluten analysis (51). At that time, this method had a limit of quantification (LOQ) of 160 ppm of gluten, which is not compatible with present regulations. Nowadays, the performance of currently available ELISA test kits based on this **antibody** has been greatly improved. The limit of detection (LOD) is now as low as 5 ppm of gluten. However, only the original and less sensitive method has received the designation of AOAC Official Method (55).

4.1.1.2 R5 Antibody

The R5 **antibody** was developed by Méndez to target the ω-secalins of rye. This monoclonal **antibody** also strongly reacts with α- and γ-gliadins and presumably with ω-gliadins (52). Limited reactivity with glutenin fractions was observed, but R5 sometimes binds to some LMW glutenin fractions, which could lead to overestimation of the total gluten content in wheat (55). It also overestimates hordeins of barley (56). Analysis revealed that the R5 antibody mainly reacts with the QQPFP **epitope** but also recognizes other potential toxic celiac **epitopes** such as LQPFP, PQPFP, QLPFP, QLPYP, QQTFP and QQQPFP, although with weaker reactivity (5) (4). It was first found to cross-react with soy and lupin proteins (53), but **extraction** protocols have been adapted by using a cocktail solution instead of an ethanol solution and false positive are no longer observed (5).

The sandwich assay R5 ELISA RIDASCREEN® Gliadin has been endorsed by the **Codex Alimentarius** Commission for gluten determination in gluten-free foods since 2006 (57). In 2012, it was also approved by the AOAC Research Institute as the Official First Action method following an interlaboratory study (58). It is particularly recommended for gluten analysis in maize matrices (50). It has also received approval from the American Association of Cereal Chemists International (**AACCI**).

4.1.1.3 G12 Antibody

The monoclonal G12 **antibody** specifically recognizes the 33-mer (LQLQPFPQPQLPYPQPQLPY) of the gliadin protein. This **peptide** was shown to be one of the principal contributors to gluten immunotoxicity (8). Test kits using the G12 **antibody** were manufactured after some researchers recommended updating the concept of gluten detection with respect to the immunotoxicity of gluten. More specifically, this **antibody** recognizes the sequences QPQLPY (wheat), QPQQPY (rye) and QPQLPF (barley) (5). It has shown no cross-reactivity with soy, maize or rice (53). It differs from other antibodies because it gives a positive response to some oat varieties that are suspected of triggering a response in people affected by celiac disease (53). It has even been suggested that the G12 would be a reliable tool for detecting oat varieties that are potentially safe for people affected by celiac disease (43).

The AgraQuant® ELISA Gluten G12 test has been accepted by AOAC INTERNATIONAL as an Official First Action method and has received approval from the American Association of Cereal Chemists International (AACCI) (59). However, the **Codex Alimentarius** Commission Committee agreed not to include the ELISA G12 method in the *Standard for Foods for Special Dietary Use for Persons Intolerant to Gluten* (CODEX STAN 118-1979), as the results of the comparability study with R5 are still lacking. This method could be considered at a future date when the results from the ongoing comparability studies by the International Working Group on Prolamin Analysis and Toxicity become available (60).

4.1.1.4 Polyclonal Antibodies

In contrast with monoclonal **antibodies**, polyclonal **antibodies** detect more than one **epitope** on the same **antigen**. This makes them less specific, which could lead to inaccurate results. On the other hand, they are usually very sensitive. For example, the wheat/gluten (gliadin) ELISA kit from the Morinaga Institute of Biological Science, Inc. has a limit of detection as low as 0.26 ppm of gluten. This **antibody** specifically binds to the gliadin of wheat, but it can also recognize hordeins of barley and secalins of rye to a lesser degree and with a weaker response. This assay has been validated in an interlaboratory study supported by the Japanese Ministry of Health, Labour and Welfare. It is used and recommended by the FDA in tandem with RIDASCREEN® Gliadin as a method for regulatory enforcement of gluten-free products.

4.1.2 Reference material

The PWG-gliadin produced under the Prolamin Working Group (PWG) is currently the most internationally recognized standard. The PWG-gliadin was produced by extracting gliadin from the 28 most frequently cultivated European wheat varieties (61). Many other standards were calibrated against this reference material, such as Sigma-Aldrich's G3375-SIGMA Gliadin from Wheat.

The choice of an accurate reference material is one of the biggest challenges in gluten quantification, as gluten is a complex group of proteins and its composition can vary significantly in different food matrices. Some authors have suggested using specific standards among applications (62). For example, barley standards, available in some test kits, were found to provide better quantification in samples contaminated with barley. However, most of the time the type of contamination in a sample is unknown and contamination does not necessarily come from a single variety.

Processes can also modify the gluten content of certain products. Selecting a proper reference material is particularly complicated for hydrolyzed and fermented products due to differences in the type and degree of **hydrolyzation**. A specific standard was recently developed to quantify partially hydrolyzed gluten in fermented wheat, rye and barley products in competitive-type assays. The sample was prepared from a mixture of isolated **prolamin** fractions of barley and rye flours and the reference material PWG-gliadin, which were successively digested. However, some authors suggested that the calibration material might not yet perfectly mimic the fragmented or partially hydrolyzed gluten found in beers and that further improvements are required (28) (63). Other reference materials were also prepared to better quantify barley or rye contamination in samples.

4.1.3 Extraction Protocols

Gliadin can easily be extracted using ethanol solutions in non-processed foods. However, this type of **extraction** was found to be substantially less efficient for processed product samples. Different cocktail solutions were therefore developed by test kit manufacturers to improve recovery. For example, the R-Biopharm cocktail is composed of a mixture of β-mercaptoethanol, a reducing agent, and guanidine hydrochloride, a dissociating agent, diluted in a phosphate buffered saline solution (64). Similar cocktails or additives are also used by other test kit manufacturers.

Unfortunately, the use of cocktail solutions also permits the **extraction** of some glutenins, which can lead to an overestimation of the final gluten content if the **antibody** used also binds to these fractions (49). Another drawback of cocktail **extraction** is that it is not compatible with competitive-type methods. Nevertheless, the UPEX (Universal Prolamin and Glutelin Extractant Solution) has recently shown promising results for the resolution of this situation (51) and might soon be commercialized.

4.1.4 The Importance of Validation

Foods are complex matrices made of various components, and the interaction between ingredients can have an impact on **extraction** efficiency and detection of gluten proteins. Processing steps such as cooking, extrusion, drying, purification, **fermentation**, and **hydrolyzation** can have an effect on protein structure. Moreover, some food matrices, such as food containing high amounts of polyphenols or tannins (e.g. tea, hops, cocoa products, coffee, spices, chestnut flour, buckwheat, millet), can interfere with the determination of gluten in foods due to their interaction with proteins. Underestimation of the **prolamin** content can be avoided by adding **extraction** additives such as fish gelatine, skim milk powder, polyvinylpyrrolidone (PVP) or urea to the **extraction** solution (3). These additives disrupt gluten protein-polyphenol interactions. Other elements may affect gluten quantification such as cross-reactivity with other cereal grains, the presence of a strong acid or alkali, high salt content, high fat content or the presence of food gum (65). Cross-reactivity studies are often available from test kit providers.

A **validation** should always be conducted for every type of matrix before applying a detection method to avoid misinterpretation (50). Test kit manufacturers can assist you in developing **extraction** and **validation** protocols for special matrices. Sample **validation** involves spiking a known amount of gluten to a matrix and attempting to recover the same quantity (66). Suggested acceptable recoveries vary between 80% and 120%. However, in the case of gluten, recoveries between 50% and 150% are usually considered acceptable as long as they are shown to be consistent (66). It is also important to consider that although the AOAC has recognized many test kits in recent years, **validation** studies have only been conducted on a limited number of food matrices.

4.2 Genomics-based Methods

DNA-based methods rely on the amplification of specific deoxyribonucleic acid (DNA) fragments by the conventional polymerase chain reaction methods (PCR) or Real-time PCR (49). DNA is extracted from the sample and target genes are selected for detection. PCR methods then amplify small fragments of the target DNA until a sufficient number of copies are obtained for visualization or quantification. Fluorescent dye can be added for better quantification (66).

These methods were developed in the 1980s and are relatively fast and sensitive. (66). For example, the SureFood® ALLERGEN QUANT Gluten test kit reports a detection limit as low as 0.04 ppm (67). This Real-time PCR test kit costs a little more than ELISA test kits.

A comparison between immunochemical methods and PCR methods gives a good correlation between the results obtained (68). Since DNA-based methods do not detect the gluten protein directly but rather the gene that encodes the protein, false positive and false

negative results are possible for certain matrices (69). In fact, DNA can exist without the presence of any proteins, for instance in wheat starch samples (5). In addition, DNA content may not correlate with the concentration of gluten proteins since the ratio of protein to DNA typically varies depending on the degree of gene expression (49). Another major drawback is that highly processed or hydrolyzed samples cannot be detected by this method due to the massive destruction and degradation of DNA, which makes its amplification difficult or impossible (70) (50).

However, DNA-based methods are useful in determining the nature of gluten in cases of contamination where immunochemical methods are not suitable. For example, these methods were designated as an acceptable methods to detect cross-contamination with wheat in oats samples (71). Finally, some techniques were shown to detect several allergens in a single assay (69).

4.3 Proteomics Approach

The proteomic methods are based on the separation of proteins and the identification of individual proteins (4). First, a sample is mixed with an **extraction** buffer, usually containing reducing agents to break up the disulfide bonds of proteins (66). Proteins are extracted from other components that could have an effect on the resolution of the protein profile, such as salts, proteases, polysaccharides, nucleic acids, lipids, phenols and others (4). The total protein content is then measured and the protein mixture is enzymatically digested into small **peptide** fragments that are subsequently separated. **Peptide** separation can be achieved using many different techniques, including liquid chromatography, electrophoretic techniques or high-performance liquid chromatography (HPLC) (4). Finally, mass spectrometry is conducted to collect data on each **peptide**, which will be compared to databases in order to identify the **amino acid** sequence of **peptides.**

Separation techniques are useful for characterizing and identifying **peptides** with high sensitivity (49). For example, electrophoresis studies have allowed scientists to separate gliadin fractions into four groups according to their molecular weight and electric charge (α, β, ϒand ω). Mass spectrometry has been used to identify the toxic **peptides** associated with celiac disease (72). However, proteomic methods rely on information published in databases and a limited number of wheat, barley and rye sequences are presently registered (3). Further research is still required before this methodology can be used effectively to analyze a wide range of gluten-free products. Until recently, it was difficult to calculate gluten content in the units specified by the legislation (ppm or mg/kg) (50).

However, methods combining mass spectrometry with liquid chromatography are particularly promising for the accurate quantification of gluten. The use of these methods has increased in recent years and has shown great potential for gluten analysis applications (49).

For example, it has been possible to detect wheat in gluten-free oat flours at a concentration of approximately 1 ppm (72). The method was also used to measure **peptides** in fermented beverages such as beer, whereas some ELISA methods failed (56). This method can attain a very high degree of accuracy (66) and screen many allergens using a single analysis run. It has proven to be an effective tool for confirming ELISA results when there are doubts as to its capacity to target the protein itself. However, this method is more labour-intensive than other approaches used in gluten analysis and requires a high level of expertise in addition to specialized and expensive laboratory equipment (49).

4.4 Non-specific Surface Testing Method

4.4.1 Detection of Total Protein

Detection of total protein tests help food manufacturers to verify the cleanliness of surfaces by measuring proteins left after cleaning. The test is based on the Biuret reaction in which copper ion forms a coloured complex in the presence of **peptide** bonds under alkaline conditions. The colour can be matched on a colour chart to evaluate the degree of contamination.

Methods based on total protein analysis are sensitive, cheap, easy to transport and to use, fast and available from different companies. However, reducing sugars, uric acid, ascorbic acid, tannins and some sanitizers can cause interference, giving false positive or false negative results. Since these methods are not specific to gluten, they should only be used as indicators. These methods can be used for **verification** of overall cleanliness. However, they should always be validated using specific methods.

4.4.2 ATP Bioluminescence

ATP bioluminescence is also a method that allows to quickly screen the cleanliness of surfaces. It is based on the detection of adenosine triphosphate (ATP), a chemical molecule used to transport energy in the cell and found in all living organisms. In other words, ATP methods measure organic residues left on a surface. An **enzyme** converts the ATP residues found on surfaces after cleaning into a light signal, which is measured by a luminometer. The results are expressed in Relative Light Units (RLU).

This method is widely used by the industry since it quickly demonstrates the effectiveness of cleaning processes and the overall cleanliness of surfaces. It is sensitive and less expensive than ELISA methods. However, since ATP methods cannot distinguish between different sources of ATP, they should not be favoured over other more gluten-specific methods. In addition, test results should be interpreted carefully since it is difficult to set an acceptable limit. These methods should therefore only be incorporated in addition and complementary to other gluten-specific methods into a complete **verification** program.

4.5 Emerging Technologies in Gluten Detection

Since gluten detection remains a challenging analytical problem, multiple efforts have been implemented in recent years. Emerging technologies have shown that it is possible to quickly detect multiple allergens at the same time. For example, a magnetic bead-based assay has provided the simultaneous detection of 14 different allergens, including gluten, in a single Microtiter plate within six hours (73).

Biosensors have also provided promising results using various biological recognition elements (69). Multiple devices were developed based on an **antibody-antigen** reaction or sometimes a DNA approach. These devices are mostly easy to use, cheap and provide quick results (74). For example, the Nima test is a portable device that uses electronic sensors to detect gluten in less than five minutes. You only need to insert a small food sample into a disposable test capsule. The capsule is then loaded into the Nima device, where the sample is automatically ground into small particles with a buffer extraction solution. Once the sample is ready, the solution passes onto a test strip preloaded with antibodies, making a chemical reaction possible. A pair of antibodies that bind to the 33-mer fragment of gluten is used, similarly to other ELISA test kits. The qualitative test result is displayed directly on the device and synchronized to the mobile app. This is a fast technology that enables test users to get analysis results without any laboratory equipment. According to the manufacturer, this device can detect a very low level of gluten (i.e. below 2 ppm) in food products. However, the Nima test kit manufacturer has not yet published any validation reports in the scientific literature. Furthermore, false negative and false positive results were obtained with this test kit when compared with other validated methods (75). Although this technology presents multiple advantages, its use by food manufacturers has not been recommended.

In spite of everything, several scientific advances have made it possible in recent years to develop promising new testing methodologies and to improve existing methods.

Conclusion

Gluten is an important source of protein that can be used in several applications because of its physicochemical properties. Gluten is found in wheat, barley, rye, oats and their cross-bred varieties. However, the allowance of gluten-free oats (pure oats) varies around the world since it is considered safe for most persons with celiac disease.

Gluten is sometimes hidden in unexpected sources such as drugs, sausages, sauces and spices. Cross-contamination can also occur when harvesting, storing, transporting and processing products otherwise considered to be inherently free from gluten. Appropriate detection methods must therefore be selected to ensure proper detection and quantification of gluten in a wide range of food products. Each method carries its own advantages and disadvantages.

Immunochemical methods are the most popular for gluten detection since they offer specific results in a short period of time. Many of these methods have been validated and recognized by the AOAC. Quantitative methods are generally more sensitive than qualitative methods. ELISA quantitative methods are mostly used in commercial laboratories because the person conducting the test must have a certain degree of technical knowledge and use certain laboratory equipment. On the other hand, lateral flow devices offer a quick, cheap and easy to use solution for manufacturers of gluten-free products. Moreover, the analyses can be carried out on their own premises. However, these qualitative methods are less reliable for some matrices, less sensitive and have higher limits of detection.

It is important to note that ELISA test kits may produce distinct test results from one kit to another. The discrepancy in test results is explained by the different types of antibodies used, the different reference materials and different extraction methods. In addition, some matrices can cross-react with certain antibodies, while other components may cause interference. Thus, each matrix should ideally be validated to verify the gluten recovery.

In conclusion, there is currently no perfect testing method for detecting and quantifying gluten in all types of foods. In people with celiac disease, gluten triggers a chain reaction similar to the antigen-antibody reaction used in immunochemical methods. However, gluten is an extremely complex group of proteins that can cause a wide range of disorders involving different mechanisms. This complexity presents a major challenge for developing methods that can accurately quantify gluten in all food products. More research is required to improve methods of quantifying gluten.

ELISA test kits remain suitable and are among the best tools for detecting and quantifying gluten in a wide variety of food matrices. Complementary methods such as PCR and mass spectrometry can be used to confirm the source of contamination, which cannot be achieved using ELISA methods.

Sample analysis must always be included as part of a food safety system to improve confidence in the expected results from beginning to end. Manufacturers should develop documented and validated sampling plans as part of their gluten control system to support their gluten-free claims.

Glossary

AACCI: AACC International is a global, non-profit association of more than 2,000 scientists and food industry professionals working to advance the understanding and knowledge of cereal grain science and its product development applications through research, leadership, education and superior technical service.

Albumin: Albumin forms a group of globular proteins. Albumin is not toxic to people affected by celiac disease but can cause allergic reactions if it binds with human IgE antibodies. It is soluble in water solutions and moderately soluble in concentrated salt solutions. It becomes denatured when heated.

Amino acid: Amino acids are molecules containing a carboxylic group, a hydrogen atom, an amino group and an organic side group attached to a carbon atom. Amino acids are attached together through peptide bonds, forming a long chain of what are called proteins. There are 22 different amino acids in nature.

Antibody: An antibody is a large Y-shaped protein produced by B cells after stimulation by an agent called antigen. Antibodies, also known as immunoglobulins (Ig), are part of the immune system and specifically recognize a particular epitope on an antigen, allowing the antibody and antigen to bind together. Antibodies are also used with ELISA test kits to detect the presence of a specific substance.

Antigen: An antigen is a substance capable of inducing an immune response. In this instance, the host will specifically produce antibodies against an antigen after cells in the immune system come in contact with the antigen.

Codex Alimentarius: The Codex Alimentarius is a group of internationally recognized standards, codes of practice, guidelines and other recommendations relating to food. Its texts are developed and maintained by the Codex Alimentarius Commission, established in 1963 by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) to protect the health of consumers and ensure fair practices in the international food trade.

Deamination: Deamination is a process characterized by the removal of an amine group to modify the structure of a protein.

Enzymes: An enzyme is a type of protein that accelerates or catalyzes a chemical reaction.

Epitope: An epitope is the specific segment of an antigen to which an antibody binds. It is composed of a series of amino acids.

Extraction: Extraction is the action of removing a substance from another substance with the help of a solvent. Extracting gluten from food is the first step in detecting gluten. Since gluten is not soluble in water, a special extraction process is required to separate gluten from a food matrix.

Fermentation: Fermentation is a metabolic process that converts sugar into acids, gases and alcohol. This process is widely used to produce foods and beverages (e.g. pickles, kimchi, cheese, beer, yoghurt, etc.). During fermentation, if gluten proteins are present, they will be broken down into smaller peptide fragments.

Globulins: Globulins are a group of globular proteins present in animal species and plants. Globulin proteins are not toxic to people affected by celiac disease, but they can cause allergic reactions if they bind with human IgE antibodies. They are insoluble in pure water but dissolve in dilute salt solutions.

Glutelins: Glutelins are a group of plant storage proteins found in cereal grain seeds. They form gluten, together with prolamins. Glutelin in wheat is the most common glutelin, but glutelins are also found in barley and rye. Glutelin is mostly responsible for the bread baking performance of wheat. Glutelins are rich in phenylalanine, valine, arginine, leucine and proline amino acids. They are soluble in dilute acids or bases, detergents or reducing agents.

Hydrolyzation: Hydrolyzation is a chemical process in which a molecule of water is added to a substance, causing the cleavage of chemical bonds and thus the degradation of a substance. The hydrolysis of proteins results in smaller molecules such as amino acids.

Peptides: Peptides are short chains of amino acid monomers linked by peptide bonds.

Prolamins: Prolamins are a group of plant storage proteins found in cereal grain seeds such as wheat (gliadin), barley (hordein), rye (secalins), corn (zein), sorghum (kafirin) and oats (avenin). Prolamins form gluten, together with glutelins. Prolamins are characterized by a high glutamine and proline content. They are generally soluble only in strong alcohol solutions.

Transamination: Transamination is the process of producing amino acids. Transglutaminase enzymes are widely used in various manufacturing processes to improve the firmness, viscosity and elasticity of foods such as cheese, dairy, meat and baked goods.

Validation: The collection and evaluation of scientific, technical and observational information to determine whether control measures are capable of achieving their specified purpose in terms of hazard control. Validation involves measuring performance against a desired food safety outcome or target with regard to a required level of hazard control.

Verification: The application of methods, procedures, tests and other evaluation methods in addition to monitoring to determine whether a control measure is or has been operating as intended.

References

1. **Health Canada.** Celiac Disease. [Online] [Cited: 01 20, 2017.] http://www.hc-sc.gc.ca/fn-an/securit/allerg/cel-coe/index-eng.php.

2. **Codex Alimentarius Commission. Joint Food and Agriculture Organization of the United Nations / World Health Organization Food Standards.** *Standard for foods for special dietary use for perosns intolerant to gluten (CODEX STAN 118-1979).* 2008.

3. **Mena, M Carmen and Sousa, Carolina.** *Analytical Tools for Gluten Detection. Policies and Regulation.* In Arranz E, Fernandez-Banares F. Rosell CM, Rodrigo L, Pena AS, editors. Advances in the understanding of Gluten related Pathology and the Evolution of Gluten-Free Foods. Barcelona, Spain : OmniaScience, 2015. pp. 527-564.

4. **Haraszi, Reka, et al.** Analytical Methods for Detection of Gluten in Food - Method Developments in Support of Food Labeling Legislation. *Journal of AOAC INTERNATIONAL.* 2011, Vol. 94, pp. 1006-1025.

5. **Kanerva, Päivi.** *Immunochemical analysis of prolamins in gluten-free foods.* Helsinki, Finland : Department of Food and Environmental Sciences, University of Helsinki, 2011.

6. **Jacek, Waga.** Structure and Allergenicity of Wheat Gluten Proteins - A Review. *Polish Journal of Food and Nutrition Sciences.* 2004, Vol. 13/54, 4, pp. 327-338.

7. **Wieser, Herbert.** Chemistry of gluten proteins. *Food Microbiology.* 2007, Vol. 24, pp. 115-119.

8. **de Lourdes Moreno, María, et al.** Selective Capture of Most Celiac Immunogenic Peptides from Hydrolyzed Gluten Proteins. *Food Chemistry.* 2016, Vol. 205, pp. 36-42.

9. **Dostálek, Pavel, et al.** Determination of gluten in glucose syrups. *Journal of Food Composition and Analysis.* 2009, Vol. 22, pp. 762-765.

10. **Taylor, Steve L. and Baumert, Joseph L.** Achieving Gluten-Free Status. *Food Safety Magazine.* December 2013/January 2014, pp. 30-66.

11. **U.S. Food and Drug Administration.** Proposed Rule for Gluten-Free Labeling of Fermented or Hydrolyzed Foods. *Questions & Answers.* [Online] 06 29, 2016. [Cited: 09 07, 2016.] http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/Allergens/ucm472735.htm.

12. **Meresse, Bertrand, Malamut, Georgia and Cerf-Bensussan, Nadine.** Celiac Disease: An Immunological Jigsaw. *Immunity Review.* 2012, Vol. 36, pp. 907-919.

13. **Food and Drug Administration. The Threshold Working Group.** *Approaches to Establish Thresholds for Major Food Allergens and for Gluten in Food.* March 2006.

14. **Armstrong, David, Don-Wauchope, Andrew C and Verdu, Elena F.** Testing for Gluten-related disorders in Clinical Practice: The Role of Serology in Managing the Spectrum of Gluten Sensitivity. *The Canadian Journal of Gastroenterology & Hepatology.* 2011, Vol. 25, 4, pp. 193-197.

15. **Food and Drug Administration. Office of Food Safety.** *Health Hazard Assessment for Gluten Exposure in Individuals with Celiac Disease: Determination of Tolerable Daily Intake Levels and Levels of Concern for Gluten.* May 2011.

16. **Waga, Jacek.** Structure and Allergenicity of Wheat Gluten Proteins - A Review. *POLISH JOURNAL OF FOOD AND NUTRITION SCIENCES.* 2004, Vol. 13/54, 4, pp. 327–338.

17. **Vader, Willemijn, et al.** The gluten response in children with celiac disease is directed toward multiple gliadin and glutenin peptides. *Gastroenterology.* 2002, Vol. 122, 7, pp. 1729–1737.

18. **Bruins Slot, Ilona D., et al.** Evaluating the Performance of Gluten ELISA Test Kits: The Number of Do Not Tell the Tale. *Cereal Chemistry.* 2015, Vol. 92, 5, pp. 513-521.

19. **Celiac Disease Foundation.** Dermatitis Herpetiformis. [Online] [Cited: 08 09, 2016.] https://celiac.org/celiac-disease/understanding-celiac-disease-2/dermatitis-herpetiformis/.

20. **Canadian Celiac Association.** Dermatitis Herpetiformis. [Online] [Cited: 08 05, 2016.] http://www.celiac.ca/?page_id=889.

21. **Hadjivassiliou, M, et al.** Dietary Treatment of Gluten Ataxia. *Journal Neurol Neurosurg Psychiatry.* 2003, Vol. 74, pp. 1221-1224.

22. **Gluten-free Society.** Ataxia - Another Symptom of Gluten Induced Damage. [Online] [Cited: 09 08, 2016.] https://www.glutenfreesociety.org/ataxia-another-symptom-of-gluten-induced-damage/.

23. **Yeager, David.** Gluten Ataxia. *Today's Dietitian.* [Online] March 2014. [Cited: 08 08, 2016.] http://www.todaysdietitian.com/newarchives/030314p16.shtml.

24. **CFIA & Health Canada.** Wheat: One of the ten priority food allergens. [Online] 2012. [Cited: 09 08, 2016.] http://www.hc-sc.gc.ca/fn-an/pubs/securit/2016-allergen_wheat-ble/index-eng.php.

25. **Canadian Celiac Association.** Non-Celiac Gluten Sensitivity: How to Diagnose and Differentiate it from Celiac Disease. [Online] [Cited: 09 08, 2016.] http://www.celiac.ca/?page_id=883.

26. **Volta, Umberto, et al.** Non-celiac gluten sensitivity: questions still to be answered despite increasing awareness. *Cellular & Molecular Immunology.* 2013, Vol. 10, pp. 383-392.

27. **U.S. Government.** Federal Register - Rules and Regulations. *Vol. 78, No. 150.* [Online] 08 05, 2013. [Cited: 09 08, 2016.] https://www.federalregister.gov/articles/2013/08/05/2013-18813/food-labeling-gluten-free-labeling-of-foods.

28. **Don, C and Koehler, P.** Enzyme-Linked Immunosorbent Assays for the Detection and Quantitation of Gluten in cereal-Based Foods. *Cereal Foods World.* 2014, Vol. 59, 4, pp. 171-178.

29. **Canadian Food Inspection Agency.** *Plan d'action pour assurer la sécurité des produits alimentaires. Allergènes non déclarés dans des sachets d'arômes.* Enquêtes ciblées 2010-2011.

30. **Minister of Justice.** *Food and Drug Regulations, C.R.C., c. 870.* 2013.

31. **Health Cananda.** Health Canada's Position on Gluten-Free Claims. *Bureau of Chemical Safety, Food Directorate, Health Products and Food Branch.* June 2012.

32. **U.S. Food and Drug Administration.** Food Labeling; Gluten-Free Labeling of Foods, Final Rule. Fed. Regist. 78:47154–47179. [Online] 2013. [Cited: 09 09, 2016.] https://www.federalregister.gov/articles/2013/08/05/2013-18813/food-labeling-gluten-free-labeling-of-foods.

33. **Commission of the European Communities.** Commission Implementing Regulation (EU) No 828/2014 of 30 July 2014 on the requirements for the provision of information to consumers on the absence or reduced presence of gluten in food. *Official Journal of the European Union.* [Online] [Cited: 01 25, 2017.] http://eur-lex.europa.eu/legal-content/FR/TXT/?uri=CELEX%3A32014R0828.

34. **Australian and New Zealand Government.** Australia New Zealand Food Standards Code – Standard 1.2.7 – Nutrition, health and related claims. *Federal Register of Legislation.* [Online] [Cited: 09 09, 2016.] https://www.legislation.gov.au/Details/F2016C00161.

35. **Lee, Hyun Jung, Anderson, Zach and Ryu, Dojin.** Gluten Contamination in Foods Labeled as ''Gluten-free '' in the United States. *Journal of Food Protection.* 2014, Vol. 77, 10, pp. 1830–1833.

36. **FDA.** *Questions and Answers: Gluten-Free Food Labeling Final Rule.* [Online] [Cited: 01 24, 2017.] http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/Allergens/ucm362880.htm.

37. **La Vieille, Sébastien, et al.** Estimated Levels of Gluten Incidentally Present in a Canadian Gluten-Free Diet. *Nutrients.* 6, 2014, pp. 881-896.

38. **Koerner, T. B., et al.** Gluten contamination in the Canadian commercial oat supply. *Food Additives & Contaminants: Part A.* 2011, Vol. 28, 6, pp. 705-710.

39. **Gélinas, Pierre, et al.** Gluten contamination of cereal foods in Canada. *International Journal of Food Science and Technology.* 2008, Vol. 43, pp. 1245–1252.

40. **Rashid, Mohsin, et al.** Consumption of Pure Oats by Individuals with Celiac Disease: A Position Statement by the Canadian Celiac Association. *Can Journal Gastroenterol.* 2007, Vol. 21, 10.

41. **Health Canada.** Celiac Disease and the Safety of Oats. *Health Canada's Position on the Introduction of Oats to the Diet of Individuals Diagnosed with Celiac Disease (CD).* [Online] 2007. [Cited: 09 09, 2016.] http://hc-sc.gc.ca/fn-an/securit/allerg/cel-coe/oats_cd-avoine_e.html.

42. —. Celiac Disease and Gluten-Free Claims on Uncontaminated Oats. [Online] May 2015. [Cited: 09 09, 2016.] http://www.hc-sc.gc.ca/fn-an/consult/2014-cel-oats-contam-avoine-coel/document-consultation-eng.php.

43. **Comino, Isabel, et al.** Diversity in oat potential immunogenicity: basis for the selection of oat varieties with no toxicity in coeliac disease. *Gut.* 2011.

44. **de Souza, M. Cristina P., et al.** Pure Oats as Part of the Canadian Gluten-Free Diet in Celiac Disease: The Need to Revisit the Issue. *Canadian Journal of Gastroenterology and Hepatology.* 2016, pp. 1-8.

45. **Lundin, K. E. A., et al.** Oats induced villous atrophy in coeliac disease. *Gut.* 2003, Vol. 52, pp. 1649–1652.

46. **Health Canada.** Gluten-free labelling claims for products containing specially produced "gluten-free oats". [Online] May 19, 2015. [Cited: 09 09, 2016.] http://www.hc-sc.gc.ca/fn-an/securit/allerg/cel-coe/avoine-gluten-oats-eng.php.

47. **Thompson, Tricia.** Gluten Contamination of Commercial Oat Products in the United States. *The New England Journal of Medicine.* 2004, Vol. 351, 19, pp. 2021-2022.

48. **Koerner, Terry B, et al.** Validation procedures for quantitative gluten ELISA methods: AOAC allergen community guidance and best practices. *Journal of AOAC international.* 2013, Vol. 96, 5, pp. 1033-1040.

49. **Diaz-Amigo, Carmen and Popping, Bert.** Gluten and gluten-free: issues and considerations of labeling regulations, detection methods and assay validation. *Jounal of AOAC International.* 2012, Vol. 95, 2, pp. 337-348.

50. **Scherf, Katharina Anne and Poms, Roland Ernest.** Recent developments in analytical methods for tracing gluten. *Journal of Cereal Science.* 2016, Vol. 67, pp. 112-122.

51. **Mena, Maria C. , et al.** Comprehensive analysis of gluten in processed foods using a new extraction method and a competitive ELISA based on the R5 antibody. *Talanta.* 2012, Vol. 91, pp. 33-40.

52. **van Eckert, R., et al.** Reactivity of gluten detecting monoclonal antibodies to a gliadin reference material. *Journal of Cereal Science.* 51, 2010, pp. 198–204.

53. **Hammer, Elisabeth.** Gluten-Free : How can you Prove it? *Food Quality Magazine.* [Online] August 2012. [Cited: 09 12, 2016.] http://www.foodsafetymagazine.com/signature-series/gluten-free-how-can-you-prove-it/.

54. **Allred, Laura K. and Ritter, Bruce W.** Recognition of Gliadin and Glutenin Fractions in Four Commercial Gluten Assays. *JOURNAL OF AOAC INTERNATIONAL.* 2010, Vol. 93, 1, pp. 190-196.

55. **Diaz-Amigo, Carmen and Yeung, Jupiter.** Critical Evaluation of Uncertainties of Gluten Testing: Issues and Solutions for Food Allergen Detection. *Pathogens and Toxins in foods: challenges and Interventions.* 2010, pp. 286-300.

56. **Colgrave, Michelle L, et al.** Using mass spectrometry to detect hydrolysed gluten in beer that isresponsible for false negatives by ELISA. *Journal of Chromatography A.* 2014, pp. 105–114.

57. **Codex Alimentarius Commission. Joint Food and Agriculture Organization of the United Nations / World Health Organization Food Standards.** *Report of the Twenty-sixth session of the Codex Committee on Methods of Analysis and Sampling.* 2005.

58. **Koehler, Peter, et al.** AACCI Approved Methods Technical Committee Report: Collaborative Study on the Immunochemical Determination of Intact Gluten using an R5 Sandwich ELISA. *Cereal Foods World.* 2013, Vol. 58, 1.

59. **Don, Clyde, et al.** AACCI Approved Methods Technical Committee Report: Collaborative Study on the Immunochemical Quantitation of Intact Gluten in Rice Flour and Rice-Based Products using G12 Sandwich ELISA. *Cereal Foods World.* 2014, Vol. 59, 4.

60. **Codex Alimentarius Commission.** Report of the thirty-eighth session of the Codex Committee on nutrition and Foods for special dietary uses. *Hamburg, Germany.* 5-9 December 2016.

61. **van Eckert, R., et al.** Towards a new gliadin reference material–isolation and characterisation. *Journal of Cereal Science.* 2006, Vol. 43, pp. 331–341.

62. **Kanerva, Päivi M., et al.** Analysis of barley contamination in oats using R5 and ω-gliadin antibodies. *Journal of Cereal Science.* 2006, Vol. 44, pp. 347–352.

63. **Panda, Rakhi, et al.** Detection and Quantification of Gluten during the Brewing and Fermentation of Beer Using Antibody-Based Technologies. *Journal of Food Protection.* 78, 2015, Vol. 6, pp. 1167–1177.

64. **Méndez, Enrique, et al.** Report of a collaborative trial to investigate the performance of the R5 enzyme linked immunoassay to determine gliadin in gluten-free food. *European Journal of Gastroenterology & Hepatology.* 17, 2005, pp. 1053-1063.

65. **Halbmayr-Jech, Elisabeth, et al.** Gluten in Rice Flour and Baked Rice Products by G12 Sandwich ELISA: First Action 2014.03. *Journal of AOAC International.* 98, 2015, Vol. 1, pp. 103-111.

66. **Rogers, Adrian, Brunner, Kurt and Kraus , Jasmin.** Challenges in Allergen Testing: Spiking and Recoveries. *Spot On: Diagnostic Solutions.* Issue 2.

67. **R-Biopharm.** SureFood® ALLERGEN ID Gluten. [Online] [Cited: 01 25, 2017.] http://www.r-biopharm.com/products/food-feed-analysis/allergens/gliadin-gluten/item/surefood-allergen-gluten.

68. **Dahinden, Isabelle, von Büren, Michael and Lüthy, Jürg.** A quantitative competitive PCR system to detect contamination of wheat, barley or rye in gluten-free food for coeliac patients. *Eur Food Res Technol.* 212, 2001, pp. 228–233.

69. **Monaci, Linda and Visconti, Angelo.** Immunochemical and DNA-based methods in food allergen analysis and quality assurance perspectives. *Trends in Food Science & Technology.* 21, 2010, Vol. 6, p. 272e283.

70. **Koppel, Esther, Stadler, Markus and Luthy, Jurg.** Detection of wheat contamination in oats by polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA). *Z Lebensm Unters Forsch A.* 206, 1998, pp. 399-403.

71. **Sandberg, Martin, et al.** Real Time PCR for the detection and discrimination of cereal contamination in gluten-free foods. *Eur Food Res Technol.* 217, 2003, pp. 344–349.

72. **Fiedler, Katherine L., et al.** Characterization of Grain-Specific Peptide Markers for the Detection of Gluten by Mass Spectrometry. *Journal of Agricultural and Food Chemistry.* 2014, Vol. 62, p. 5835−5844.

73. **Cho, Chung Y., et al.** Multiplex detection of food allergens and gluten. *Anal Bioanal Chem.* 407, 2015, pp. 4195–4206.

74. **Immer, Ulrike and Haas-Lauterbach, Sigrid.** Gluten Detection (Chapter 19). *Molecular Biological and Immunological Techniques and Applications for Food Chemists.* s.l. : John Wiley & Sons, Inc., 2010.

75. **Gluten-free Watchdog Position Statement on Consumer Use of the Nima Sensor to Test Food for Gluten.** ***Tricia Thompson.*** **[Online] 04 23, 2017. [Cited: 08 08, 2017.] https://www.glutenfreewatchdog.org/news/gluten-free-watchdogs-position-statement-on-consumer-use-of-the-nima-sensor-to-test-food-for-gluten/.**

APPENDIX 1 - Comparison of commercially available ELISA test kits

Test kit	Aller-Tek Gluten ELISA	Wheat/Gluten (Gliadin)	Veratox® for gliadin	Veratox® for Gliadin R5	RIDASCREEN® Gliadin
Manufacturer	**ELISA Technologies**	**Morinaga Institute**	**Neogen**	**Neogen**	**R-Biopharm**
Method	Sandwich ELISA	Sandwich ELISA	Sandwich ELISA	Sandwich ELISA	Sandwich ELISA
Type of results	Quantitative	Quantitative	Quantitative	Quantitative	Quantitative
Reference material	Wheat gluten and barley standard available	NIST SRM 1567a - Wheat Flour	Gliadin G3375 (Sigma-Aldrich)	Gliadin G3375 (Sigma-Aldrich)	PWG gliadin
Antibody	Skerritt monoclonal (401.21)	Anti-gliadin polyclonal antibody	Skerritt monoclonal (401.21)	R5 monoclonal	R5 monoclonal
Extraction protocol	40% ethanol with extraction mix	Specific extraction solution	40% ethanol or cocktail solution	60% ethanol or cocktail solution	60% ethanol or cocktail solution
Interval of quantification (ppm of gluten)	5–80 ppm	0.26–68 ppm	10–100 ppm	5–80 ppm	5–80 ppm
Limit of detection (LOD) (ppm of gluten)	5 ppm	0.26 ppm	10 ppm	2.2 ppm	1 ppm
Validation	AOAC-RI 081202	Interlaboratory study supported by the Japanese Ministry of Health, Labour and Welfare	Not available	AOAC-RI 061201	AAOAC-OMA 2012.01 AOAC-RI 120601 AACCI 38.50.01 Codex Alimentarius Reference Method (Type I)
Usage	Processed and unprocessed food products, environmental surfaces	Processed and unprocessed food products, environmental surfaces	Processed and unprocessed food products, environmental surfaces	Processed and unprocessed food products, environmental surfaces	Processed and unprocessed food products, environmental surfaces
Other specifications	- It underestimates barley.	- High recovery of gluten for processed foods. - Used by the FDA for gluten-free products (in tandem with RIDASCREEN® Gliadin kit). - It underestimates barley and rye.	- It is generally no longer in use, since it has been replaced by the R5 test kit.	- It overestimates barley and rye.	- It overestimates barley. - Used by the FDA for gluten-free products (in tandem with Morinaga Wheat/Gluten [Gliadin] kit).

Test kit	AgraQuant®	RIDASCREEN® Gliadin competitive	GlutenTox® Pro	GlutenTox® Sticks Plus	EZ Gluten®
Manufacturer	**Romer Labs®**	**R-Biopharm**	**Biomedal Diagnostics**	**Biomedal Diagnostics**	**ELISA Technologies**
Method	Sandwich ELISA	Competitive ELISA	Lateral flow device	Lateral flow device	Lateral flow device
Type of results	Quantitative	Quantitative	Semi-qualitative	Semi-quantitative or quantitative	Qualitative
Reference material	VWG gliadin	Hydrolysate mixture of wheat, rye and barley	N/A	N/A	N/A
Antibody	G12 monoclonal	R5 monoclonal	G12 monoclonal	G12 monoclonal	Skerritt monoclonal (401.21)
Extraction protocol	60% ethanol or cocktail solution	60% ethanol	Universal Gluten Extraction Solution (UGES)	Universal Gluten Extraction Solution (UGES)	Extraction buffer
Interval of quantification (ppm of gluten)	4–200 ppm	10–150 ppm	N/A	8–85 ppm	N/A
Limit of detection (LOD) (ppm of gluten)	2 ppm	2.7 ppm	Samples: 5, 10, 20 or 40 ppm Swabs: 16 ng/16 cm^2	Samples: 3, 10, 20, 30 or 100 ppm Swabs: 16 ng/16 cm^2	Samples: 10 ppm Swabs: 1 µg/25 cm^2
Validation	AACCI 38.52.01 AOAC-OMA 2014.03	AACCI 38.55.01 AOAC-OMA 2015.05	AOAC-RI 061502	Validated by FAPAS and AESAN only (Spain)	AOAC-RI 051101
Usage	Processed and unprocessed food products, environmental surfaces	Hydrolyzed and fermented food products	Lightly processed and unprocessed food products, environmental surfaces	Lightly processed and unprocessed food products, environmental surfaces	Lightly processed and unprocessed food products, environmental surfaces
Other specifications	- It detects some oat varieties suspected to trigger a response in people with celiac disease.	- It can also be used to detect intact and unprocessed proteins, but it will be less specific. - It cannot be used with the cocktail solution.	- It should not be used for matrices with a high content of polyphenols and tannins. - It detects some oat varieties (suspected to trigger a response in people with celiac disease).	- It detects some oat varieties (suspected to trigger a response in people with celiac disease). - Quantitative results can be obtained in combination with the GlutenTox® Reader.	- It underestimates barley.

Test kit	AllerFlow Gluten	Reveal® 3-D	Alert® for Gliadin R5	RIDA®QUICK Gliadin	AgraStrip®
Manufacturer	**Hygiena**	**Neogen**	**Neogen**	**R-Biopharm**	**Romer Labs®**
Method	Lateral flow device	Lateral flow device	Lateral flow device	Lateral flow device	Lateral flow device
Type of results	Qualitative	Qualitative	Qualitative	Qualitative	Semi-qualitative
Reference material	N/A	N/A	N/A	N/A	N/A
Antibody	G12 monoclonal	R5 monoclonal	R5 monoclonal	R5 monoclonal	G12 monoclonal
Extraction protocol	Extraction solution with reducing and dissociating agents	Extraction solution	80% ethanol or cocktail solution	60% ethanol or cocktail solution	60% ethanol
Interval of quantification (ppm of gluten)	N/A	N/A	N/A	N/A	N/A
Limit of detection (LOD) (ppm of gluten)	Swabs: 5 µg/100 cm^2	Samples: 5 ppm Swabs: 80 µg/100 cm^2	Sample: 20 ppm Swabs: 1–2 µg/100 cm^2	Samples: 5 ppm Swabs: 2–4 µg/100 cm^2	Samples: 5, 10 or 20 ppm Rinse water: 35 ppb Swabs: 4 µg/25cm^2
Validation	Internal validation	Internal validation	Internal validation	AOAC-OMA 2015.16 (AACCI in process)	AOAC-RI 061403
Usage	Environmental surfaces and rinse water	Environmental surfaces and rinse water	Lightly processed and unprocessed food products, environmental surfaces and rinse water	Lightly processed and unprocessed food products, environmental surfaces	Lightly processed and unprocessed food products, environmental surfaces and rinse water
Other specifications	- Similar to Biomedal's GlutenTox®. - It detects some oat varieties (suspected to trigger a response in people with celiac disease).				- It detects some oat varieties (suspected to trigger a response in people with celiac disease).