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Nutritional and Analytical Approaches of Gluten-Free Diet in Celiac Disease

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Chapter 1
Celiac Disease and Gluten-Related Disorders

Idoia Larretxi, Virginia Navarro, and Itziar Churruca

Abbreviations

AGA	Antigliadin antibodies
CD	Celiac disease
DGP	Deamidated antigliadin
EMA	Endomysial antibodies
ESPGHAN	European Society for Pediatric Gastroenterology, Hepatology and Nutrition
FDEIA	Food-dependent, exercise-induced anaphylaxis
FODMAPS	Fermentable oligo-di-mono-saccharides and polyols
GF	Gluten-free
GFD	Gluten-free diet
GFP	Gluten-free products
HLA	Human leucocyte antigen
IEL	Intraepithelial lymphocytes
IL	Interleukin
NCGS	Non-celiac gluten sensitivity
NGF	Naturally gluten-free
TG	Transglutaminase; peptides
WA	Wheat allergy
WDEIA	Wheat-dependent, exercise-induced anaphylaxis

Gluten is a complex mixture of proteins found in the endosperm of cereal, kernels, and grains in the *Triticeae* family (wheat, barley, rye, and their varieties) and, probably, from the *Aveneae* family (oats). Gluten comprises approximately 80% of these cereal proteins and includes two fractions: glutenins and

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prolamins. Although prolamins, soluble in alcohol, are the toxic fraction for celiac sufferers, celiac disease (CD) is usually known as gluten sensitivity instead of prolamin sensitivity.

A description of prolamins depends on the cereals' origin. In the case of wheat, they are called gliadins; regarding barley and rye, they are called hordeins and secalins, respectively, and the prolamins from oat are known as avenins.

Gluten is used as a gelling agent and emulsifier that binds water molecules and therefore works as a structural element; among other functions, it acts as a binding element that provides elasticity. It also provides adequate sensorial and technological characteristics, such as cohesiveness, firmness, moisture, and uniformity, to baked products. Because of these characteristics and its value as a source of protein in human nutrition, gluten is one of the essential ingredients for the food industry.

1.1 Celiac Disease

The definition of CD has been modified over the past two decades. Regarding the latest European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) guidelines, CD is defined as a systemic immune-mediated disorder caused by gluten and related prolamins in genetically predisposed individuals [1]. In CD a combination of gluten-dependent clinical manifestations, specific antibodies, certain antigen-presenting molecules (HDL-DQ2 and HDL-DQ8 haplotypes), and enteropathy merge, with varying degrees of severity. This definition modified the prior concept of rare enteropathy, and CD has come to be considered a common and extended pathology, with multi-organ manifestations.

1.1.1 Epidemiology

In recent years, the epidemiology and symptomatology of CD have both considerably changed. Historically, this rare gastrointestinal disorder mainly affected the Caucasian population, and it usually appeared during the early years of life [2]. Nowadays, it is known that CD affects all age groups, with a female-to-male ratio of 2:1. Available data suggest that its prevalence has significantly increased in the past 20 years, and it is currently one of the most common life-long heritable food intolerances worldwide [3]; this could, in part, be not only because of better diagnostic tools, but also because of the deeper understanding of the disease and its symptoms by health professionals.

The prevalence of CD is not well established, but in most of the Western world it affects approximately 1–2% of the population – even if wide differences have been found among countries [2, 4]. Peña and Rodrigo [4] found significant differences between Northern Europe and Mediterranean countries, with a greater prevalence in the former (0.016–2.4%). The literature has shown that the average prevalence of CD in other developed countries, such as the United States, Australia, and New Zealand,

is similar to that seen in Europe [2, 4]. On the other hand, Catassi et al. [5] found a prevalence of about 5.6% in the Western Sahara population in 1998. This might be linked to higher gluten consumption and to higher frequencies of HLA-DQ2 and HLA-DQ8 haplotypes in this population [6]. On the contrary, the prevalence of this condition in the Far East is almost anecdotal, probably because of the low consumption of gluten and the low prevalence of HLA-predisposing genotypes [2, 4, 6]. The epidemiology of CD is considered to have the characteristics of an iceberg, where symptomatic (both gastrointestinal and extraintestinal) CD is on the top, and a significant percentage of patients with silent or atypical CD are still undetected [7, 8].

It has been observed that the probability of developing CD increases in certain risk groups, such as first-degree relatives and patients with genetic alterations such as Down, Turner's, and William's syndromes, or selective immunoglobulin A (IgA) deficiency. It has also observed an increased CD prevalence in people suffering from autoimmune diseases, such as type 1 diabetes mellitus, autoimmune thyroid disease, chronic autoimmune liver disease, youthful chronic arthritis, Sjögren's syndrome, kidney disease, or idiopathic dilated cardiomyopathy. The understanding of these risk groups is important, and even though the conditions may not be pathogenically related to CD, the high prevalence of the disorder in these groups makes testing them serologically very necessary.

1.1.2 Pathogenesis and Clinical Presentation of CD

CD development requires the interaction of genetic, immunological, and environmental factors.

When gluten that is present in food is ingested, it is metabolized into peptides (prolamin). Taking wheat as an example, the gliadin crosses the epithelial barrier, which has an increased permeability in these patients, and it reaches the lamina propria. At this level, the peptide is deamidated by a tissue transglutaminase (tTG2), which allows its recognition by HLA DQ-2 or DQ8. The antigen-presenting cell presents deamidated gliadin to CD4 T cells. These immune cells, on the one hand, secrete pro-inflammatory cytokines that cause damage (villous atrophy and crypt hyperplasia) to the enterocytes but, on the other hand, activate B cells that produce antibodies against gliadin or other toxic prolamin. At the same time, deamidated gliadin induces IL15 production by the enterocyte, which stimulates the proliferation of intraepithelial lymphocytes (IEL) (Fig. 1.1).

Various factors have been proposed as possible triggers of later development of CD. On the one hand, feeding during early childhood has been studied. There is not enough evidence to provide general recommendations about breastfeeding (duration and breastfeeding at the time of gluten introduction) to prevent CD [10, 11]. With regard to the association of the timing of gluten introduction and later development of CD, various results have been proposed [10]; even so, the ESPGHAN recommends avoiding both an early (<4 months) and late (≥7 months) introduction of gluten, and introducing it while the infant is still being breastfed.

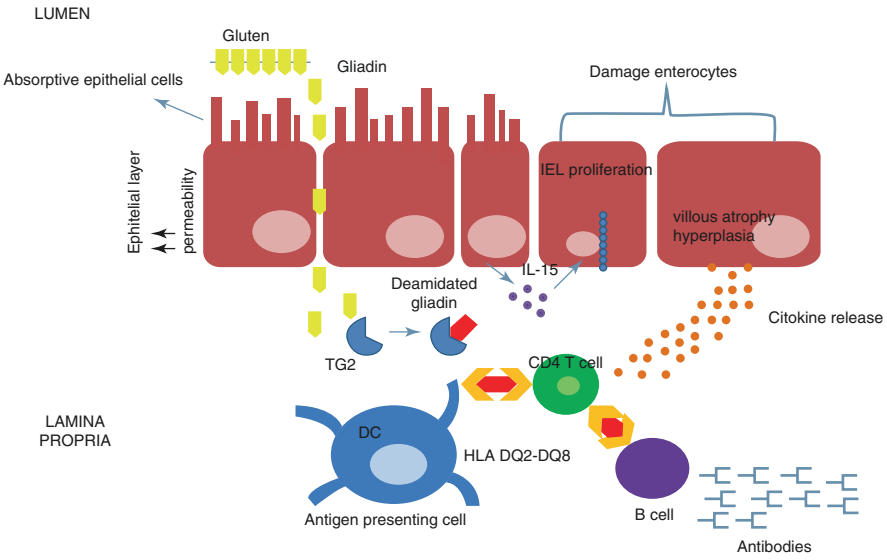

Fig. 1.1 Illustration of CD pathogenesis (Adapted from Moscoso and Quera [9]). *IEL* intraepithelial lymphocytes, *TG2* transglutaminase 2, *DC* dendritic cell

Although a gluten-containing diet only supposes a risk of developing CD for persons carrying at least one of the CD risk alleles, recommendations are applied to all infants. With regard to the amount of gluten, avoiding the consumption of large quantities of gluten at the beginning of gluten introduction has been proposed; however, optimal amounts have not yet been established [11]. On the other hand, the relationship between CD and some gastrointestinal infections have been assessed; it seems that such infections increase the risk of celiac disease autoimmunity in people with a genetic susceptibility to CD [12], yet are reduced in people vaccinated against rotavirus. In the case of *Helicobacter pylori*, it has been suggested that gastric infection can confer protection against CD [13]. In summary, the literature indicates potential interactions among infections, genetic factors, and diet in the etiology of this pathology, but more studies are needed to shed light on this issue.

There is a wide variety of signs and symptoms, which makes it difficult for physicians to diagnose the disorder early and correctly. There are even no symptoms [3] in some cases and, when they appear, they can be distinguished as either gastrointestinal or extra-intestinal symptoms.

Symptomatic forms differ considerably, depending on the age, with gastrointestinal signs more characteristic in childhood, whereas adults endure extraintestinal symptoms (Table 1.1). In fact, less than 25% of the adult population diagnosed with CD presented with gastrointestinal symptoms [14].

As mentioned, symptomatic forms are just the tip of the iceberg regarding the broad spectrum of gluten sensitivity, as several epidemiological studies determined that CD without classic symptoms is more common than the symptomatic form [15].

Table 1.1 Signs and symptoms of symptomatic CD

Gastrointestinal symptoms		Extraintestinal symptoms	
Childhood	Adulthood	Childhood	Adulthood
Lack of appetite Chronic diarrhea Constipation Recurrent abdominal pain Vomiting Abdominal distension	Irritable bowel syndrome Constipation Recurrent abdominal pain Dyspepsia Chronic diarrhea Malabsorptive diarrhea	Arthritis Follicular keratosis Irritability/apathy Menstrual irregularities/ delayed menarche Stunted growth Introversion/sadness Failure to thrive Osteopenia Enamel hypoplasia Dermatitis herpetiformis Cold sores Headaches Malnutrition/anemia Muscle hypotrophy	Anemia Early menopause Osteoporosis/arthritis Paresthesia Ataxia/epilepsy/peripheral neuropathy Irritability/apathy Dermatitis herpetiformis Cold sores/peripheral edema

Some years ago, at the bottom of the iceberg, previously unknown symptoms were characterized as silent CD, latent CD, or potential CD [1].

Symptomatic CD is characterized by the onset of digestive, extra-digestive symptoms and/or associated diseases such as dermatitis herpetiformis. Silent CD or asymptomatic CD is a form of the disease in which the patient presents positive CD-specific antibodies, compatible HLA, and pathogenic small bowel as in classic CD. However, there are not enough symptoms to warrant clinical suspicion of CD. These cases are normally discovered by a serum analysis when belonging to any of the risk groups, such as being first-degree relatives.

Latent CD is defined as the presence of compatible HLA but without enteropathy (found in a normal small bowel biopsy) in a patient who has had, or will have, a gluten-dependent enteropathy at some point in his or her life. The patient may or may not have symptoms and may or may not have CD-specific antibodies.

Potential CD is characterized by the presence of positive specific antibodies and compatible HLA, but without abnormalities in duodenal biopsies. The patient may or may not have signs and symptoms and may or may not develop a gluten-dependent enteropathy later on.

1.1.3 Diagnosis and Treatment

Despite the increasing number of positive diagnoses for CD, this condition is frequently unrecognized. Information on variable clinical presentations has shown that only a clinical diagnosis (symptoms, associated diseases, and risk groups) is not enough for a suitable diagnosis of the disorder [16]. The advances and accuracy of new immunological diagnosis tests and the information on the genetic predisposition to CD have allowed the ESPGHAN to review the diagnostic criteria for this

Table 1.2 ESPGHAN diagnosis criteria [1]

CD forms	Genetic (HLA DQ2, DQ8)	Pathological anatomy	Antibodies (anti-tTG, EMA, or anti-DGP)	Symptoms
Symptomatic	Compatible	+	+	+
Silent	Compatible	+	+	−
Latent	Compatible	−[a]	±	±
Potential	Compatible	−	+	±

[a]Could be + at some point of the evolution

disease [1]. ESPGHAN has determined that not only clinical suspicion is necessary for the diagnosis of CD, but also serological tests, genetic studies, and intestinal biopsy and histology (Table 1.2).

1.1.3.1 In Clinical Practice, CD Is Diagnosed Through Various Pathways Divided into Four Groups: Clinical Suspicion

Suspicion of CD emerges when the patient shows any of the wide range of symptoms and signs of the disease, as well as when she/he has a CD-associated disease or belongs to a high-risk group.

1.1.3.2 Serological Tests

CD-specific antibody tests are useful as indicators of CD. IgA autoantibodies against tissue transglutaminase are the test of choice for most populations. Currently, the IgA-type anti-tissue transglutaminase (anti-tTG) and IgA-type antiendomysial (EMA) antibodies, which are highly sensitive and specific for the diagnosis of CD, are the two most often-used biomarkers. In the last few years, these two tests have replaced the one that was based on anti-gliadin antibodies (anti-AGA), with less specificity.

Together with these serological tests, it is important to assess the total IgA of serum, given that up to 2–3% of patients with CD show a selective IgA deficiency, which may lead to false negatives – especially in adults. In these cases, an IgG-based test should be used, in particular IgG-type anti-tTG or IgG-type deamidated antigliadin peptide (anti-DGP) antibodies. Its use is also justified in toddlers less than 2 years old, as they also show a high rate of false positives for IgA antibodies [9].

Anti-DGP antibodies were introduced because they showed a similar sensitivity and specificity to the IgA-type anti-tTG antibodies. They are useful mainly for detecting CD in cases of IgA deficiency and in pediatric patients up to 2 years old, whose IgA antibody levels may lead to false negatives [17]. Moreover, it has been suggested that anti-DGP IgG assays may be useful to monitor mucosal damage and histological improvement in CD patients on a strict GFD [18].

Finally, it is noteworthy that negative results of these markers do not rule out the diagnosis; sometimes it is necessary to perform other tests when the suspicion is high. For example, a symptomatic first-degree relative with negative serology should be examined via a duodenal biopsy.

1.1.3.3 Genetic Test

Ninety-five percent of patients with celiac disease are HLA-DQ2 positive, and 5% HLA-DQ8 positive, while in the general population only 20–30% of individuals express these haplotypes. Therefore, the absence of HLA-DQ2 and HLA-DQ8 makes the diagnosis of CD unlikely [19].

1.1.3.4 Histological Analysis of Duodenal Biopsies

A biopsy of the proximal duodenum or jejunum has been used for the definitive diagnosis of CD. This test involves identifying structural changes and cell abnormalities of small intestine mucosa. According to ESPGHAN's latest guidelines, it is recommended to take at least five samples (one from the bulb and four from the second or third portion of the duodenum) because the lesions may be patchy [1]. Biopsies must be taken either when patients are on a gluten-containing diet (at least 3 g of gluten/day for 2 weeks) or after a gluten challenge [20].

It is highly recommended that a pathologist´s report include a description of the orientation of the biopsy [21]; the presence/absence of normal villi; the degree of atrophy and crypt elongation; the villus-crypt ratio; the number of intraepithelial lymphocytes (IELs); and the interpretation according to the Marsh-Oberhuber classification, which is widely used in clinical practice [15, 22].

Marsh classification of gluten-induced small-intestinal damage has four stages; Stage 0 indicates a normal mucosa, whereas Stage 3 includes a destructive lesion of a villus, including atrophy. This stage is the classic CD lesion. Stage 4 shows hypoplastic lesions that include full atrophy with crypt hypoplasia. This 4th stage is not described by World Gastroenterology Organization´s Global Guidelines [23]. These Guidelines described only first three stages.

Histological presentation is compatible with the disease, but it is not specific. Hence the serological test and genetic study are needed to verify the diagnosis.

Although biopsy today is still major evidence of the diagnosis, the possibility of making a correct diagnosis of CD without intestinal biopsy is nevertheless being studied. In 2012, the ESPGHAN, in its new diagnostic guidelines, suggested that it is possible to avoid the biopsy in children whenever some requirements are achieved: suggestive clinic of CD; IgA antibodies (IgA anti-tTG) that exceed 10 times the used reference value; verification with anti-endomysial (EMA) antibodies in a different sample; and a positive genetic test (DQ2/DQ8 markers) [1].

The CD can be either silent or latent for several years. Therefore, careful clinical monitoring is required, mainly for first-degree relatives of patients and people in high-risk groups, including serological markers (IgA antibodies) and, if appropriate, an intestinal biopsy.

1.1.4 Treatment

Currently, the only effective treatment for CD is a strict, lifelong gluten-free diet (GFD). GFD consists of excluding wheat, barley, triticale, and rye from the diet, as well as all the food derived from these cereals. Some oat varieties do not have a harmful effect on celiac patients, but other varieties can produce the toxicity [24]. Furthermore, many products containing oats can be contaminated by traces of flour of other cereals, which suggest limiting their use. For these collectives, a balanced diet should be based on naturally gluten-free (NGF) cereals such as rice, corn, quinoa, millet, and other pseudo-cereals and pulses. The diet will be rounded out with other NGFs such as vegetables and fruits, meat, fish, eggs, and milk and dairy products. It is recommended not to overuse the specific gluten-free products (GFP), as they used to have a different nutritional profile compared to their gluten-containing homologues. We found that these GFPs are usually fattier and contain less fiber [25].

Following a GFD is of vital importance, because after a few weeks or months of dietetic treatment, the symptoms of the disease are improved or even disappear in some cases. Between 6 and 12 months later, serological normalization is achieved and around 2 years are needed to attain complete recovery of the intestinal villus. In adult patients, the clinical improvement is usually slower than in children. Besides, it has been shown that following a strict GFD minimizes the risk of long-term complications such as osteoporosis, cancer of the intestines, and other associated autoimmune disorders (e.g., type 1 diabetes or thyroid disease). Clinical monitoring of patients is also important in order to observe the evolution of symptoms, control growth in children, and assessment of adherence to GFD. Developing complications when GFD is strictly followed is very unlikely; indeed, transgressions on the GF diet or undiagnosed conditions can arise, as can complications in a medium or long term, such hyposplenism, exocrine pancreatic insufficiency, osteoporosis, chronic ulcerative jejunoileitis, microscopic colitis, bacterial overgrowth, non-Hodgkin's lymphoma, and gastrointestinal carcinomas.

It is important to point out that even small amounts of gluten can be harmful to the intestinal villus. This shows the importance of avoiding gluten in diet, but that following a strict GFD is not easy, as gluten-containing grains and food are widespread. Moreover, aspects such as higher cost, poor sensorial quality, and low availability of gluten-free products and cross-contamination with gluten-containing cereals may enhance the difficulty in sticking to a GFD. As will be seen in Chap. 2, labeling rules differ from country to country, even if huge advances have been made during the last few years that reflect the awareness of authorities for the regulation of the labeling of manufactured products.

1.2 Gluten-related Disorders Other Than Celiac Disease

Even if during the last decade, gluten-related disorders have been found to have no relation to the diagnosis of CD, it is necessary to take into account the current knowledge about the toxic effects of gluten and the various disorders it is responsible for.

Gluten is not only the causative agent of CD, but it may be also for other pathologies such as wheat allergy (WA), dermatitis herpetiformis, gluten ataxia, or peripheral neuropathy [26]. Besides, in recent years a disease called "non-celiac gluten sensitivity" (NCGS) has gotten more attention. While CD, gluten ataxia, and peripheral neuropathy have an autoimmune basis, wheat allergy is based on altered immune reaction and the source of NCGS is still unclear [27]. A diagram for gluten-related disorders' nomenclature is depicted in Fig. 1.2.

1.2.1 Wheat Allergy (WA)

WA is an adverse immunologic reaction to wheat proteins. Depending on the underlying mechanisms and the exposure type, WA could be classified into classic food allergy; wheat-dependent, exercise-induced anaphylaxis (WDEIA); occupational asthma (baker's asthma) and rhinitis; and contact urticaria (Fig. 1.2) [27]. The most frequent form of the disease is respiratory (baker's asthma), while the dietary allergy form is less widespread.

Allergens responsible for the respiratory form are slightly different from those related to food allergy. α-amylase inhibitors are the group of proteins responsible mainly for the respiratory form. However, other proteins have been reported to bind IgE from patients with baker's asthma, like germ agglutinin, peroxidase, and non-specific lipid transfer proteins (LTPs). The latter two are also related to the food

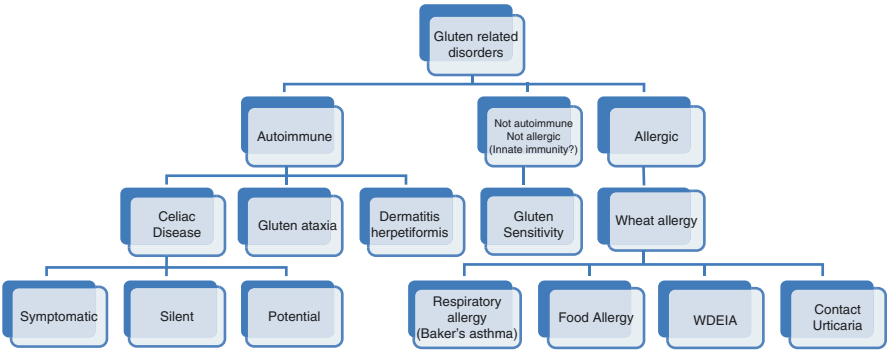

Fig. 1.2 Nomenclature and classification of gluten-related disorders (Adapted from Sapone et al. [27])

allergy form [28]; nevertheless, most patients with respiratory allergy to grasses consume cereals normally. It is interesting to note that deamidated forms of gluten (produced for their special technological properties) cause allergies too, but they are considered separate entities from WA [29].

Wheat-induced food allergies can be classified into two groups: WDEIA, and other allergic responses, such as urticaria, atopic dermatitis, and anaphylaxis. The major allergen of WDEIA is a specific type of gliadin (ω_5-gliadin), whereas other allergic responses seem to be related to a range of wheat proteins [27]. Studies of purified proteins using IgE-specific assays with patients' sera showed that all patients with anaphylaxis or WDEIA, and 55% of those with urticaria, produced IgE to ω_5-gliadins [28]. Other relatively well-documented allergens include α-amylase inhibitors, the response to which is associated with food allergy in children with atopic dermatitis. Wheat allergy prevalence varies, depending on the age and region, from 0.4% to 1% [30].

Food-dependent, exercise-induced anaphylaxis (FDEIA) is a peculiar form of food allergy in which food intake alone does not induce any symptom. Wheat is the predominant causative agent for FDEIA, and then it is called WDEIA; in this case, it is an allergic reaction caused by ingesting wheat, followed by physical exercise. Some other variables could be implicated, such as non-steroidal anti-inflammatory drugs (NSAIDS), alcohol, stress, or infections [31]. Since both the amount ingested and the extent of exercise may vary from individual to individual, diagnosis becomes difficult. The reaction lasts several hours, and symptoms can change significantly. These include local or generalized [31]. Even if WDEIA is rare, it is difficult to diagnose, and the fatal consequences it causes deserve attention.

On the other hand, classic wheat allergy symptoms may include itching in the mouth; swelling of lips and tongue; hives; eczema; rhinitis; tightening of the throat or trouble breathing; drop in blood pressure; gastrointestinal symptoms such as vomiting, diarrhea, or abdominal cramps and pain; and, if severe, anaphylaxis [32].

Skin-prick tests are first-level diagnostics for WA. Nevertheless, they are prone to technical errors and false positives in some circumstances. The measurement of specific IgE levels is an alternative for the prediction of symptomatic allergies, but wheat-specific IgE is highly non-specific, detects only part of the wheat protein fractions, and the level required for clinical response is high [30]. Figure 1.3 shows the algorithm for the differential diagnosis of gluten-related disorders, adapted from Sapone et al. [27].

1.2.1.1 Non-celiac Gluten Sensitivity (NCGS)

Although this disorder was first described in 1978 [34], nowadays it is gaining more interest, since GFD has been seen to be useful for the alleviation of symptoms of some non-celiac and non-wheat allergy disorders. In fact, it has been suggested that NCGS is a broad term that includes various clinical entities related to gluten. It is possible that innate immunity and intestinal permeability are implicated in this

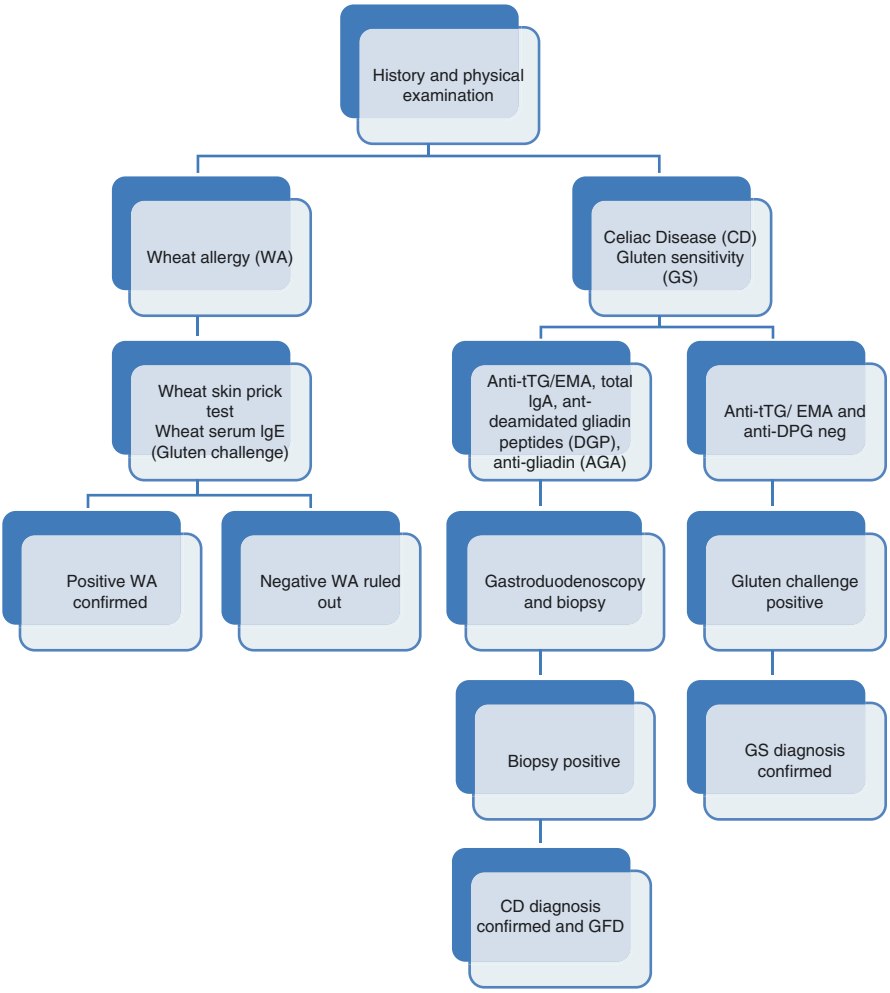

Fig. 1.3 Algorithm for the differential diagnosis of gluten-related disorders [33]. *EMA* endomysial antibodies, *tTG* tissue transglutaminase, *DPG* deamidated gliadin peptides

disorder [27, 35]; nevertheless, it may be that gluten is not the only responsible agent, or not responsible at all, as there is also some debate about the role that non-gluten molecules such as FODMAPS (fermentable oligo-di-mono-saccharides and polyols) play in this syndrome [26, 36]. It is not easy to identify the agent, since gluten-rich products are also usually rich in FODMAPS. In fact, some authors propose replacing the term "NCGS" with "wheat intolerance syndrome" [26, 32].

So far, the term NCGS applies to patients who do not meet the criteria for CD or WA, but react to gluten intake by intestinal (similar to irritable bowel syndrome), and/or extra-intestinal symptoms that mostly occur soon after ingesting gluten-containing foods and disappearing quickly with a strict GFD [26]. In Table 1.3, differences

Table 1.3 Main differences between CD, NCGS and wheat allergy [27, 30, 37]

	Celiac disease	NCGS	Wheat allergy
Onset of symptoms since exposure	Week to years	Hours to days	Minutes to hours
Basis	Disturbances in the acquired immune response to gluten depend on the combination of HLA-DQ2 and HLA-DQ8	Innate immunity?	IgE-mediated
Symptoms	Classical intestinal (e.g., chronic diarrhea, weight loss) or non-classical extra-intestinal (e.g., anemia, osteoporosis, neurological disturbances) features. Silent forms have been described	Resemble CD but with prevalence of extra-intestinal symptoms, such as behavioral changes, bone or joint pain, muscle cramps, leg numbness, weight loss, and chronic fatigue	Wheat-dependent, exercise-induced anaphylaxis (WDEIA); occupational asthma (baker's asthma) and rhinitis; and contact urticaria, atopic dermatitis, gastrointestinal symptoms and anaphylaxis
Morbidity	1%	Unknown (possibly 0.6–6%)	0.4–1%
Antibodies in serum	tTG, EMA, DGP, AGA primarily in the IgA class, less frequently in the IgG class	In 50%: IgG-AGA	sIgE for wheat sIgE for w5-gliadin (in anaphylaxis) in 25%: IgG-AGA
Histology of duodenal mucosa	Marsh I-IV, prevalent Marsh III - IV	Marsh 0,I	Marsh 0,I,II
Atrophy of duodenal villi	Present	Absent	May be present

tTG tissue transglutaminase, *EMA* endomysium antibodies, *DGP* deamidated gliadin peptide, *AGA* antigliadin antibodies

between CD, wheat allergy, and NCGS are detailed. Extra-intestinal symptoms vary from study to study, but some of them are unspecific, such as the lack of well-being or fatigue; others are psychiatric or neurological (anxiety, depression, schizophrenia, autism, attention deficit disorder, hyperactivity, sleep problems, etc.); and still others as dissimilar as skin rash, headache, or muscular problems [26].

It is difficult to assess the prevalence of NCGS due to the lack of objective diagnostic criteria. There are no specific biomarkers, and diagnosis is made by the exclusion of wheat allergy and CD, and the effect of the withdrawal of gluten from the diet (Fig. 1.3). Moreover, many patients are self-diagnosed and start a GFD without medical advice, so some authors think that the incidence is even higher than in CD and WA [37]. Nevertheless, it is possible that a GFD followed with no exclusion diagnosis underestimates CD prevalence. What seems to be clear is that it is more common in females and in young/middle-aged adults [38]. As mentioned, the expression of HLA-DQ2 or DQ8 genes are directly related to celiac disease (they

are present in 95% of celiac patients). In the case of NCGS, 50% of patients express them, whereas 30% of healthy people do [37].

More research is needed for the clear classification of wheat- and gluten-related diseases and for the identification of the causative agents. NCGS overlaps with CD, WA, and irritable bowel syndrome, lacking diagnostic criteria or biomarkers. Besides, epidemiology, diagnosis, and the efficacy of a GFD are still controversial. Moreover, labelling minor forms of CD as NCGS is a matter of concern, as both diseases have different levels of dietary restriction and prognosis if untreated [36].

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Chapter 2
Gluten: General Aspects and International Regulations for Products for Celiac People

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Abbreviations

CD	Celiac disease
FALCPA	Food Allergen Labelling and Consumer Protection Act of 2004
FDA	U.S. Food and Drug Administration
Gln	Glutamine
GMP	Good manufacturing practice
HMW	High molecular weight
kDa	kilodalton
LMW	Low molecular weight
MALDI-TOF MS	Matrix-Assisted Laser Desorption Ionization – Time of Flight Mass Spectrometry
ppm	parts per million (mg/kg)
Pro	Proline
PWG	Working Group on Prolamin Analysis and Toxicity
RP-HPLC	Reversed-phase high-performance liquid chromatography
TTB	Alcohol and Tobacco Tax and Trade Bureau
USDA	United States Department of Agriculture

2.1 Introduction

Gluten is a complex mixture of proteins found in cereal kernels from the *Triticeae* family. This group includes wheat (*Triticum aestivum L.*), barley (*Hordeum vulgare L.*), and rye (*Secale cereale L.*), which are evolution-related and contain homologous peptide

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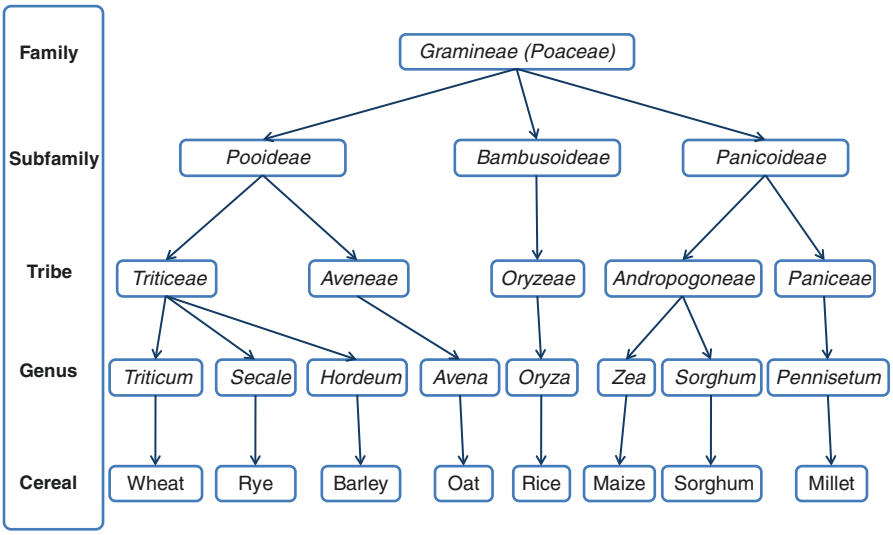

Fig. 2.1 Taxonomic relationship of cereals (Modified from Belitz et al. [2])

Table 2.1 Protein distribution (%) and designations of fractions separated by Osborne

Fraction	Wheat	Rye	Barley	Oat	Corn	Rice	Millet
Albumins	14.7 Edestin	44.4	12.1	20.2	4.0	10.8	18.2
Globulins	7.0 Leukosin	10.2	8.4	11.9 Avenalin	2.8	9.7	6.1
Prolamins	32.6 Gliadin	20.9 Secalin	25.0 Hordein	14.0 Avenin	47.9 Zein	2.2 Oryzin	33.9 Cafirin
Glutelins	45.7 Glutenin	24.5 Secalinin	54.5 Hordenin	53.9	45.3 Zeanin	77.3 Oryzenin	41.8

Adapted from Belitz et al. [2]

groups. Although oats belong to the same sub-family, they come from the *Aveneae* family (Fig. 2.1). There is an ongoing debate concerning the toxicity of oat prolamins, which present some characteristics that are different than those from *Triticeae* [1].

In 1907 Osborne [3] divided cereal proteins into four groups according to their solubility: water-soluble albumins; salt-soluble globulins (0.4 M); 60–70% aqueous ethanol-soluble prolamins; and glutelins, which are soluble in dilute acids or bases, detergents, and reducing agents. Each fraction takes different names, depending on the cereal from which it comes, and the content varies from one cereal to another (Table 2.1). Rye has the highest albumin content, and the prolamin content is highest in corn and wheat, whereas oat and rice have the lowest content, and glutelins are the major property in rice and oats.

Albumins and globulins are derived from the original cytoplasm of the cell and from other sub-cellular fractions, and they have metabolic and structural functions because of the presence of enzymes. Prolamins and glutelins are both proteins whose function is storing nitrogen, carbon, and sulfur in the endosperm of the kernel [2, 4]. The term "prolamin" is used due to its high content in the amino acid

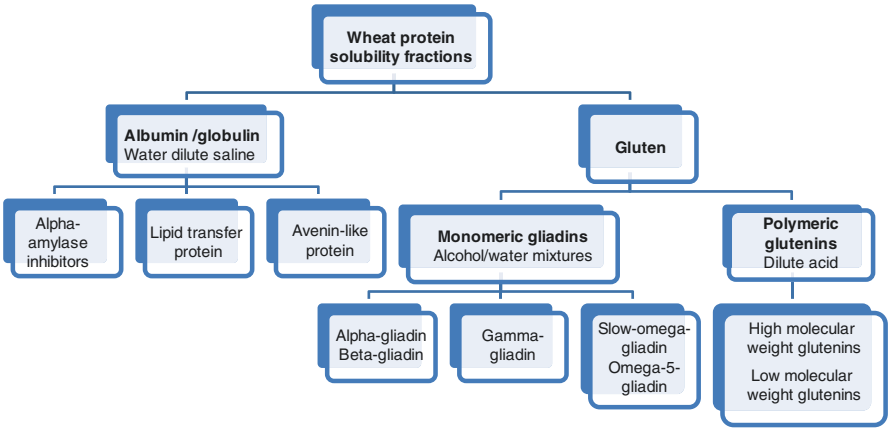

Fig. 2.2 Wheat protein classification

proline (Pro), and "glutelin" is used because of the content of glutamine (Gln). Proline has the ability to make β-turns, which has been shown to be more efficient than α-turns in packing proteins into a small space. This characteristic is very useful for storing amino acids, but the tight structure that is created hinders the hydrolyzation of the prolamin [5].

Gluten is a complex protein formed by a mixture of prolamins and glutelins at a ratio of 1:1 that represents around 80% of the total proteins of most cereals. These proteins can be found in cereals either as monomers or as oligomers and polymers linked by disulphide bonds – something that causes different physicochemical properties.

Wheat prolamin and glutelin are responsible for the rheological characteristics of dough; while gliadin brings viscosity, glutenin provides elasticity and strength for the dough. Gliadins form monomers with intramolecular disulphide bonds, and molecular weights ranging from 30 to 60 kDa that can be divided into α-, β-, γ-, and ω-gliadins (Fig. 2.2). Gliadins are also grouped by their N-terminal sequence: S-rich α/β-, γ-, and S-poor ω-gliadins that contain no cysteine residue [6]. On the other hand, glutenins are found as polymers through interchain disulfide bonds as well as intramolecular bonds, with molecular weights ranging from 80 kDa to several million kilodaltons. These proteins are divided into two groups: low–molecular weight glutenins and high–molecular weight glutenins, as a consequence of their molecular weight range [7, 8]. In other grains, like rye, gluten fractions are sub-grouped into ω-secalins, γ-secalins, and HMW glutelins. In barley, this heterogeneous protein is divided into B-, C-, and D-hordeins.

2.2 Toxicity Factors in Cereal Proteins

A great heterogeneity of peptides seems to be involved in the pathogenesis of celiac disease, although the characterization of all the relevant epitopes has not yet been achieved [9, 10]. Furthermore, the chemical diversity resulting from the various amino acid compositions makes the quantification of immunogenic peptide sequences difficult.

In order to develop an ideal antibody for gluten analysis in foods, it is important to take into account the fact that besides being able to determine various cereal prolamins, it should also recognize the specific intramolecular regions responsible for toxicity in CD [1]. The toxicity of prolamins depends on their amino acid sequences and molecular properties. Celiac-harmful proteins are rich in glutamine and proline [11–13] and these aminoacids are located in the protein repetitive domains. Alfa- and ω-gliadin-derived peptides are involved in immunological responses in adults, whereas other peptides, such as low–molecular weight glutenins and γ-gliadins, have been responsible for toxicity in children and occasionally in adults [14, 15]. In this context, many protein regions had been proposed by their immunogenic properties, and even today some of them remain unidentified.

Accordingly, several approaches have been developed to identify the gluten peptides that can be recognized by T cells from the celiac population. The proline-rich repetitive region of gliadins is responsible for carrying epitopes for a respective lymphocyte receptor and is connected to CD. One of the most popular is 33-mer from α-gliadin, which contains three overlapping glutamine (Gln)-Pro rich epitopes (12,52,95): PFPQPQLPY, PQPQLPYPQ (3 copies), and PYPQPQLPY (2 copies) [16].

A 33-amino acid peptide (LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF) from α/β-gliadin has been shown to be resistant to gastric and pancreatic hydrolysis and to exhibit celiac disease toxicity *in vivo* and *in vitro*. Furthermore, this 33-mer peptide acts as a strong stimulator to intestinal T-cells [16]. Nevertheless, other potential immunogenic peptides had been described (Table 2.2).

2.3 The Gliadin Standard

It is crucial for celiac patients to know the gluten content of the foods they eat, but detecting the toxic fraction of these molecules is not easy. Currently, immunochemistry is the most-used technology for food analysis, but some technical aspects remain to be solved. A critical point in this assay is the availability of a suitable reference standard in order to assess the results. The Working Group on Prolamin Analysis and Toxicity prepared the European Gliadin Standard (called PWG standard or PWG gliadin) from the 28 most common wheat cultivars grown in Europe [27]. These prolamins (gliadins) were separated from albumins and globulins using 0.4 M NaCl and then extracted with 60% ethanol. The gliadin extracts were finally concentrated, desalted by ultrafiltration, freeze-dried, and homogenized. This standard has been characterized by polyacrylamide gel electrophoresis, capillary electrophoresis, RP-HPLC, MALDI-TOF MS, and immunoassays, and its solubility and stability have been also evaluated.

However, the number of proteins in cereals is greater than those present in the PWG gliadin standard. Moreover, a great number of foodstuffs (produced using processes of fermentation and hydrolysis, like sourdough products, starch syrup,

Table 2.2 Some immunogenic peptides found in wheat prolamins (modified from Kanerva [5])

Peptide	Amino acid sequence[a]	Reference
In vivo		
α-gliadin	LGQQQPFPPQQPY	Marsh et al. [17]
	LGQQQPFPPQQPYPQPQPF	Sturgess et al. [18]
	PQPQPFPSQQPY	Marsh et al. [17]
	LQLQPFPQPQLPYPQPQLPY	Fraser et al. [19]
	LGQGSFRPSQQN	Mantzaris and Jewell [20]
In vitro		
α-gliadin	VRVPVPQLQPQNPSQQQPQEQVP LVQQQQF	De Ritis et al. [21]
	VPVPQLQPQNPSQQQPQEQVPL	Wieser et al. [22]
	QLQPFPQPELPY	Arentz-Hansen et al. [23]
	PQPELPYPQPQLPY	Arentz-Hansen et al. [23]
	QYPSGQGSFQPSQQNPQA	Van de Wal et al. [24]
	QYPSGQGSFQPSQQNPQA	Mazzarella et al. [25]
γ-gliadin	QPFPQPQLPY, QPFPQPQQTF	Arentz-Hansen et al. [26]
	LQPQQPFPQQPQQPYPQQPQ	Arentz-Hansen et al. [26]
LMW glutenin	QQQQPPFSQQQQSPFSQQQQ	Vader et al. [16]
	QQPPFSQQQQQPLPQ	Vader et al. [16]
HMW glutenin	GQQGYYPTSPQQS	Van de Wal et al. [15]
ω-prolamins	FPLQPQQP	Vallejo-Diez et al. [11]

A alanine, *E* glutamic acid, *F* phenylalanine, *G* glycine, *L* leucine, *P* proline, *Q* glutamine, *S* serine, *T* threonine, *Y* tyrosine

malt extracts, or beer) can suffer a partial or total hydrolysis of proteins. In these cases, using a hydrolyzed standard and a competitive immunoassay to determine gluten in foods has been suggested. This hydrolyzed standard has been prepared with prolamin fractions from wheat (gliadin), barley (hordein), and rye (secalin) [28]. Although the results obtained with the PWG gliadin standard are comparable to those obtained using the partially enzymatically digested prolamin standard in a competitive assay, it is more difficult to prepare a reproducible hydrolyzed standard [29]. Other immunotoxic peptides of prolamin are being proposed as standard with a high degree of repeatability, reproducibility, and stability [30, 31], but they are still not widely used in routine analysis.

2.4 International Food Laws for Gluten-Free Products

Even if the promoter of CD was discovered in the twentieth century, with diet as the only treatment possible for these patients, food safety and food laws were not completely revised to meet their necessities until the twenty-first century. This is due, fundamentally, to the fact that CD and food allergies are becoming more prevalent

with time. In the case of CD, it is probably because nowadays the diagnosis is more efficient, even if there is still a high percentage of under-diagnosed people; moreover, many potential celiac sufferers start diet treatment on their own, with no official diagnosis and without the supervision of doctors.

Food allergen management and control (including gluten) has become a food safety issue in recent years. In the case of gluten, it has been brought to light that even if naturally gluten-free ingredients are used, the final product could be cross-contaminated, and products apparently free of gluten could be made from ingredients that in the end bring a non-admissible gluten level not always reflected on the label. This situation leads to diet transgressions that ultimately affect the health of celiac sufferers and give little chance to reach nutritional balance with a true gluten-free diet. Many products labelled gluten-free have been launched on the market recently, and such a claim has been attempted to be regulated. Laws have been passed all over the world trying to make adequate labelling of gluten-free products mandatory in order to increase the variety of food these patients can use, thereby ensuring their safety.

The Codex Alimentarius (an international organization founded by the Food and Agriculture Organization and the World Health Organization) was established to develop coordinated international food standards. In 1979 this institution adopted a standard for foods for special dietary use for persons intolerant to gluten; the standard was revised and corrected both in 2008 and 2015 [32]. The document set the definition of gluten-free foods and foods specially processed to reduce gluten content as follows:

1. Gluten-free foods are dietary foods

   (a) Consisting of or made only from one or more ingredients that 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 crossbred varieties, and the gluten level does not exceed 20 mg/kg in total, based on the food as sold or distributed to the consumer, and/or

   (b) 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 various trademarks such as Kamut), rye, barley, oats, or their crossbred varieties, which have been specially processed to remove gluten, and the gluten level does not exceed 20 mg/kg in total, based on the food as sold or distributed to the consumer.

2. Foods specially processed to reduce gluten content to a level from above 20 to 100 mg/kg

   are foods that consist 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 crossbred varieties, which have been specially processed to reduce the gluten content to a level from above 20 to 100 mg/kg in total, based on the food as sold or distributed to the consumer.

Oats, or at least some varieties [33, 34] of oats, can be tolerated by most but not all people who are intolerant to gluten. Taking this into consideration, the allowance of oats that are not contaminated with wheat, rye, or barley in foods covered by this standard may be determined at the national level. According to the document, nations are responsible for the decisions on the marketing of products, and the decision on whether or not to use the term "gluten-free" on the label lies with each manufacturer, so the product is only subject to the respective regulatory framework if a voluntary gluten-free claim is made.

Most nations have based their laws on this standard. Nevertheless, there are some differences between nations at the level of labelling.

In the European Union (EU), before the revision of the Codex Standard in 2008, the Council Directive 89/398/CEE of May 3, 1989, included gluten-free foods as food for particular uses [35]. This directive forced the approval of other directives that would regulate any of these foods. In the case of gluten-free foods, Directive 2000/13/EC included by amendment a list of allergens in its annex IIIa [36], in which appeared cereals containing gluten (i.e., wheat, rye, barley, oats, spelt, Kamut, or their hybridized strains) and products thereof. The food containing any of the allergens listed in that annex had to be clearly labelled in the list of ingredients or by an indication that will include the word "contains" followed by the names of the ingredient(s) concerned. This list of allergens was recently modified in other directives in order to give details of the non-allergenic derivatives of the ingredients for which there were exemptions from the labelling requirement in force at that moment, such as wheat-based maltodextrins or glucose syrups based on barley.

Lately, and considering the fact that gluten is not introduced into an infant's diet until several months of age, Commission Directive 2006/141/EC of December 22, 2006, on infant formulas and formulas for infants when appropriate complementary feeding is introduced, prohibited the use of gluten-containing ingredients in such foods [37]. This prohibition extended to the use of the terms "gluten-free" or "low content of gluten" in these products. Even if the directive will be repealed in 2020, the prohibition on using gluten-containing ingredients in these foods will remain in the new regulation (Commission Delegated Regulation (EU) 2016/127 of September 25, 2015 [38]).

After the revision of the Codex Standard, and based on it, the European Union adopted Commission Regulation (EC) No. 41/2009 of January 20, 2009 [39]. This regulation was recently repealed and its framework is now under the Regulation (EU) No. 1169/2011, of October 25, 2011, *on the provision of food information to consumers* [40], and under the Commission Implementing Regulation (EU) No. 828/2014, which lays out the specific requirements for the provision of information to consumers on the absence or reduced presence of gluten in food [41]. These regulations considered that gluten levels defined in the Codex were scientifically set and they regulated the indications related to gluten content that will be made in labelling in the EU, as seen before. They allowed the use of "very low gluten" and "gluten-free" wordings that enable celiac patients to find on the market a variety of foods suitable to their needs and to their level of sensitivity to gluten (Table 2.3). Moreover,

Table 2.3 Statements on the absence or reduced presence of gluten in foods that are allowed to be made and conditions thereof by Regulation (EU) No 828/2014

Gluten-free	The label *gluten-free* may only be applied where the food as sold to the final consumer contains no more than 20 mg/kg of gluten
Very low gluten	The label *very low gluten* may only be applied where the food, consisting of or containing one or more ingredients made from wheat, rye, barley, oats or their crossbred varieties that have been specially processed to reduce the gluten content, contains no more than 100 mg/kg of gluten in the food as sold to the final consumer
Specifically formulated for people intolerant to gluten or specifically formulated for celiacs	If the food is specially produced, prepared, and/or processed to: (a) reduce the gluten content of one or more gluten-containing ingredients (b) substitute the gluten-containing ingredients with other ingredients naturally free of gluten.
Suitable for people intolerant to gluten or suitable for celiacs.	If requirements for *gluten-free* and *very low gluten* statements are compiled naturally.

Additional requirements for food containing oats
Oats contained in a food presented as gluten-free or very low gluten must have been specially produced, prepared, and/or processed in a way to avoid contamination by wheat, rye, barley, or their crossbred varieties, and the gluten content of such oats cannot exceed 20 mg/kg

according to this regulation, it is permitted to distinguish between foods that are naturally free of gluten and products that are specially formulated for celiac patients.

Outside the EU, before 2013 there were no standards for the food industry to use in labelling products as "gluten-free" in the U.S. That year, the Food and Drug Administration (FDA) established, among other criteria, a gluten limit of less than 20 ppm for foods that carry the label "gluten-free," "no gluten," "free of gluten," or "without gluten" [42]. The rule implements part of the Food Allergen Labelling and Consumer Protection Act of 2004 (FALCPA) [43]. According to FDA opinion, messages such as "low gluten" or "very low gluten" are misleading, and if used, they will be studied case by case (Guidance for Industry, FDA, 2014). The rule excludes those foods whose labelling is regulated by the U.S. Department of Agriculture (USDA) and the Alcohol and Tobacco Tax and Trade Bureau (TTB), but includes supplements. Generally, the USDA regulates the labelling of meats, poultry, and certain egg products, while the TTB regulates the labelling of most alcoholic beverages, including all distilled spirits, wines that contain 7% or more alcohol by volume, and malted beverages that are made with both malted barley and hops.

The U.S.'s neighbor, Canada, for example, does not allow expressions such as "low gluten" or "reduced gluten" for the same reason, as seen in Section B.24.018 of the Canadian Food and Drug Regulations [44]. The U.S., however, does not allow food that has less than 20 ppm gluten but contains a gluten-containing grain to use the claim "gluten-free," while Canada does. This claim will always be made if the manufacturer includes additional processing steps that are shown to be effective in removing gluten [45].

Furthermore, and as an example of the permanent evolution of the regulation, the FDA is at the moment proposing a rule to establish requirements concerning

"gluten-free" labelling for foods that are fermented or hydrolyzed or that contain fermented or hydrolyzed ingredients [46]. The FDA proposes evaluating the compliance of such fermented and hydrolyzed foods that bear a "gluten-free" claim with the gluten-free labelling rule based on records that are made and kept by the manufacturer of the food with the "gluten-free" claim and made available to the agency for inspection. This is undoubtedly good news, as there is confusion in interpreting the results of current gluten test methods for fermented and hydrolyzed foods, as mentioned in Chap. 3. This could also be a good guideline for international legislators to broaden the variety of food that celiac patients could consume safely.

According to this proposed rule, the records would need to provide adequate assurance that the food is "gluten-free" in compliance with the gluten-free food-labelling final rule before fermentation or hydrolysis. In addition, the proposed rule would require the manufacturer of fermented or hydrolyzed foods bearing the "gluten-free" claim to document that it has adequately evaluated the potential for gluten cross-contact and, if identified, that the manufacturer has implemented measures to prevent the introduction of gluten into the food during the manufacturing process. Likewise, manufacturers of foods that contain fermented or hydrolyzed ingredients and bear the "gluten-free" claim would be required to make and keep records that adequately show that the fermented or hydrolyzed ingredients are "gluten-free" according to their regulations. Finally, the proposed rule would state that the FDA would evaluate the compliance of distilled foods by verifying the absence of protein using scientifically valid analytical methods that can reliably detect the presence of protein or protein fragments in the distilled food.

Other countries that are culturally close, such as Australia and New Zealand, regulate gluten-related labelling in their Food Standards Codes. This regulation states that while gluten-free mentions can only be made for products with no gluten-containing ingredients (even if they have been malted or hydrolyzed – including oats), "low gluten" mentions could be made for products with less than 200 ppm of gluten, which doubles the limit of the Codex Standard [47].

Other countries, such as Argentina, are stricter than the Codex, and set the limit for gluten-free products to 10 ppm. Moreover, Argentina defines gluten-free products as those that are naturally free of gluten, and manufacturers must apply GMP (Good Manufacturing Practices) in order to assure the absence of cross-contamination as set by Article 1383 of Chapter XVII of the Argentinian Food Code [48]. In Japan, seven items are mandatory for labelling: shrimp/prawns, crab, wheat, buckwheat, eggs, milk, and peanuts, and another 20 are recommended for labelling. They are aware of allergies, but they do not regulate gluten content-related expressions regarding celiac disease. In fact, manufacturers must declare the presence of these allergens when their content is above 10 ppm, and precautionary expressions such as "may contain" are prohibited [49]. Nevertheless, changes are rapidly being made all over the world.

Other countries have no regulations yet for gluten-free labelling or labelling for allergies and intolerances. Even if legislators have been working hard over the last few years, there is still much work to be done. Hydrolyzed and/or fermented products are an example, as previously mentioned, but industrial processes are complex

and not always totally controlled to avoid cross-contaminations; this promotes the use of precautionary labelling (terms such as “may contain,” “made in a factory that also uses...”, or “not suitable for celiac patients”), prohibited in some countries for any allergen. This precautionary labelling is not properly regulated in most countries. It is confusing and turns over to the patients the responsibility of having a potential risk if they consume the product, or it narrows down the availability of products for this group even if, in some cases, the products are probably safe for them. Moreover, the inconsistent use of these messages leads to mistrust in the label and they are often ignored [50, 51].

For oats, the regulation is slightly different. The aforementioned Codex standard pointed out that even if most people with intolerance to gluten can include oats in their diets without any adverse effect on their health, the allowance of oats that are not contaminated with wheat, rye, or barley in foods may be determined at the national level. In the EU, Regulation (EU) No. 828/2014 includes additional requirements for products containing oats [41]. These cereals, if used in products labelled as gluten-free or low-gluten content, must have been specially produced, prepared, and/or processed in a way to avoid contamination by wheat, rye, barley, or their crossbred varieties, and the gluten content of such oats cannot exceed 20 mg/kg. In the U.S., oats are not considered to be a “gluten-containing grain” [41], so oats can be used as an ingredient in foods labelled “gluten-free” as long as the oats contain <20 ppm gluten. Nevertheless, the rule encourages “manufacturers of foods labelled ‘gluten-free’ that use an oat-derived ingredient where the word ‘oat’ does not appear in the ingredient list to indicate in their labelling that an oat-derived ingredient is present.”

It is necessary to label food products adequately, and it is obvious that important efforts have been made in recent years all over the world. Nevertheless, the “homogenization” of the rules worldwide will greatly enhance the quality of life for celiac sufferers. Globalization allows travelling and working in various parts of the globe, and it is difficult for celiac sufferers to follow a safe and varied diet if labelling rules remain different in each country. Nevertheless, it is very important to highlight that GMP in the food industry is fundamental for the production of food without any risk of cross-contamination. It is not only labelling, but also a new inclusive perspective of food-production that takes into account the reality for celiac and allergy sufferers, an ever-expanding group. For this new perspective, new technologies for gluten detection play a crucial role in the industry. Rapid, cost-effective, and reliable methodologies may allow manufacturers to detect gluten in any of the steps of the processes, without any inconvenient time lapses in their production.

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Chapter 3
Techniques for Analyzing Gluten

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Abbreviations

AACCI	American Association of Cereal Chemists International
Ab	Antibody
AOACI	Association of Official Analytical Chemists International
A-PAGE	Electrophoresis at acid pH
ELISA	Enzyme-linked immunosorbent assays
mAb	Monoclonal antibody
MALDI-TOF-MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MS	Mass spectrometry
pAb	Polyclonal antibody
PCR	Polymerase chain reaction
RP- or GP- HPLC	Reversed-phase or gel permeation high-performance liquid chromatography
SDS-PAGE	Single- and two-dimensional gel electrophoresis
UV	Ultraviolet

3.1 Introduction

Reliable and accurate methods for quantifying gluten in food are necessary to protect the gluten-sensitive consumer against exposure to this protein. According to the standard for gluten-free foods [1], the quantitative determination of gluten in food samples and ingredients must be based on an immunologic or other method that provides at least equal sensitivity and specificity.

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In that sense, currently used analytical methods in gluten-free assessment and legal compliance testing are based on enzyme-linked immunosorbent assays (ELISAs). Nevertheless, other molecular techniques, such as mass spectrometry and chromatography, have also been used [2, 3]. In addition, polymerase chain reaction (PCR) techniques are used as a complementary tool to identify the presence of gluten using a DNA pathway, and novel approaches such as aptamers, microarrays, and multi-analyte profiling are being developed [4, 5].

3.2 Immunological Techniques

Immunological methods are based on the antibodies (Abs) raised against the different prolamin fractions or specific sequences found in them. These assays should be able to measure the harmful proteins and peptides, regardless of the food matrix or manufacturing process. Nevertheless, as we will explain in the final section of this chapter, cereal proteins are modified during the elaboration process and they could make the detection analysis difficult [6].

ELISAs are most commonly used for routine gluten analysis, not only because of their specificity, sensitivity, and reproducibility, but also because of a lack of an independent reference method. Despite this, ELISA is the method recommended by the Codex [1], and other immunology-based methodologies are being developed, such as lateral flow devices, dipsticks, immunosensors, or immunomagnetic beads for multiplex analyses [7–10].

ELISA test kits provide rapid results, are easy to handle and, compared with other techniques, are usually cheaper and require simpler laboratory equipment. The gluten analysis assays are developed in several formats, although sandwich and competitive are more common than indirect methodologies. Either monoclonal or polyclonal Abs could be useful, and whereas monoclonal Abs (mAbs) recognize a single epitope that allows fine detection and quantification, polyclonal is often used to gather as much of the antigen as possible.

The sandwich ELISA quantifies prolamins – antigens – between two layers of Abs (i.e., capture and detection Abs) and they can be either monoclonal or polyclonal and can be the same or a different Ab. The capture Ab is well bound to the bottom of the microplate, and toxic fractions of prolamins are attached to this coating Ab. Later, a detection antibody conjugated to an enzyme (substrate) is added to the wells in order to join the capture Ab-prolamin mix together.

The prolamin to be measured must contain at least two antigenic epitopes capable of binding to both antibodies at the same time, since at least two antibodies take part in the sandwich. The Ab-linked enzyme used is usually horseradish peroxidase or alkaline phosphatase, and its purpose is to induce a color reaction involving chromogen, which can be measured by spectrophotometric methods. Figure 3.1 summarizes these steps.

The competitive ELISA method is based on the competition between foodstuffs – i.e., a sample – prolamins and standard prolamins. This method uses only

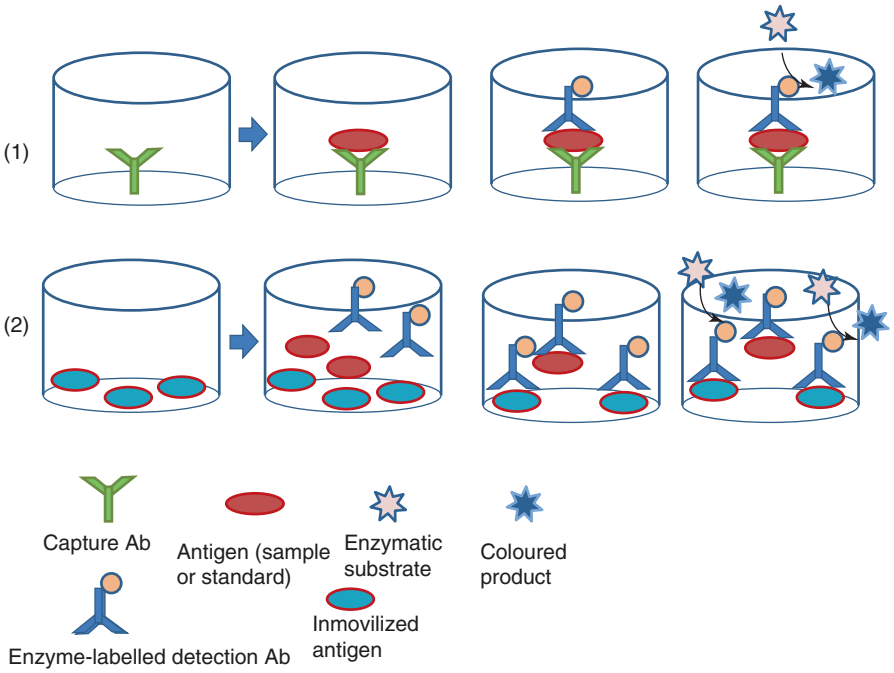

Fig. 3.1 The Principles of ELISA: (1) Sandwich method; (2) Competitive method

one antibody, which makes it suitable for detecting small, hydrolyzed proteins and peptides as well. Taking into account that nonspecific binding is more likely to occur when only one binding site is needed for detection, this method may be less specific than the sandwich format (Fig. 3.1).

ELISA methods mostly use either gliadins or hordeins as reference standards, and antibody-specific for gliadins; they do not consider other possible toxic proteins, such as glutenins [11]. Furthermore, based on an assumption that gliadins and glutenins are similar in proportion, ELISA estimates the total gluten content in foodstuffs using a conversion factor equal to 2 [12].

3.2.1 Antibodies and ELISA Test for Gluten Detection

Since the late 1980s, several immunochemical gluten analysis methods have been developed. They are based on mono- and polyclonal antibodies against prolamin proteins. Despite the fact that many of them have been built against wheat gliadin or its sub-fractions, and due to the structural homology of prolamins, they also recognize all the other prolamin fractions (hordein and secalin) to almost the same extent [13, 14]. Nowadays, the most common are the monoclonal antibodies raised against multiple potential prolamin-specific antibodies. Several antibodies had been

developed, but only a few of them have been tested as ELISA and approved by a successful inter-laboratory study in accordance with AOAC guidelines.

3.2.1.1 Skerrit ELISA: Antibody Against ω-gliadin

This monoclonal mouse antibody (mAb), also referred as 401.21, was raised against ω-gliadins of the Australian Timgalen wheat variety [15]. This mAb was produced by Skerrit [16] and is able to recognize a gliadin fraction that does not denature during heating. This was an advantage to making it highly suitable for gluten detection in processed foods.

After a successful collaborative study, the sandwich ELISA based on this antibody was adopted as Official Method 991.19 by the Association of Official Analytical Chemists International (AOACI) [16]. It underwent a very important development in the 1990s, but the use of ELISA based on this mAb is becoming less frequent because of the low validated sensitivity (limit of quantitation (LOQ): 160 mg gluten/kg), and also due to the high variability in the amount of ω-gliadins between cereal species and varieties. Consequently, the quantitative analysis can vary, depending on the relative ω-gliadins content; afterwards, it was replaced by other antibodies. Another disadvantage is that this method has only a low reactivity to barley hordeins.

3.2.1.2 R5 ELISA: Antibody Against Pentapeptide QQPFP (R5)

An mAb called R5 was developed against an ω-secalin extract [17] from rye, and it mainly recognizes the epitope QQPFP. The ELISA assay based on this antibody recognizes gliadins, hordeins, and secalins to a similar degree but does not recognize avenins [18]. As QQPFP occurs in the repetitive domains of prolamins and has been found to occur numerous times in m-type prolamins [19], the antibody is a very good candidate for the detection of prolamins.

Since 2008, Codex Alimentarius [1] recommends ELISA methods based on the R5 antibody for gluten analysis. This antibody does not cross-react with proteins from inherently GF grains, but in some ethanol extraction conditions, not only harmful prolamins but also soy and lupin proteins [20] might be detected. Later on, the use of a cocktail extraction, including reducing agents, solved these false positives.

The sandwich R5 ELISA, together with cocktail extraction, was validated by collaborative studies [18, 21] and subsequently adopted as an AACCI Approved Method 38–50.01 [22]. A competitive R5 ELISA was developed for the determination of partially hydrolyzed gluten and, after a collaborative trial, it was accepted as AACCI Approved Method 38–55.01 [23, 24].

3.2.1.3 G12 and A1 ELISA Antibodies Against 33-mer (G12 and A1)

Two antibodies, G12 and A1, have been raised against 33-mer. The G12 antibody was developed by Morón et al. [25]; it particularly recognizes the hexapeptide QPQLPY. The antibody is highly selective for 33-mer and similar peptides found in

barley and rye. The A1 antibody recognizes the heptameric sequence QLPYPQP, which is also a part of 33-mer. A1 has a higher sensitivity for gluten detection than the G12 antibody, but G12 has a better affinity for 33-mer [25, 26].

Moreover, the advantage of these antibodies is their ability to recognize oat avenins [26] and this reactivity was proportional to the potential immunotoxicity of the oat cultivar [27]. The weakness came because it makes the G12 mAb unsuitable for detecting wheat, rye, or barley contaminations in oats. The sandwich assay was adopted as AACCI Approved Method 38–52.01 [28] after a collaborative study.

3.2.1.4 Others

Other immunological ELISA systems based on various antibodies have also been developed.

The Morinaga wheat protein ELISA method is based on the use of a polyclonal antibody to wheat – gliadin – that detects multiple epitopes. The antibody also cross-reacts with hordeins and secalins to a lesser degree than with wheat and, consequently, can underestimate barley and rye protein contamination.

Gabosská et al. (2006) [29] developed a gliadin ELISA kit based on two monoclonal antibodies against two different epitopes of gliadin and one polyclonal antibody. It recognizes wheat, rye, and spelt with the equivalent efficiency, but is lower for barley, where only about 20–30% is detected [30].

Ellis et al. [31] developed a competitive ELISA based on the PN3 antibody. PN3 is a monoclonal antibody raised against an epitope of 19 amino acids of A-gliadin (19mer), a harmful fragment of prolamins. The main recognition epitope is QQQPFP, and this mAb reacts strongly with a- and y-gliadins, but only weakly with ω-gliadins [11, 32, 33]. The antibody also detects LMW glutenins, secalins, and hordeins, but not HMW glutenins, avenins, or zeins. These and many other antibodies and ELISA methods have been assayed but none of them is commercially available.

3.3 Non-immunological Methods

3.3.1 Proteomic-Based Methods

3.3.1.1 SDS Polyacrylamide Gel Electrophoresis and Western Blotting

Gel electrophoresis has been largely used in protein analysis and, therefore, in cereal protein identification. Single and two-dimensional gel electrophoresis (both SDS-PAGE and A-PAGE) have been widely used to characterize prolamin subfractions based on their mobility.

The qualification of proteins by SDS-PAGE is further improved by adding another dimension based on isoelectric focusing, creating a two-dimensional system. Nevertheless, these techniques have not achieved enough sensibility for quantifying gluten at low levels. For this reason, complementary techniques have been

associated as SDS-PAGE and Western blotting. Therefore, after electrophoretic separation by a one-dimensional SDS-PAGE, the proteins were transferred to polyvinylidene difluoride membranes where proteins are adsorbed. After transfer, the membranes were blocked with BSA (bovine serum albumin) and incubated with any of the monoclonal or polyclononal antibodies raised against toxic prolamins, already exposed. The western blot techniques allow a qualitative or semi-quantitative analysis of these immunogenic proteins [34, 35].

3.3.1.2 RP or GP-HPLC

The protein profile has been characterized by reversed-phase or gel permeation high-performance liquid chromatography (RP- or GP-HPLC) with UV detection [19]. Nevertheless, this one-dimensional separation analysis is deficient for detecting gluten traces in complex food matrices due to low selectivity and sensitivity [5].

In that sense, in order to improve the prolamin analysis, new approaches have been designed that combine chromatography with mass spectrometry (MS) using soft ionization such as matrix-assisted laser desorption/ionization (MALDI) or electrospray ionization (ESI) followed by time-of-flight (TOF), ion trap, or triple quadrupole detection [19, 36, 37].

3.3.1.3 Mass Spectrometry, MALDITOF and LC/MS

As seen before, applying proteomics is interesting to complement immunological techniques. But when gluten-free products are being analyzed, where very low quantities of gluten are expected compared with the other major proteins, analysis becomes difficult [38]. Due to its high sensitivity for identification, characterization and quantification of proteins and peptides, mass spectrometry (MS) is one of the more important physical methods used in this field. MS is based in an ionization of the molecules of interest followed by ion separation. This separation is made according to their relation mass/charge, and finally, detection of the separated ions [39].

A type of MS method, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), has been proven to be an efficient tool for wheat gluten analysis [18, 36]. Compared with the common separation methods, such as electrophoresis or HPLC, the MALDI-TOF-MS technique appears to be much more accurate and sensitive, requiring only a few minutes per sample to perform the measurement [40].

MALDI-TOF MS was the first technique used to identify toxic prolamins involved in celiac disease [19, 36] and to recognize the different patterns of toxic prolamins in grains, depending on the type of cultivar and variety studied. Nevertheless, as Mujico et al. have published [41], this system does not detect prolamins below 20–25 mg/kg and is, therefore, not appropriate for confirming the ELISA results in food samples with low gluten content. On the other hand, the complexity of the protein mixture, hydrolysis, or heating process carry out this

methodology weakness, due to a possibly ambiguous gluten protein identification. The insufficient accuracy could be solved by a proteomic approach involving tandem mass spectrometry (MS/MS) or multi-stage MS experiments.

The classical workflow approach consists of separating protein mixtures by electrophoresis, digesting the sample by the enzyme trypsin breaking down proteins into peptides, and, finally, identifying those using MS. Other scientists overcome the protein separation stage by digesting the entire protein mixture into peptides and dividing them with one or two liquid chromatography (LC) steps [6]. Furthermore, newly emerging technologies combine MS and proteomics, as well as the expanding field of bioinformatics tools and interactive databases (called "*in silico*"). These methods encompass the analysis of wheat protein extracts by MS with statistical methods such as artificial neural networks, partial least-squares regression, and principal component analysis in order to predict the variety or quality of unknown wheat samples [42].

3.3.2 Genomics-Based Methods

Genomics-based methods do not target gluten proteins, but rather a marker indicative of the presence of gluten: DNA or RNA. Methods relying on DNA have attracted great attention due to the high stability of DNA molecules.

As in protein-based methods, the success of the DNA-based methods for quantifying gluten in foods depends on the sensitivity and selectivity of the analysis. Moreover, in these kinds of methods, the efficacy of the DNA extraction, which should be valid for all food matrices and compatible with the selected analytical technique, and the use of a correct standard for calibration, are crucial [43].

DNA presents the advantage that it is efficiently extracted under harsh conditions, as well as being less affected by extraction from food matrices than proteins [44]; moreover, DNA analysis is more sensitive than protein analysis. However, it should be pointed out that genomics-based methods are indirect. This means that precise correlation between a DNA concentration or copy number and gluten protein concentration would be needed to use DNA-based methods for quantification purposes. Along these lines, legislation only establishes thresholds in terms of gluten concentration. Therefore, genomic-based methods are considered to be a complement or confirmation of the protein-based methods [43, 45].

Food processing must be also taken into consideration. Highly processed foods are usually subjected to thermal and/or enzymatic transformations in which the DNA can be altered or even degraded. Nevertheless, DNA is much more stable than proteins, which is why DNA-based methods are a promising alternative in the analysis of these types of food.

Various DNA-based methods have been developed for detecting gluten; the PCR stands out among others. PCR is a technique that makes multiple copies of a segment of DNA using the ability of DNA polymerase to synthesize new strands of DNA complementary to a target sequence. DNA polymerase needs pre-existing

nucleotides to which to add new ones. Thus, short oligonucleotides, called primers, are used to initiate the amplification; this allows the use of complementary primers in order to detect specific DNA regions that they want to amplify, making PCR a very precise and specific technique. At the end of the reaction, the specific target sequence will be exponentially amplified in billions of copies.

After PCR, the amplified product can be completely identified by sequencing, or detected by gel electrophoresis subsequent staining and hybridization to a labeled version of the target DNA (southern blot) [10]. To quantify the target DNA, real-time PCR is used, which does not require post-PCR detection. In this case, fluorescent dyes that intercalate in double-stranded DNA, or probes specific to target DNA that are labeled with a fluorescent reporter, are used to detect the amplification of the DNA in real time [5]. The increase in fluorescence occurs at the same time that PCR is being carried out, and it is proportional to the amount of target DNA present in the sample [10].

With regard to gluten detection, the target DNA is any species-specific component of cereals that contain gluten and functions as a marker for the presence of a particular food ingredient. Commercial PCR kits for gluten detection mention that they include specific primer sequences developed for the amplification of DNA fragments solely present in gluten-containing cereals (wheat, spelt, kamut, rye, barley, triticale, and oat). However, they do not provide detailed information about their sequence.

Several targets have been proposed in the literature. Dahinden et al. [46] developed a PCR system as an indicator of celiac-toxic cereals' contamination of gluten-free food, simultaneously detecting a non-coding region of chloroplast *trnL* gene of wheat, barley, and rye. Afterwards, Olexová et al. used those primers satisfactorily, proving their suitability for the detection of gluten-containing cereals in flours and GF bakery products [47]. Similarly, in 2008, the applicability of those primers was validated in several food samples from various food groups (such as cereals and derivatives, chocolate, jam, meat extracts, commercially available gluten-free products, baby foods, and soy products), as well as heat-treated foods and partially hydrolyzed products [45].

The allergen-multiplex ligation-dependent probe amplification (MLPA) method for the detection of eight allergens – one of them gluten – proposed by Mustorp et al. must be also pointed out [44]. It consists of a 10-plex quantitative and sensitive ligation-dependent probe amplification method, in which ligated probes are amplified by PCR, and amplicons detected by capillary electrophoresis. Gluten probes were also based on a Dahinden-proposed sequence and tested positive for wheat, rye, barley, and oat.

Zeltner et al. [48] used primers specific for homologue sequences encoding high-molecular-weight glutenin sub-units in the case of wheat, spelt, kamut, and rye, the Hor3 gene for barley, and the gene-encoding 12S seed storage protein for oat. They obtained a satisfactory ruggedness in the detection, with a sensitivity of 2.5 mg/kg of wheat in vegetable food matrices, 5 mg/kg of wheat in meat, and 10 mg/kg for barley and oat. On the other hand, Mujico et al. [41] used a fragment of 51 base pairs of the intergenic region limited by the wheat ribosomal 25S and 18S genes to

quantify wheat contamination. They obtained a PCR assay to confirm the presence of wheat in food even more sensitive than R5 ELISA. Martín-Fernández et al. [49] developed the real-time PCR method to assess three DNA sequences encoding wheat proteins (α2-gliadin, agglutinin isolectin, and thioredoxin h). Results revealed high specificity to detect not only wheat, but also other gluten-containing cereals such as barley and rye. The most sensitive one was the α2-gliadin marker sequence-based PCR.

Various outcomes have been published comparing DNA-based PCR methods results to those obtained by the Codex-approved protein-based R5 ELISA. Sandberg et al. [50] used primers targeting prolamin genes of wheat, rye, barley, and oats in food samples. The PCR method provided a good correlation with the protein assay, as it was also rapid and sensitive. Similarly, a high correlation between PCR and R5 ELISA was noted by Mujico et al. [41]; however, while in some samples, prolamins were detectable by PCR and not by ELISA, in others, the DNA content was lower or higher than expected (high-temperature-treated samples and starch-based flours and foods respectively). Churruca et al. [45]observed a positive linear correlation between the gluten DNA and protein content in different food samples, including processed foods such as hydrolyzed beverages and heat-treated foods.

Proficiency tests were applied by Scharf et al. [51] to assess the suitability of these methods. A total of 45 laboratories submitted PCR results and 170 laboratories submitted ELISA results between 2006 and 2011, showing agreements for both methods. Nevertheless, ELISA methods – but not PCR ones – gave 2% of false negatives in complex matrices such as pastries and sausage meat. Nevertheless, it must be pointed out that both methods were generally suitable for the detection of different gluten amounts in complex matrices, such as infant food.

Overall, these results show that PCR can be recommended as a highly sensitive screening method for the presence of gluten-containing cereals in food analysis, being confirmatory as well as complementary to the enzyme-immunoassay. In fact, DNA-based methods offer higher sensitivity and specificity than ELISA. However, PCR must be more deeply studied and developed for gluten detection in highly processed or hydrolyzed samples due to DNA degradation [5].

3.3.3 Novel Methods

3.3.3.1 Electrochemical Genosensors

DNA hybridization biosensors, also known as “genosensors,” are analytical devices for the detection of specific DNA “target” sequences in solution, upon hybridization of the targets with complementary “probes” immobilized on a solid substrate [52]. This is measured by the use of a reporter molecule and an electrode-based platform as transducer [53]. Electrochemical genosensors have been proposed as an

alternative to real-time PCR, yielding a rapid and specific amplicon post-PCR detection [54]. The development of genosensors applied to complex biological samples, including food samples, has begun [52, 53].

For celiac disease, an electrochemical genosensor to detect the oligonucleotide sequence encoding the CD immunogenic peptide 33-mer of gliadin in wheat was proposed, with an electrochemical sandwich assay [54, 55]. It was able to selectively detect different varieties of wheat, barley, rye, and oats from other cereals, as low as 0.001% (10 mg/kg) of wheat flour in an inert matrix. Furthermore, even in highly processed food samples, a good correlation with the official immunoassay has been demonstrated [53].

3.3.3.2 SELEX

The systematic evolution of ligands by exponential enrichment, SELEX, is an *in vitro* selection process in which aptamers are identified from combinatorial libraries. Aptamers are structured, single-stranded nucleic acids [56] that bind with high affinity and specificity to their protein target. Later, aptamer-peptide complexes are detected by various techniques, such as PCR amplification, enzyme-linked assays, etc. Aptamers have been proposed as biomolecular recognition elements, an alternative to the use of antibody-detecting methods for many reasons: high affinity and specificity; high stability; they are chemically synthesized in a cost-effective way and with high reproducibility (obviating the requirement for host animals, as in the case of antibodies); and they can be easily combined with various chemical labels or groups for their adaptation to different analytical techniques [43, 57].

There are few reports of aptamers selected against food allergens – in fact, not all targets are prone to generating useful aptamers. Gluten is the case, due to its hydrophobicity, that does not go well with the hydrophilic nature of nucleic acids [43]. Nevertheless, some researchers have developed a SELEX-based process for the selection of a DNA aptamer against the gliadin, called G33 aptamer, and its subsequent application for the detection of gliadin [57]. A competitive real-time apta-PCR was developed, where the bound aptamer was amplified for the detection of gliadin. Another SELEX process against α2-gliadin led to different aptamers called anti33-mer gliaptamers. Among them, Gli4 and Gli1 were selected. Gli4 was the aptamer with the highest affinity towards the target in the SELEX pool, while Gli1 was the most abundant one.

In 2014 Gli4 was used as a receptor for an electrochemical competitive enzyme-linked apta-assay on magnetic particles. The results showed that besides gliadin, also detected were hordeins, secalins and avenins, with no cross-reactivity to corn, soy, or rice. Furthermore, this method was more sensitive than the reference immunoassay for detecting the same target [58]. The assay mentioned is compatible with the cocktail protein extraction method, allowing the quantification of gluten in heated foods. In 2015 the research group conducted a study with a Gli1 aptamer assay that allowed the detection of the allergen in different kinds of food samples, including hydrolyzed ones [59]. The results revealed a good correlation with antibody-based assays, and G12-based and R5-based ELISAs.

3.3.3.3 Next-Generation Sequencing

Current commercial gluten analysis methods do not differentiate immunogenic and non-immunogenic CD epitope variants. It has been suggested that next-generation sequencing (NGS) may be used as a screening tool to classify wheat varieties according to phylogeny and their CD-immunogenic potential. Although it is not directly applicable to gluten detection, NGS could be a promising tool for differentiating between CD immunogenic epitopes and gluten sequences that are not CD active. This means that NGS could provide a correct selection of wheat varieties with low potential to cause CD [60].

Wheat α-gliadin proteins contain three major CD immunogenic peptides: p31–43, 33-mer, and DQ2.5-glia-α3 epitopes. An NGS study of α-gliadin genes from diploid and polyploid wheat revealed six types of α-gliadins with strong differences in the presence and abundance of these CD immunogenic peptides. Indeed, one of six contained all immunogenic peptides and epitopes [61]. A 454 RNA-amplicon NGS was developed for α-gliadin transcripts encompassing the three major CD epitopes and their variants in various durum wheat varieties, all of which showed CD epitopes, but a few plants showed lower CD immunogenicity [60].

Taking into account all the published information, although novel methods are promising, more studies are needed to confirm their positive expectations.

3.4 Factors Affecting Gluten Analysis

Wheat gluten, obtained as a byproduct of the wheat starch industry, is a ubiquitous and relatively inexpensive source for the food industry. In the food processing industry sector, proteins like gluten are enzymatically modified as an effective tool to increase the functional properties, such as structure stabilization, and to enhance protein applications [62]. High temperatures during food processing could induce new intra- and intermolecular bonds, affecting toxic peptide detection. Furthermore, proteins can be modified by various techniques, such as deamidation, transamidation, and degradation by different types of hydrolysis. These modifications, which also occur naturally due to enzymatic mechanisms in cereal seeds, might mask or keep gluten hidden to some analytical tools [39, 63].

3.4.1 Heating

During the processing of some foods, proteins are treated at high temperatures that induce new disulphide bonds between lysine and asparagines (Asp) and glutamine (Gln) residues. The α- and γ-gliadins contain high amounts of sulphur, increasing the heat-induced changes compared to those with a low sulphur content, as in

ω-gliadin. Furthermore, the heat-treatment of cooked and baked products produces protein aggregates forming an insoluble matrix that makes the extraction of gluten difficult, and, consequently, their analysis [64, 65].

Some extraction systems have been developed in order to solve these structural changes and ensure a complete recovery of prolamins and glutelins. In fact, ethanol solution extractions are insufficient, and solutions such as the cocktail that contains reducing and disaggregating agents, e.g., 2-mercaptoethanol and guanidine hydrochloride, are used. As mentioned, most spread is called cocktail [64], but other similar compounds are suitable for that purpose. Nevertheless, β-mercaptoethanol can not be used in various tests -such as *ELISA competitive* test- because it interferes with the specific binding of the antibodies [64, 65]. Despite these approaches, a complete protein extraction cannot be guaranteed because they can form large aggregates that are hardly reducible and form new bonds to other types of polymers, such as starch or lipids [65, 66].

3.4.2 Interference of Ingredients

Certain food ingredients may interfere in the gluten detection and prolamin analysis, giving lower or higher values than the real gluten content. It is known that polyphenol-containing foods such as chocolate, tannins, etc., may interfere in the gluten extraction process and, consequently, have detected less prolamin than was expected. This happens, for example, when spiked samples are prepared in order to determine recovery percentages, and the amount detected can be lower than assumed. It can be justified because these polyphenols are able to bind and precipitate proteins such as gliadins, and these complexes cannot be extracted for further analysis.

Cocoa and chocolate are one of the most common unwanted traps, but polyphenol sources are very varied among vegetables foods and includes spices and herbs (pepper, curry, oregano, parsley…), some nuts (peanut, pistachio…), pulses (lentils, beans, or peas), dried fruits (blueberries, plum…) and vegetables (artichoke, swiss chard leaves…). This problem has been solved by adding proteins to the extraction buffer that can capture these compounds, thereby avoiding catching gliadin or other prolamins. For this purpose, various options are available; these include adding fish gelatin and polyvinyl pyrrolidone (PVP) or skimmed milk to the cocktail solution.

Another conflictive matrix is soy; studies have shown that soy-based foods can give an overestimation of gluten content. Some authors have suggested that some soy epitopes could be recognized by gluten against raised antibodies [20]. As a result, these sample analyses could carry some false positives or an increased recovery time when spiked controls are prepared with this matrix. In that sense, in our laboratory we also found that after a hydroalcoholic extraction at 60% in soy matrices, the ELISA sandwich method detected around 50 mg/kg of gluten but, surprisingly, these samples became negatives after adding the cocktail solution or after placing the samples at 80 °C for 24 hours and then carrying out the extraction.

3.4.3 Hydrolysis

In some hypersensitive conditions, such as allergies, the use of extensively hydrolyzed formulas might be an option instead of an allergenic protein substitution [67]. Proteic hydrolysis during the processing of food might be a useful tool to reduce or abolish the harmfulness of prolamin proteins. Other strategies, such as fermentation of sourdough by lactic acid bacteria, might diminish prolamin toxic fractions because the majority of ethanol-soluble polypeptides could be mainly hydrolyzed and as a result, T cell stimulatory peptides would be broken [68]. Apart from adding lactic bacteria or fungal enzymes, endogenous cereal enzymes are most likely responsible for prolamin degradation during fermentation.

Other technological processes, like brewing, induce partial hydrolysis that produces smaller peptides and less secondary structure than the original proteins. Comparing them to the intact protein, these partial hydrolysates usually have enhanced physicochemical properties, whereas excessive hydrolysis might reduce some functionality [69–71].

In food processing, the enzymatic hydrolysis of wheat gluten is capable of improving its solubility and developing the emulsifying and foaming properties. In short, hydrolysis may decrease the toxicity of gluten [72], but this peptide fragmentation might complicate the analysis of gluten in these foods.

Reviewing some gluten analysis techniques, the ELISA sandwich is based on the requirement that at least two specific epitopes are recognized by the antibody. However, it is not appropriate when foods and beverages are treated with proteolytic enzymes, or when they are fermented because there may not be two of this sequence. Consequently, small hydrolyzed products with a single epitope cannot be reliably determined by using sandwich R5 ELISA [23, 24, 73]. As competitive R5 ELISA requires only one antibody-binding epitope, it is more suitable for the detection of hydrolyzed gluten than sandwich R5 ELISA. The weakness of competitive assessment might appear because nonspecific binding is more likely to occur when only one binding site is needed for detection.

The hydrolyzed gliadin extraction process is also complex because some reducing agents used in the hydro-alcoholic solution interfere with the assay compounds. In order to solve this problem, another extraction solution called UPEX (universal prolamin and glutelin extractant solution) has been designed; it improves this analysis procedure, avoiding the problems previously mentioned [64]. In other genomics-based techniques, such as the polymerase chain reaction (PCR) method, DNA extraction in hydrolyzed samples is difficult, and gluten-containing cereal DNA presence is not always related to gluten protein presence, probably due to the technological treatment of foods [45].

3.4.4 Deamidation and Transmidation

Wheat gluten hydrophobic characteristics are due to the high percentage of uncharged amino acid residues such as glutamines (Gln) and asparagines (Asn). These amino acids are easily deamidated, changing to glutamic and aspartic acid, respectively. After deamidation, the solubility, emulsification and foaming properties of these proteins are improved under mild acidic-heating conditions [74]. Apart from the chemical approach, enzymatic reactions based on transglutaminase enzymes (tTG) are used in gluten deamidation processes. Furthermore, tTG could catalyze cross-linking between the glutamine residue and lysine, or another amine donor, by covalent bonding, in a process known as "transamidation." Taking these characteristics into account, in the food industry, the microbial TG (mTG) has become widely used as a food glue as well as in order to improve the baking qualities of weak wheat flours [75–77].

Although some authors suggested that the addition of microbial TG to wheat flours does not affect the prolamin analysis, [78], other researchers found that deamidation of toxic peptides decreases the antibody affinity in gluten analysis assays [63]. Indeed, gluten deamidation drastically depressed the antibody recognition compared to the intact gluten proteins [79, 80].

On the other hand, the tTG present in the intestines induces deamidation and transamidation of prolamin toxic peptides, increasing their binding to HLA-DQ2 or -DQ8 molecules, and then the T-cell stimulating activity is increased [81, 82]. In that sense, celiac sufferers develop increased levels of autoantibodies against tTG that become important in diagnosis. In this way, Lerner et al. [75] have suggested that the increased use of mTG in food processing may promote celiac pathogenesis *ex vivo*, possibly explaining the surge in the incidence of celiac disease. By contrast, other authors [83] have found that if cultured duodenal biopsies from celiac patients are tested, the enzymatic modification of gluten by transglutaminase could prevent the immunologic effects of CD.

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Chapter 4
Gluten Content Change Over the Two Last Decades

Jonatan Miranda and Edurne Simón

Abbreviations

CD	Celiac disease
GF	Gluten-free
ppm	parts per million

In recent years, the gluten-free (GF) food industry has become one of the most thriving industries. According to the Mintel Report on GF foods, in the U.S., the production of these foods has risen 136% between 2013 and 2105, with sales reaching $11,609,000,000 [1]. However, the most remarkable aspect of this report is its GF market forecast of $14,175,000,000 for 2018 in the worst-case scenario, while the best predictions could be around $31,128,000,000 [1]. Reports by other companies portend a similar projection in Europe for 2015–2020 [2]. In the case of Canada, its government reported that 15.5% of newly launched food products in 2013 were GF [3]. According to the registered data, among GF food, snacks, bakery products, sauces and seasonings were the top categories in GF food launches. In addition to foodstuffs, around 200 new GF beverages were put on the market in Canada between 2007 and 2013 [3].

A clear example of this GF product expansion is easy to observe. While a few years ago, selling GF products was limited to specialty shops, today these products fill the shelves of grocery stores and supermarkets everywhere. However, this upsurge in the production and variety of GF products is not accompanied by an increase in the prevalence or awareness of celiac disease (CD). As Reilly [4] describes, searches for comments related to CD by Google Trend in the last decade remained constant, whereas those related to GF foods increased exponentially. Indeed, nowadays the search ratio is 1:8; that is, for each search for information on CD, there have been eight for gluten-free products [4].

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Most American consumers of these products are not people who suffer from CD [5]. In these particular cases, their interest in GF foods appears to be similar to organic food conventionalization [6]. GF food for people without symptoms associated with gluten is motivated by a lifestyle based on cultural, ecological, civic, historical, ethical or health-related interest [6]. The use of ancient GF grains, for instance, may contribute to a product's enchanting appeal.

Along these lines, a worldwide survey carried out in 2015 among 30,000 adults in 60 countries indicated that 21% of the interviewees found the term "GF" to be very important in their choice of buying food [7]. However, it should not be forgotten that these types of products are more expensive than their counterparts that contain gluten, which also affects the purchasing decision [8]; this has a great impact on the age of the potential buyers of gluten-free food. The same survey revealed how around a third of respondents under age 34 are willing to buy food without gluten, despite the higher price, while only 12% of the over-65s would do so [7].

As mentioned in the chapter of this book concerning legislation, there are many countries that have accepted the definition of GF and the threshold of 20 mg of gluten per kg of food (or parts per million: ppm), proposed by the Codex Alimentarius to establish the foods that can be labeled "GF" (Chap. 2). It is important to understand that many consumers rely on the labeling or claims of GF when making their purchases; nevertheless, gluten can be unintentionally introduced into the food through direct contact with raw materials containing gluten (wheat, barley, and rye). This gluten contamination can happen to product development beforehand (during harvest, transport, or storage of raw materials), during processing (adding meat sauces, beans or soups that are intended to improve the sensory and technological characteristics of the dish), or after product embellishment (e.g., a serving dish that has been cross-contaminated with gluten-containing products). Although for some GF product consumers there would not be any repercussions, it can lead to reactions and severe symptoms in patients with CD or related pathologies. For this reason, a precise and routine control of gluten content in food is necessary to ensure its safety in people who can have a reaction resulting from the intake of this protein.

Several studies have been carried out concerning gluten contamination in GF-labeled products (Table 4.1). In 2010, two interesting Brazilian researches revealed that around 13% of gluten-free samples contained more than 20 mg/kg of gluten [9, 10]. Altogether, both studies analyzed 185 GF products, among them bread, flours, dough, sauce, cereal bars, and cereal-snack food groups.

Since 2010, the USA has been the country where most of the studies evaluating gluten content in GF labeled products have been carried out. Between 2011 and 2015, Thompson and Simpson (2015) collected 158 samples to analyze their gluten content [11]. The results concluded that gluten was detected in almost 13% of the samples (>5 mg/kg of gluten). Furthermore, it is important to point out that 5.1% of the samples had over 20 mg/kg. Other research conducted by the same group indicated a similar ratio of samples over the pre-set threshold (4:112) [12].

Table 4.1 Summary of publications related to gluten-free labeled products

Publication	Used kit	Sample number	Sample classification	Gluten detection	Positive samples	Country	Period
Valdes et al. (2003) [22]	R5	3088	Not classified by food group	3.2–20 ppm, 628 samples (20.3%) 20–100 ppm, 754 samples (24.4%) 100–200 ppm, 120 samples (3.9%) >200 ppm, 197 samples (3.9%)	–	Spain and other European counties	2003[a]
Gelinas et al. (2008) [19]	R5	77	Variety of food groups not defined	>20 ppm, 16 sample (20.8%)	Breakfast cereals, cookies, pancake, flour and sauce	Canada	2008[a]
Laureano (2010) [9]	R5	70	Bread, flours, dough, sauce, cereal bars, and snacks	5–20 ppm, 11 samples (15.7%) >20 ppm, 9 sample (12.9%)	Bread, Flours, dough, sauce, cereal bars, and snacks	Brazil	2010[a]
Plaza-Silva (2010) [10]	R5	115	Variety of food groups	>20 ppm, 15 sample (13.0%)	–	Brazil	2010[a]
Daniewski et al. (2010) [21]	R5	22	Variety of food groups	≥20 ppm, 6 samples (27.3%)	Pasta, bread, biscuits, bakery,	Poland	2010[a]
Agakidis et al. (2011) [20]	Skerritt	26	Flours, dairy, sweets, miscellaneous	>20 ppm, 2 sample (7.7%)	Flours, dairy	Greece	2012[a]
Thompson and Simpson (2015) [11]	R5	158	–	5 to ≤10 ppm, 6 samples (3.8%) >10 to <20 ppm, 7 samples (4.43%) ≥20 ppm, 8 samples (5.1%)	–	USA	2011–2014
Gibert et al. (2013) [13]	R5	205	Bread, pasta, pastry, biscuits, pizza and breakfast cereals	>5 ppm, 16 samples (6%) >20 ppm, 1 sample (0.5%)	Bread, pasta, pastry, biscuits, and breakfast cereals	Italy, Spain, Germany and Norway	2013[a]

(continued)

Table 4.1 (continued)

Publication	Used kit	Sample number	Sample classification	Gluten detection	Positive samples	Country	Period
Thompson and Grace (2013) [12]	R5	112	–	≥ 20 ppm, 4 samples (3.6%)	–	USA	2013[a]
Sharma et al. (2015) [23]	R5	275	Grains/seeds/nuts/ legumes, condiments/ sauces, curry/soup/soup mixes, baking mixes, baked foods, pasta products, breakfast cereals, snack foods, granola/bars/ energy bars, beverages/ice-creams/ frozen desserts, meat/meat substitutes/refrigerated or frozen foods and others	>5 ppm, 10 samples (3.6%) >20 ppm, 3 sample (1.1%)	Grains/seeds/nuts/ legumes, condiments/ sauces, ,pasta products, breakfast cereals, snack foods and meat/meat substitutes/ refrigerated or frozen foods	USA	2015[a]
Bustamante et al. (2017)	R5Skerritt	1652	Flours, breakfast cereals/ bars, bakery, pastry/ dough, bread, pasta, cereal snack and yeast	5–10 ppm, 25 samples (1.5%) 11–20 ppm, 32 samples (1.9%) 21–100 ppm, 39 samples (2.4%) 100–200 ppm, 5 samples (0.3%) >200 ppm, 9 samples (0.5%)	Flours, breakfast cereals/bars, bakery, pastry/dough, bread, pasta, cereal snack	Spain	2004–2016

[a]Corresponds to the publication year, due to the lack of information about when the study took place

Unfortunately, none of the authors gave information on the food groups studied or positive samples classification.

With regard to Europe, during this same time period, an analysis of bread, pasta, pastry, biscuits, pizzas, and breakfast cereals from Italy, Spain, Germany and Norway was conducted, in which the researchers noted minimal gluten contamination [13]. Although some traces were detected [17] in food samples (>5 mg/kg), only one sample (of pastry) from 205 GF products showed gluten levels over 20 mg/kg.

Some customers attribute the GF assumption of a product to reading the list of ingredients. As a consequence, research in which GF products were not labeled but appeared to be free of gluten-containing ingredients (no wheat, barley, or rye) have been carried out (Table 4.2). In the case of these kinds of foodstuffs, it can be said that Thompson promoted their analysis when she advised them about oats contamination with gluten in the USA [14]. In almost one-third of 12 studied oats, gluten levels were over 20 mg/kg. Later on, two studies conducted in two different countries (Brazil and Poland), declared similar percentages of gluten contamination above the standard limit (of 9.3 and 10.5%, respectively). The same year, Thompson et al (2010) published a study with 22 grains, seeds and flours [15]; according to the findings, millet flour and grain, white rice flour, buckwheat flour, sorghum flour, and soy flour were the most-contaminated raw materials [15].

With regard to bakery products, in research conducted in Brazil, positive samples were found in 6.1% of the products [16], and another study showed a cross-contamination by beans served in restaurants there [17]. Their results showed that 16% of the samples and 45% of the restaurants suffered from gluten contamination. A recent study performed in the USA, involving 101 non-gluten-free-labeled samples concluded that five samples (breakfast cereals, spices, snacks, seasoning mixes, and oat fiber) contained more than 20 mg/kg of gluten [18].

In view of the above-mentioned results, as well as according to logic, it seems that gluten level control has been higher in GF labeled foods than in those that are apparently GF (by checking food labels or composition). In this regard, research conducted simultaneously in Canada and Greece with GF-labeled and non-labeled foods (but apparently GF) showed similar percentages of samples above 20 mg/kg gluten in both groups (about 20% in Canada and 10% in Greece) [19, 20]. It is important to note that the sample sizes of both studies did not exceed 150 foodstuffs. On the contrary, in another study with a moderate sample size, but that took place in Poland, there was a higher ratio of positive samples in GF-labeled samples than in those that were apparently GF [21].

The first large-scale study evaluating gluten content not only in GF-labeled products but also in non-labeled ones, was carried out by Valdes et al (2003) [22] in which they analyzed 3,088 GF-labeled samples from several European countries. Their results indicated a high gluten detection (1,699 samples with >3.2 mg/kg) in these kinds of products, with almost one-third of the total samples having gluten over 20 mg/kg. For non-GF-labeled samples, this analysis found that in 1,366 samples, 66% were contaminated with gluten (>3.2 mg/kg of gluten) and 570 with more than 20 mg/kg. Moreover, in the samples studied, the authors observed that maize was the raw material most contaminated with gluten; therefore, it is possible to

Table 4.2 Summary of publications related to non-gluten-free labeled products

Publication	Used kit	Sample number	Sample classification	Gluten detection	Positive samples	Country	Period
Valdes et al. (2003) [22]	R5	1366	Not classified by food group	3.2–20 ppm, 341 samples (25.0%) 20–100 ppm, 325 samples (23.8%) 100–200 ppm, 95 samples (7.0%) >200 ppm, 150 samples (1.1%)	–	Spain and other European counties	2003*
Thompson (2004) [14]	R5	12	Rolled or steel-cut oats	>20 ppm, 9 sample (32%)	Rolled or steel-cut oats	USA	2004
Gelinas et al. (2008) [19]	R5	71	Variety of food group not defined	> 20 ppm, 16 sample (22.5%)	Cereals bars, flours, chips, breakfast cereals, bran, bean mix, oatmeal	Canada	2008*
Plaza-Silva (2010) [10]	R5	86	Variety of food group	>20 ppm, 8 sample (9.3%)	–	Brazil	2010*
Daniewski et al. (2010) [21]	R5	19	Variety of food group	≥ 20 ppm, 2 samples (10.5%)	Flakes, flour	Poland	2010*
Thompson et al. (2010) [15]	R5	22	Grains, seeds and flours	>5 ppm, 9 samples (41%) >20 ppm, 7 sample (32%)	Millet flour and grain, White rice flour, buckwheat flour, sorghum flour and soy flour.	USA	2010*
Agakidis et al. (2011) [20]	Skerritt	15	Flours, dairy, sweets, miscellaneous	>20 ppm, 2 sample (13.3%)	Flours	Greece	2012*
Oliveira et al. (2014) [17]	Skerritt	60	Beans	>20 ppm, 10 sample (16.7%)	Beans	Brazil	2014*
Farage et al. (2017) [16]	R5	130	Bakery products	>20 ppm, 8 sample (6.1%)	Bakery	Brazil	2014

Sharma et al. (2015) [23]	R5	186	Grains/seeds/nuts/ legumes, condiments/ sauces, curry/soup/soup mixes, pasta products, breakfast cereals, snack foods, granola/bars/ energy bars, beverages/ ice-creams/frozen desserts, meat/meat substitutes/ refrigerated or frozen foods, and others	>5 ppm, 48 samples (25.8%) >20 ppm, 36 sample (19.3%)	Grains/seeds/nuts/legumes, curry/soup/soup mixes, pasta products, breakfast cereals, snack foods, granola/bars/ energy bars, beverages/ ice-creams/frozen desserts, meat/meat substitutes/ refrigerated or frozen foods, and others	USA	2015*
Thompson et al. (2016) [18]	R5	101	–	>5 to<20 ppm, 9 samples(8.9% ≥20 ppm, 5 samples (4.9%)	Breakfast cereal, spices, snacks, seasoning mix, green tea leaves, oat cereal, legume, oat fiber	USA	2016*
Bustamante et al. (2017) [27]	R5 Skerritt	962	Flours, breakfast cereals/ bars, bakery, pastry/ dough, bread, pasta, cereal snack and yeast	5–10 ppm, 28 samples (3.1%) 11–20 ppm, 17 samples (1.9%) 21–100 ppm, 26 samples (2.7%) 100–200 ppm, 13 samples (1.4%) >200 ppm, 38 samples (4.0%)	Flours, breakfast cereals/ bars, bakery, pastry/dough, bread, pasta, cereal snacks and yeast	Spain	2004–2016

*Correspond to the publication year, due to the lack of information about when the study took place

conclude that according to Valdes et al (2003), around 30% of analyzed samples were over 20 mg/kg in GF-labeled products, as well as non-labeled ones.

By contrast, two recently published studies conducted on Spanish (Bustamante et al. 2017 [27]) and North American [23] on GF claimed products indicated that highly gluten-contaminated samples were more common in non-GF-labeled products. Indeed, the positive samples percentage of apparently GF products doubles the percentage of GF-labeled ones (around 10% vs. around 5%). The explanation that could justify the discrepancy between these two studies and that carried out by Valdes et al (2003) [22] could be the Codex Alimentarius revision, that was done in 2008. Since that year, the threshold proposal of 20 mg/kg or ppm of gluten to declare a food as GF was implemented by various government regulations. This guided the food industry toward stricter hazard analysis and critical control point implementation and, probably, to the development of new composition formulas. Figure 4.1 was created by the data collected from the gluten analysis of GF research previously mentioned.

Tables 4.1 and 4.2 show how before 2008 there was a marked reduction in gluten-positive samples (over 20 ppm of gluten). It is true that the decrease was even greater for GF- labeled products (reaching 3% of samples over 20 ppm of gluten) – which is logical, taking into account that the food industry must guarantee the GF claim. However, it must be emphasized that the "apparently GF" products (those without any gluten-containing ingredient shown on the label) have lowered the gluten-positive samples to half from 2008 until 2016. With regard to gluten-contaminated samples, an overview (from 1998 to 2013) of nearly 10,000 food products [24] sold in Spain, confirmed the presupposition that commercially GF-rendered food groups

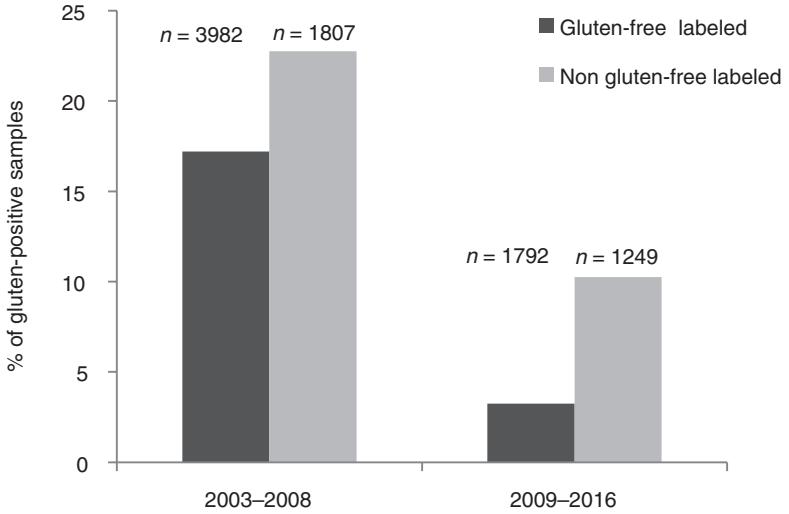

Fig. 4.1 Evolution of gluten-positive samples (>20 mg/kg) sorted by 2003–2008 and 2009–2016 time periods for gluten-free labeled and non-labeled products. Data are expressed as the percentage of the total sample analyzed (*n*) in each period

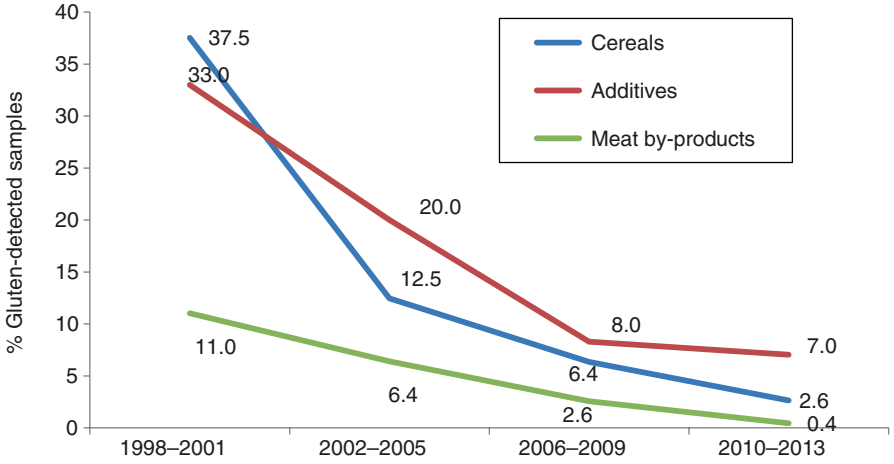

Fig. 4.2 Evolution of gluten-detected samples, expressed as % of the total analyzed, in three food groups: cereals, additives, and meat derivatives (Modified from Bustamante et al. [24])

represented by cereal-based foods, was the more contaminated with this prolamin than naturally gluten-free food in other food categories, represented by meat by-products) [24] (Fig. 4.2). Taking into account the positive samples' description of Table 4.2, this was also seen in other research. Apart from gluten contamination, it is well known that commercially GF-rendered products were poor from an organoleptic point of view, as well as extremely expensive when compared with homologous gluten-containing breads or baked products. It is worth noting that another frequently contaminated food group is that of additives, such as paprika, cinnamon, or curcumin powder, among others.

The different standards and regulations led the food industry toward stricter hazard analysis and critical control point implementation and, probably, to new composition formulas. After the Codex Alimentarius's proposed threshold [25], detected gluten was significantly down (Fig. 3.1). In that sense, gluten traces containing cereal-based foods were reduced to around 3% and then safer GF-labelled products were manufactured. Moreover, after the EU No. 1169/2011 standard was enforced, apparently GF, but not GF-labeled products, also became less contaminated.

The achievements attained have been essential to promote safe food consumption for celiac patients and groups with gluten-related disorders. Nevertheless, work must continue in this direction. Data shows that wheat consumption per capita has increased [26], which may contribute to gluten cross-contamination of "probably safe" foods (GF-labeled products and products apparently not containing gluten). In this sense, it is important to properly train food handlers, because sometimes their knowledge of the subject is not adequate enough. Moreover, it should keep on controlling the gluten content of the "apparently" GF food, because owing to economical aspects, many people include these products in their diet instead of GF-labeled ones.

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Chapter 5
Nutritional and Sensorial Aspects of Gluten-Free Products

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Abbreviations

GF Gluten-free

5.1 Introduction

Gluten, as a general concept, is a protein responsible for elasticity and viscosity of dough, enhancing the retention of gas, and making structured, baked end-products [1]. Whereas the forced removal of this protein in gluten-free (GF) products can lead to reduced palatability and acceptance, many alternative proposals are available to keep this from happening.

During the creation of GF products, it is common to use GF raw materials. In most cases, gluten-free cereals (mainly corn and rice) are the major ingredients of these products. However, pseudo-cereals, vegetables, or other ingredients (milk and egg protein) represent another possibility. When using GF cereals, substances that mimic the properties of gluten are often added. Among these, hydrocolloids (guar and xanthan gums, alginate, carrageenan, hydroxypropyl methylcellulose, carboxymethyl cellulose), emulsifiers, isolated proteins (from egg, legumes, or dairy products) or enzymes (cyclodextrin glycosyl transferases, transglutaminase, proteases, glucose oxidase, and laccase) are those that are most often used [2].

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In the particular case of bread, other approaches have been undertaken to improve its organoleptic quality; among them, sourdough technology and high hydrostatic pressure are the most important. Although these mechanisms have been designed for the organoleptic improvement of GF products, they can also affect the nutritional composition. Throughout this chapter, in many cases bread will be used as a reference. It is important to point out that among all GF foods, bread is the most widely studied [3].

On the other hand, some foods originally contained gluten, but after various treatments in the elaboration process, are no longer toxic for celiac patients; this is the case in GF wheat starches or enzyme-treated barley beers, among others. Although there is growing scientific and academic interest in the improvement of the organoleptic characteristics of GF products [4, 5], studies of the celiac population show that the degree of satisfaction of these people with products actually available on the market is poor [6].

5.2 Non-gluten-Containing Grains and Pseudo-cereals

Two main food groups are mostly used for replacing cereals containing gluten: (1) GF grains or true cereals (maize, rice, sorghum and teff), and (2) pseudo-cereals (amaranth, quinoa, buckwheat, etc.).

Corn (*Zea mays* L.) and cornstarch are commonly used in the preparation of GF Foodstuffs alone or with xanthan gum. In the case of bread, the product obtained has an appropriate volume, but lacks of taste and has an unsuitable crumb structure [7]. Because of the absence of color, easy digestion and hypoallergenic effect, rice (*Oryza sativa*) is one of the most appropriate cereal grains for GF foods. However, rice lacks the elastic and plastic properties to retain the gas formed during fermentation.

Tables 5.1 and 5.2 show the nutritional composition of the most commonly used raw materials in GF and gluten-containing food preparation. The data were obtained from the online database of the U.S. Department of Agriculture [8].

When comparing the two traditionally used cereals in GF foodstuffs (corn and rice) with cereals containing gluten (wheat, barley, and rye), it can be observed that rice and corn have smaller amounts of protein and fat and larger amounts of carbohydrates (Table 5.1). Meanwhile, the general contribution of micronutrients is lower when comparing wheat with corn.

Sorghum (*Sorghum vulgare)* is quite similar to corn in that the primary component is starch. However, sorghum protein and starch are less digestible [9]. This GF cereal has some limited properties for bread-making; its temperature for gelatinization is high, and it forms cracks in the bread and large holes in the crumbs [7]. This cereal has the lowest caloric intake compared to wheat, due to a lower content of lipids and carbohydrates (Table 5.1).

Teff (*Eragrostis teff*) is a GF cereal used primarily in Ethiopia and Eritrea. Although its use as a raw material is not widespread due to its high protein and

Table 5.1 Macronutrient composition of gluten-containing and gluten-free grains according to USDA Food Composition Databases

		Gluten-containing grains											
	Units	Wheat, hard white	Ryegrain	Barley, pearled, raw	Oats[a]	Corngrain, yellow	Rice, white, long-grain, regular, raw, unenriched	Teff, uncooked	Amaranthgrain, uncooked	Quinoaun-cooked	Sorghumgrain	Millet, raw	Buckwheat
Water	g	9.57	10.60	10.09	8.22	10.91	11.62	8.82	11.29	13.28	12.40	8.67	9.75
Energy	kcal	342	338	352	389	361	365	367	371	368	329	378	343
Protein	g	11.31	10.34	9.91	16.89	6.93	7.13	13.30	13.56	14.12	10.62	11.02	13.25
Carbohydrate, by difference	g	75.90	75.86	77.72	66.27	76.85	79.95	73.13	65.25	64.16	72.09	72.85	71.50
Fiber, total dietary	g	12.2	15.1	15.6	10.6	7.3	1.3	8.0	6.7	7.0	6.7	8.5	10.0
Sugars, total	g	0.41	0.98	0.80	–	0.64	0.12	1.84	1.69	–	2.53	–	–
Total lipid (fat)	g	1.71	1.63	1.16	6.90	3.40	0.66	2.38	7.02	6.07	3.46	4.22	3.40
FA, total saturated	g	0.277	0.197	0.244	1.217	0.543	0.180	0.449	1.459	0.706	0.610	0.723	0.741
FA, total monounsaturated	g	0.203	0.208	0.149	2.178	1.018	0.206	0.589	1.685	1.613	1.131	0.773	1.040
FA, total polyunsaturated	g	0.750	0.767	0.560	2.535	1.759	0.177	1.071	2.778	3.292	1.558	2.134	1.039

– with no value, *FA* fatty acids

[a]Oats can be contaminated with gluten

Table 5.2 Micronutrient composition of gluten-containing and gluten-free grains according to USDA Food Composition Databases

		Gluten-containing grains				Gluten-free grains							
	Units	Wheat, hardwhite	Ryegrain	Barley, pearled, raw	Oats[a]	Corngrain, yellow	Rice, white, long-grain, regular, raw, unenriched	Teff, uncooked	Amaranthgrain, uncooked	Quinoaun-cooked	Sorghumgrain	Millet, raw	Buckwheat
Minerals													
Calcium	mg	32	24	29	54	7	28	180	159	47	13	8	18
Iron	mg	4.56	2.63	2.50	4.72	2.38	0.8	7.63	7.61	4.57	3.36	3.01	2.20
Magnesium	mg	93	110	79	177	93	25	184	248	197	165	114	231
Phosphorus	mg	355	332	221	523	272	115	429	557	457	289	285	347
Potassium	mg	432	510	280	429	315	115	427	508	563	363	195	460
Sodium	mg	2	2	9	2	5	5	12	4	5	2	5	1
Zinc	mg	3.33	2.65	2.13	3.97	1.73	1.09	3.63	2.87	3.10	1.67	1.68	2.40
Vitamins													
Vitamin C, total ascorbic acid	mg	0.0	0.0	0.0	0.0	0.0	0.0	–	0.116	–	0.0	0.0	0.0
Thiamine	mg	0.387	0.316	0.191	0.763	0.246	0.070	0.390	0.200	0.360	0.637	0.421	0.101
Riboflavin	mg	0.108	0.251	0.114	0.139	0.080	0.049	0.270	0.923	0.318	0.184	0.290	0.425
Niacin	mg	4.381	4.270	4.604	0.961	1.900	1.600	3.363	0.591	2	7.081	4.720	7.020
Vitamin B6	mg	0.368	0.294	0.260	0.119	0.370	0.164	0.482	82	0.487	0.851	0.384	0.210
Folate, DFE	µg	38	38	23	56	25	8	–	0.00	184	38	85	30
Vitamin B12	µg	0.00	0.00	0.00	0.00	0.00	0.00	–	0	0.00	0.00	0.00	0.00

Vitamin A,	µg	0	1	1	0	11	0	0	2	1	0	0	0
Vitamin A	IU	9	11	22	0	214	0	9	1.19	14	0	0	0
Vitamin E (α-tocopherol)	mg	1.01	0.85	0.02	–	0.42	0.11	0.08	0.0	2.44	0.96	0.05	–
Vitamin D (D2 + D3)	µg	0.0	0.0	0.0	0.0	0.0	0.0	–	0	0	0.0	0.0	0.0
Vitamin D	IU	0	0	0	0	0	0	–	0.0	0	0	0	0
Vitamin K (phylloquinone)	µg	1.9	5.9	2.2	–	0.3	0.1	1.9	0.0	0	–	0.9	–

–with no value, *IU* international units

[a]Oats can be contaminated with gluten

micronutrient content, new formulations based on this cereal are increasing. In this sense, it is very important to highlight its excellent balance in the amino acid composition (eight of them essential for humans). According to the literature, the amounts of isoleucine, leucine, valine, tyrosine, threonine, methionine, phenylalanine, arginine, alanine, and histidine in teff are higher than those in wheat or barley [10]. Furthermore, this amino acid profile gives teff high levels of properties for malt and beer production [10]. As described in Table 5.2, its high mineral content (of calcium, iron, magnesium or zinc) must be emphasized.

Millets are small cereal grains widely used for brewing traditional beers as well as a staple food in the form of porridges and couscous. Under "millet" are included several genera and species such as *Pennisetum typhoides*, *Pennisetum glaucum*, *Eleusine coracona*, *Setaria italica*, etc. [11]. Millets are rich in polyphenols with antioxidant capacities [12]. The protein and carbohydrate content is similar to the quantity of these macronutrients in wheat and rye, with a higher concentration of lipids, but their fat content is lower than in oat. Millet has a nutritional composition very close to teff, at least in terms of macronutrient content (Table 5.1); however, micronutrient deficiencies can be observed when comparing millet with teff (Table 5.2).

Oats (*Avena sativa*) are special cereals, and their use in the Scandinavian countries as GF cereals is widespread. Nevertheless, using oats in a GF diet is still a controversial subject. Some authors maintain that avenins can trigger an immunogenic response in celiac patients [13], and other researchers have found that the immunogenicity of oats varies, depending on the cultivar consumed [14–16]. Furthermore, in order to assure the lack of contamination from the point of view of celiac disease, it is necessary to take particular care. Some oats are grown, stored, transported, or processed with gluten-containing cereals, thereby becoming gluten-contaminated products. A clear example of this cross-contamination is reflected in the publication of Thompson et al. [17], in which the authors found that 32% of analyzed GF samples contained gluten.

However, numerous benefits have been attributed to oats in recent years. Its calcium, phosphorus, potassium, and zinc content is high, as is the contribution of unsaturated fatty acids, thiamine, or folate (Tables 5.1. and 5.2). Its high level of fiber, mainly beta-glucans, is noteworthy [18]. Meanwhile, in the production of oat bread, unlike what happens when baking other GF cereals (e.g., sorghum), the end product has a nice appearance with a proper soft crumb structure or volume [19].

As a last raw material related to GF grains, it is necessary to speak of wheat starch, a product extracted from processed wheat flour. After several optimized processes for separating the protein from the starch, a pure, and gluten-free element is obtained, which needs to have certification. Its nutritional value is minimal, as it is just a source of carbohydrates.

Unlike monocotyledonous cereals, pseudo-cereals are dicotyledonous and do not belong to the *Gramineae* family. However, its seeds are rich in starch and can be used as cereal alternatives [7]. Due to its botanical origin, their nutritional properties share similarities with legumes and grains [20].

Amaranth (*Amaranthus* spp) has been consumed by the Inca, Mayan and Aztec civilizations for many years. It has high levels of both fiber and protein, with equally

significant levels of methionine and lysine. With respect to fiber, it has a considerable percentage of insoluble fiber. Escudero et al. (2004) reported that from the total dietary fiber in *A. cruentus*, 4.2% was soluble [21]. Similar to quinoa, its fatty acid profile is beneficial for health, with a large amount of unsaturated fatty acids (Table 5.1). As is shown in Table 2, and in the literature [22], amaranth is very rich in micronutrients. Although the content of minerals as calcium, phosphorus, iron, potassium, magnesium, and zinc must be highlighted, ascorbic acid, riboflavin, or thiamine could be found at an acceptable level.

The introduction of buckwheat (*Fagopyrum esculentum*) as a raw material for GF foodstuffs has improved their nutritional qualities [23]; however, problems have been detected for these kinds of breads, limiting their use. The disadvantages include flavor intensity and brittleness after 2 days of storage [7].

Quinoa, *Chenopodium quinoa* is an *Amaranthacean*, stress-tolerant plant that has been cultivated in the Andes for the last 7,000 years. As in the case of amaranth or teff, the protein content of quinoa is higher than that of gluten-containing grains. In addition, the amino acid profile of this pseudo-cereal is very beneficial. According to Vega-Galvez's review, quinoa provides levels of FAO/WHO recommendations for histidine, isoleucine, lysine, methionine + cysteine, phenylalanine + tyrosine, threonine, tryptophan, and valine [24]. Comparing it with other GF raw materials, the carbohydrate amount in quinoa is lower (Table 5.1); however, due to its high proportion of d-xylose and maltose, and low levels of glucose and fructose, this pseudo-cereal has appropriate properties for malted drink formulation [24].

The vitamin content of quinoa is especially significant for riboflavin, vitamin E and vitamin B6 (Table 5.2). With regard to minerals, its calcium and phosphorus content is higher than that of wheat and barley. Moreover, quinoa's contribution in oleic and linoleic acid is also relevant [24].

5.3 Other Ingredients Used in the Preparation of GF Foods

The research conducted by do Nascimiento et al. (2013) relating to the composition of gluten-containing and GF products, revealed that although there were ingredients common to both formulations, there were some differences [25]. The study was carried out in Portugal with a total of 324 products (162 of them GF), and 12 terms found on the product labels were on products both with and without gluten. Meanwhile, the most common terms found only on the labels of GF foods were rice flour, egg, cassava starch, lecithin, natural corn starch, soy and rice flour, and vanilla [25]. Some of these ingredients are the basis of GF foodstuffs, but others are only assistants, to add to the characteristics of GF foods.

In baking, dairy ingredients set up nets that improve the texture and besides they reduce the staling and increase the flavor and crust color [7]. This type of ingredient can be found in various forms, with isolated milk protein or caseinate the most common. It is important to point out that the presence of lactose in celiac patients can

lead to health problems. The damage caused by celiac disease in intestinal villi, in many cases causes the patients to have temporary lactose intolerance [26].

In their publication on breads, Matos and Rosell reported that one of the main ingredients found is egg protein [27]; this has high cohesive viscoelastic power, is essential for stable foaming, and improves gas retention in bread [7]. Moreover, it improves protein content and the amino acid profile of GF foods, just as soy can do. In fact, soybean protein and lupine are two other ingredients that are very often used, as Matos and Rosell found in their study conducted on 11 GF breads [27].

The addition of starches or hydrocolloids is also quite common in GF products. In the case of starches, as shown in the study of do Nascimiento et al. (2013), one of the most often used is corn [25]. Due to the starch pastes formed that can trap air bubbles, starch gelatinization plays an important role. For example, prolamin corn (zein), combined with starch and water, form a viscoelastic mass close to that of wheat dough [28]. In the case of hydrocolloids, these are polysaccharides with a high molecular weight and hydrophilic characteristics, extracted from plants, seaweed, and bacterial sources. They are used as structuring agents to mimic the viscoelastic properties of gluten. These agents have an additional value; taking into account that they are soluble fiber, their inclusion in GF foods increases the nutritional quality of the products [2].

5.4 Gluten-Free Rendered Foods vs. Gluten-Containing Rendered Foods

5.4.1 Differences in Nutritional Composition

Researchers working in the field of celiac disease and GF diet have increased their interest in the nutritional composition of the GF diet and the adequacy of GF foods as healthful products. In fact, a detailed analysis of the nutritional composition of GF foodstuffs, and the design of databases considering these foods and their composition, have recently been carried out by several groups in Italy, Austria, Canada, Australia, and Spain [29–33].

Miranda et al. (2014) previously reported a comparison between 206 GF foodstuffs vs. 289 equivalent foods containing gluten [33]. Information from the panels and product labels was compared, and differences in calories, macronutrients, fiber, sodium, salt, and cholesterol were described. Other authors estimated the full nutrient composition of GF products by using the nutritional composition of each ingredient of a GF product and estimating the quantity of each ingredient in the final recipe of the product [29, 30, 32]. As a result, in these studies the energy content, macronutrients, fiber, cholesterol, and mineral and vitamin content were analyzed and compared to that of gluten-containing foodstuffs. Nevertheless, to compare the results obtained in different studies is difficult, due to the fact that not all of them

show the same classification of food categories. Most of the studies analyze and compare the nutritional composition of gluten-free flours, breads, bakery products, pasta, cereals, cookies, and snacks, and some also report results obtained in other food groups such as frozen foods or processed meats [31], or convenience foods (chicken nuggets, fish sticks, soups, etc.) [30].

Differences in calorie content between GF and gluten-containing counterparts were not observed in most of the analyzed food groups. Only GF flours, breads, and the dough/pastry/ pizza group showed more calories than their analogues containing gluten. According to these results, when the calorie content of GF and gluten-containing foods was compared, as well as in other studies, no differences were observed [30, 31].

However, and according to the macronutrient content, results obtained in different studies agree that GF products have higher amounts of fat [31, 33], especially saturated fats, and a lower amount of protein [30, 31, 33] than their regular counterparts. These differences may be due to the formulations of GF products. On the one hand, differences in lipid content could be due to the fact that frequently, lipid-rich ingredients, such as animal or vegetable oils and emulsifiers (mono- and diglycerides of fatty acids) are added in order to improve the palatability of GF foodstuffs [34]. Such ingredients are indeed useful in bakery products for the stabilization of gas bubbles and the reduction of kneading resistance and swelling of starch granules [35]. Moreover, emulsifiers can be used to increase the dough stiffness, improve the bread structure, and decrease the speed of staling. In pasta products, emulsifiers act as lubricants in the extrusion process and provide firmer consistency and a less sticky surface because they control starch swelling and leaching phenomena during cooking [36]. Thus, although emulsifiers can be avoided in pasta products, they are necessary for baked products. On the other hand, the lower protein content could be due to the use of starches, flours from low protein cereals such as rice or corn, or the use of gums or enzymes in their elaboration [34].

Some of the GF foods analyzed in the studies mentioned differed in their carbohydrate content as well. Miranda et al. (2014) saw a higher amount of them in flour, whereas Kulai et al. (2014) observed these results in the pasta group [31, 33]. However, a lower amount of sugar was observed in foods from the pasta group by both authors, and this suggested that starches represent the largest component of carbohydrates in GF pasta. Indeed, a low intake of non-starch carbohydrates in people following a GF diet has been reported [37, 38].

GF foodstuffs showed a low amount of fiber [31, 33], especially when various pastas or flours were analyzed; this could be due to a lack of whole grain cereals in the production of GF pastas or flours. Salt and sodium content in GF products is also higher [29, 30, 33]. The use of sodium stabilizes the structure and enhances the taste of GF products, which is especially important when the main ingredient in these products is tasteless starch.

Table 5.3 summarizes the results obtained in four different studies where GF products' nutritional composition was analyzed and compared to that of gluten-containing counterparts.

Table 5.3 Outcomes from four studies comparing the nutritional composition of GF and gluten-containing foods

	Energy	Protein	Total carbohydrates	Simple carbohydrates	Total Lipids	Saturated Lipids	Fibre	Sodium	Cholesterol
Flours=									
Miranda et al. (2014)	↑	↓	↑	=	=	=	↓	↑	–
Kulai et al. (2014)	=	=	=	=	=	=	=	NA	NA
Missbach et al. (2015)	=	↓	↑	=	=	=	=	=	=
Cereals									
Miranda et al. (2014)	↓	=	=	=	=	=	↑	↑	–
Kulai et al. (2014)	=	=	=	=	=	=	=	NA	NA
Missbach et al. (2015)	=	=	=	=	=	=	=	↑	=
Wu et al. (2015)	=	↓	NA	↓	NA	=	=	=	NA
Pasta									
Miranda et al. (2014)	=	↓	=	↓	↑	↑	↓	↑	↓
Kulai et al. (2014)	=	↓	↑	↓	=	–	↓	NA	NA
Missbach et al. (2015)	=	↓	↑	↑	=	=	↑	=	=
Wu et al. (2015)	↓	↓	NA	↓	NA	=	=	=	NA
Breads									
Miranda et al. (2014)	↑	↓	=	=	↑	↑	NA	NA	NA
Kulai et al. (2014)	=	↓	=	=	↑	=	=	NA	NA
Missbach et al. (2015)	=	↓	=	=	=	=	=	↓	=
Wu et al. (2015)	=	↓	NA	=	NA	=	=	=	NA
Dough/Pastry/Pizza									
Miranda et al. (2014)	↑	↑	=	=	↑	↑	=	↓	=

Bakery									
Miranda et al. (2014)	↓	↓	↓	=	=	↓	=	↑	↓
Kulai et al. (2014)	=	=	=	=	=	0	=	NA	NA
Missbach et al. (2015)	=	↓	=	=	=	=	=	↑	NA
Wu et al. (2015)	=	=	NA	↑	NA	↓	=	=	NA

– Data not available, *NA* not analyzed, ↑ GF products contain more than gluten-containing products, ↓ GF products contain less than gluten-containing products, = GF products and gluten-containing products contain the same amount

With regard to vitamins and minerals, Thompson was the first researcher to report that GF products (breads, pasta, breakfast cereals, and flours) had less niacin, riboflavin, thiamin, folate, and iron than their gluten-containing reference products [39, 40]. Afterwards, other studies followed the research line, and they have confirmed or suggested lower amounts of zinc, iron, calcium, phosphorus, B vitamins and folate in GF foodstuffs [29–31]. This deficiency could be partly due to the fact that in some countries, such as the U.S. and U.K., fortification of wheat flour with some minerals and B vitamins is mandatory, whereas the GF analogue products are not enriched or fortified [39]. In fact, when, for example, fortified GF cereals were analyzed and compared to those containing gluten, a similar nutrition profile was observed [31].

In the particular case of folate, its reduced presence in GF products can be due to their using the elaboration of starches or corn and rice flours, which usually had low folate content. Besides, folate is a vitamin that binds to cereal proteins and thus, when protein fractions are removed from the cereal matrix, it may cause the depletion of folates [41]. However, folate content can vary – depending on the kind of GF product. Yazynina et al. (2008) showed – after the direct analysis of eight GF foodstuffs – that breads had higher amounts of folate than the others. In the case of breads, the author attributed the higher folate value of bread to yeast, which is rich in this vitamin [41].

It must be pointed out that data concerning vitamin and mineral content of GF products were just estimations; indeed, authors from the studies mentioned used data for nutrient comparison based on the ingredient list reported on the label or the nutrition information of each product. However, Sulibursk a et al. (2013) directly analyzed the content and release of calcium, magnesium, iron, zinc, and copper from five GF products [42]. According to these authors, their results indicated that the content of the analyzed mineral was lower in GF products than that in gluten-containing oat and barley products. Additionally, they reported low bioavailability for minerals in GF foodstuffs (in pasta, from 7% for calcium to 27% for iron).

All the differences between GF and gluten-containing products would suggest that a GFD rich in these kinds of GF products could also be very different from diets containing gluten. In fact, when studies focused on analyzing the nutritional composition and eating patterns of celiac patients were carried out, differences in macronutrient and micronutrient intake between celiac and control patients were observed [43–45]. Concretely, higher fat intake and lower protein, carbohydrates and fiber intake, have been observed among celiac patients, as well as some potential deficiencies in vitamins and minerals. An unbalanced GF diet could lead to a harmful nutritional status and could determine a celiac patient's quality of life. The latest outcomes related to the evaluation and analysis of a GF diet are described in Chapter 6.

5.4.2 *Differences in Other Characteristics*

Other differences among GF and gluten-containing foods – not related to nutritional value – have been observed. Apart from the price of the products, their physicochemical properties, sensory properties, starch digestibility or glycemic index have been the focus of the attention of researchers working in this field.

Several studies have analyzed the differences between the prices of GF foods and those of their gluten-containing counterparts; all the studies conclude that GF products are between two and three times more expensive than their conventional analogues. In two of the studies mentioned above, the prices of GF products were analyzed apart from their nutritional composition [30, 31]. Missbach et al. (2015) observed that prices were higher in all GF product groups, varying from +267% in breads and bakery products to +205% in cereals. Results of a study conducted in the U.K. in 2014 are significant [46]; it not only confirms the elevated price of GF foodstuffs but also shows that budget supermarkets that tend to cater to patients from lower socioeconomic classes stocked no GF foods. According to the researcher, this could have a negative impact on GF diet adherence.

Carini et al. (2015) analyzed the physicochemical (volume, crumb grain, and color), sensory, and starch *in vitro* digestion properties in four GF breads produced using GF commercial mixes [47]. Mixes from various formulations and elaboration parameters led to the production of breads with different crumb grains, specific volume, and crust and crumb color that affected the product's sensory acceptability. In general terms, the judges preferred those with a heterogeneous and coarser crumb grain and darker color. Moreover, in that study, the authors saw that the starch's availability to hydrolytic enzymes in the GF breads was not influenced by a variation in ingredients and nutritional composition, but was related to the physical structure in terms of specific volume. When the specific volume was higher, the starch-digested fraction was also larger.

Flores-Silva et al. (2015) tried to improve starch digestibility in GF snacks by using a blend of unripe plantains, chickpeas, and maize. The authors saw that the snack with the highest amount of unripe plantain flour showed higher amounts of slowly digestible starch [48].

Only a few studies have analyzed the glycemic index of GF products, and controversial results have been obtained. It has been seen that the glycemic index changes not only according to the food formant carbohydrate content, but also when using the same GF product in healthy people as opposed to celiac sufferers [49]. In theory, GF products might cause a higher increase in glycemia due to the fact that, as stated above, starches are commonly added while GF products elaboration. Studies in the literature have shown [49, 50] that there is a higher glycemic index in GF breads or pastas than in their traditional counterparts. Nevertheless, other studies

have observed the opposite results in GF biscuits, pastas, and breads [51]. Thus new strategies in the GF food industries (especially breads and pastas) are being carried out in order to reduce the glycemic indexes in GF products – for example, increasing fiber content or using sourdough fermentation. Strategies such as substituting carbohydrates by lipids or proteins should be avoided since these foods are the main carbohydrates/contributors in the diets of celiac patients.

5.5 New Approaches to Improving the Characteristics of GF Foods

As noted in the previous section, one of the biggest differences between GF foods and gluten equivalents is the nutritional composition. In this scenario, trials with new formulations for GF foods have cropped up. Although most of the formulations were for the development of new baked products, tests on pasta, biscuits, and cakes have also been carried out.

In the first section of this chapter, it was reported that there are raw materials whose nutrient density is higher than that of the traditionally used GF cereals (rice or corn). Most research aimed at increasing nutrient density, combining in their formulations flour or traditional starches (flour and starch, rice flour and cornstarch, or starches of cassava, potato and wheat) with alternative flours such as amaranth, teff, or brown rice. The justification for this is that these kinds of formulations not only have greater nutritional density, they also have an organoleptic acceptance by the consumer [3].

A clear example can be the studies carried out by the research group of Alvarez-Jubete et al. [52, 53] replacing potato starch by flour from buckwheat, amaranth, and quinoa flour. These pseudo-cereals increased their content of vitamins, protein, fiber, and iron, showing no differences in acceptability compared to the control. Capriles et al. (2016) focus their work on the variation in the proportion of the main ingredients in their formulations (rice flour, potato starch, and flours with high nutritional value), to obtain products with good physical properties, acceptable sensory qualities, and high nutritional value [3]. Thus, the Alvarez-Jubete research team has managed to make breads with an acceptance that is equivalent to those made by wheat breads.

Apart from breads, other foods have also been tested with pseudo-cereal flours, and better nutritional properties have been observed [54]. For example, when breakfast cereals and snacks, among others, have been formulated with amaranth, teff, or quinoa, the levels of selenium increased to 10.8 μg/100 g as opposed to the 2.8 μg/100 g in traditional GF cereal formulations [55].

A study reflecting partial substitution of cornflour by amaranth and buckwheat flour to obtain cakes (sponge, coconut, and carrot cake) and biscuits showed that alternative products had better quality and nutritional value than controls made only with cornflour. In general terms, cakes and biscuits from pseudo-cereals had a higher amino acid content (in the case of biscuits, this increase was due to an elevation of

essential amino acids), and fiber and lipids content (especially polyunsaturated fatty acids), as well as increased magnesium, zinc, manganese and copper level, than corn formulations. Additionally, all products had a high degree of acceptance by consumers [54]. Other research on the elaboration of GF cookies conducted with rice, buckwheat, sorghum, and pearl millet, indicated that the resulting products increased the amount of ash, lipids, fiber, and protein content [56, 57].

It is also possible to combine the use of alternative cereals to corn and rice with another way of improving the nutrient density: the addition of dairy products. Following this line, in 2012 Lemos et al., found that cheese bread with 10% amaranth flour increased fiber and iron levels, thereby maintaining consumers' acceptance [58].

The partial substitution of cereal by legume flours has also been used to improve GF breads, especially in the amount of fiber and protein. Along these lines, Tsatsaragkou et al. (2012) conducted a study in which 15% of rice flour was replaced by carob germ [59]; the strategy was also used for pasta. Several approaches were tried in the pasta group, partially replacing cornflour with chickpea or broad bean flour [60, 61]. Both studies resulted in pasta with high fiber, lipid, and protein levels. It is important to note that the increase of dietary fiber has a direct impact on reducing the glycemic index of foods. Therefore, the formulated pasta could have great implications for diabetics. In the case of corn pasta enriched with broad bean flour, this product had also added quinoa, leading to iron and zinc content increase. Both minerals are very relevant among celiac patients because deficiencies in zinc and iron serum levels have been observed [60].

Similar research was carried out with nut flour. For instance, Brazilian almonds were used to enhance gluten-free cakes [62].The final product not only had good general acceptability (aroma, flavor, and texture similar to those of gluten), it also contained low carbohydrate values and high protein and lipid levels. Considering the deficiencies reported in the previous section of this chapter where the GF foodstuffs and their counterparts with gluten were compared, one of the most relevant findings of this study was the high content of fiber, zinc, copper, and iron that Brazilian almond cakes showed [62].

A new and hopeful horizon has opened up in the world of GF formulations, including fruit- and vegetable-based ingredients. Studies such as Korus and O'Shea's, formulating GF breads with de-fatted strawberry and black currant seeds, and orange pomace, showed an improvement in the nutritional composition of the final products [63, 64].

Apart from the addition of raw materials, there are other methods for improving the nutritional value of GF foodstuffs. One of the most promising, in the development of GF bread, seems to be the use of sourdough. This has been used since ancient times for the elaboration of wheat and rye bread, but nowadays is adjusted for GF raw materials. The technique is based on the fermentation of bread through a mixture of flour and water that is fermented by lactic bacteria and yeast that can improve the quality and nutritional value of food. In fact, in a study comparing breads made with sourdough and semolina durum wheat (the gluten was removed), gluten-containing wheat breads, and GF commercial breads, the bread with sourdough had a better availability of free fatty acids and higher protein digestibility. Additionally, breads with sourdough showed similar values of minerals, vitamins,

and fiber than gluten-containing breads (even in the case of thiamine and niacin the values were higher) [65]. Meanwhile, Wolter et al. (2014) investigated the potential uses of sourdough for reducing the predicted glycemic index [66]. According to their results, this technique can be effective for reducing the above-mentioned index for teff and sorghum breads, but not for those of quinoa or buckwheat (the use of buckwheat elevates the index).

Another potentially effective method for improving the nutritional value of GF foods is germination. Cornejo et al. (2015), saw how brown rice germination at different times varies the amount of macronutrients, as well as that of lipids or proteins [67]. According to their results, 48 h of germination provided a high content of lipids and proteins for brown rice bread. Furthermore, 2 days' germination resulted in higher antioxidant power (high levels of polyphenols and gamma-aminobutiric acid), a decrease in the phytic acid level (this anti-nutrient has a chelating effect on some minerals, and decreases the absorption of protein and starch), and a reduction of the glycemic index [67].

Gluten-containing foodstuffs' fortification is an open possibility in order to raise the nutritional value of GF foods. Taking into account the deficiencies shown by celiac patients in iron and calcium (among others) [68], studies on fortified GF bread [69, 70] began to be carried out. .

Except for the pasta category, any other food group among GF foodstuffs showed fiber deficiencies. However, several investigations have focused on making suitable bread for celiac patients as well as for people suffering from type 2 diabetes. In this kind of formulation, inulin, oligofructose, and *Psyllium* and beta-glucans have been used to improve the technological or sensory properties and nutritional qualities of GF foodstuffs. As a result, breads fortified in fiber (0.75 g beta-glucans and 4 g of inulin type fructansin 50 g of bread) were obtained [3]. It is important to point out that to achieve functional effects, such as reduced postprandial blood glucose and cholesterol levels or prebiotic effects, the recommended daily intake of these compounds is between 3 and 5 g.

5.6 Conclusions

There are marked nutritional differences between GF foodstuffs and gluten-containing counterparts. These differences are mainly due to higher lipid and lower protein content, but are also due to reduced amounts of micronutrients or fiber found in GF foods. Other characteristics, such as physicochemical properties, digestibility, or glycemic index, also diverge between GF and gluten-containing products. In view of this scenario, several strategies are being carried out to improve the nutritional value and other characteristics of GF foodstuffs. The use of alternative cereals instead of traditional ones is an interesting proposal that many publications postulate. However, there are also other options, such as the introduction of other ingredients in the formulation (hydrocolloids and other sources of protein, among others), or the use of sourdough, which can balance the reduced amounts in nutrient content.

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Chapter 6
Gluten-Free Diet: Nutritional Status and Dietary Habits of Celiac Patients

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Abbreviations

BMI	Body mass index
CD	Celiac disease
CH	Carbohydrate
FFM	Fat-free mass
FM	Fat mass
GF	Gluten-free
GFD	Gluten-free diet
GFP	Gluten-free product
ppm	Parts per million

6.1 Introduction

A strict, lifelong, gluten-free diet (GFD) is currently the only effective treatment for celiac disease (CD); it consists of the total elimination of all products containing gluten from the diet. As explained in previous chapters, gluten protein is present in some of the cereals from the *Triticeae* family such as wheat, barley, rye, triticale, kamut, spelt, and probably oats. Actually, the consumption of oats by celiac patients is controversial, and there is no unanimity to consider it as a safe protein. Even though the prolamin content in this cereal is much lower than in the other cereals recognized as toxic, many products containing oats are contaminated by traces of flours in cereals containing gluten, which represents a limitation for its use. Because of this, the most widespread recommendation is to discourage oat consumption.

Following a GFD means avoiding the toxic prolamin in order to guarantee celiac patients' health and well-being. For that purpose, it is essential to know which foods

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contain or may contain gluten, and which do not. According to its gluten content, a food is classified into one of three categories: foods that contain gluten, foods that do not contain gluten, and foods that may contain gluten.

Foods that contain gluten are considered harmful for celiac patients' health. This group includes cereals containing toxic prolamins and all their derivatives. Cereal-based products for specific use in celiac patients (gluten-free products (GFPs)) are excluded from this list as long as it is specified on the label.

Foods that do not contain gluten are adequate and secure foods in a GFD because they do not contain any toxic prolamin. This group includes cereals that do not contain gluten per se (rice, maize, quinoa, buckwheat, millet or sorghum), and foods that are not cereal derivatives (fruits, vegetables, fish, meat, etc.). There are also some cereal-based products which, even though they originally contained the toxic prolamin, have had it almost totally removed during the elaboration process and the final product does not exceed the content authorized by legislation. Thus they are suitable for celiac patients.

Foods that may contain gluten belong to a group that includes certain products that in their original composition did not have gluten, but which during the elaboration process, wheat or other harmful cereal derivatives could have been added (either deliberately or accidentally).

Table 6.1 shows a classification of the different food groups according to their potential presence of gluten [1]

It is quite remarkable that almost all fresh foods, except for certain cereals, are gluten-free. On the contrary, many processed products include or may include gluten in their composition. Actually, flours and wheat starches are widely used by food industries and certain products, such as cold meats or other manufactured products, which originally did not contain gluten, but can result in final products containing it. Therefore, labels need to be carefully read, and special attention paid to the ingredient list and to certification marks that ensure the absence of gluten. Moreover, any cross-contamination of gluten should be avoided during storage, preparation, and cooking. These rules are essential for celiac patients because even the tiniest quantities of gluten can cause damage to their intestinal villi.

It must be pointed out that the correct GFD has to be not only safe for celiac patients, but also nutritionally balanced. A balanced diet involves fulfilling all energy and nutrient requirements, avoiding nutrient deficiencies, and ensuring a healthful status. In general terms, balanced energy distribution in a diet is obtained when 55% of the energy comes from carbohydrates, 30% from fats, and 15% from protein, and when all vitamin and mineral recommendations, as well as those of water, fiber, or other compounds, are fulfilled.

When the nutritional composition of GFD has been assessed, unbalanced proportions of macronutrients and several deficiencies in vitamin and mineral content have been observed. This could be for several reasons: on the one hand, celiac patients who strictly adhere to a GFD have to exclude gluten-containing grains from their diets; this means that foods such as bread, flour, pasta, breakfast cereals, or biscuits are eliminated. These products are the major source of energy: protein, carbohydrates and micronutrients, such as iron, zinc, calcium, magnesium, and the

Table 6.1 Classification of foods from different food groups according to their gluten content [1]

	Foods containing gluten	Foods that do not contain gluten	Foods that may contain gluten
Cereals and tubers	Wheat, oats, barley, rye, spelt, triticale, wild wheat, kamut, green spelt grain, bulgur, semolina and all their flours. Other foods made with those flours (porridges, battered foods, biscuits...)	Corn, rice, millet grain, wheat, buckwheat, quinoa, amaranth beans, flour carob, tapioca, manioc and potato, and all their derivatives	Processed products (chips, mashed potatoes...), cornmeal, rice flour (pollution crusade) and their derivatives
Vegetables	Breaded or floured vegetables	All natural vegetables	Pre-cooked dishes
Fruit and nuts	Floured dry fruits (figs)	All raw fruits and nuts	Toasted nuts with salt,
Dairies	Yogurt with cereals or biscuits	Milk and dairy products: cured cheeses, cottage cheese, cream, natural yogurt and junket	creams, puddings and custards, milkshakes, prepared dairy desserts, flavored yogurt and with pieces of fruit, powered junket, processed cheese
Protein foods	Breaded or floured meat and fish and with gluten-containing sauces	All kinds of meats and fresh viscera, cured meat, Serrano ham. Fresh/frozen fish without breading, fresh seafood and oil/natural tinned fish and shellfish. Eggs Natural legumes	Cold meat, canned meat and fish with sauces, various patés and cooked ham
Miscellaneous	Béchamel sauce	Vegetable oils, butter, margarine, lard, vinegar, pure spices	Prepared sauces, soy sauces, spices preparations, bouillon cubes, dried powder or granulated barm and yeast extracts, peppers
Foods that have to be consumed sparingly	Chocolate cereals containing gluten, bakery products, beer, coffee substitutes with barley or malt, oats beverages	Honey and sugar soft drinks, coffee, tea, natural juices, fruit nectars, cava, wines, grape juice	Chocolate, candy and gumdrops, cocoa cream. Chocolate and coffee substitutes, combined drinks, liqueurs

B vitamins [2, 3]. Moreover, it has been reported that wheat, rye, and barley supply approximately 35% of the dietary fiber intake in healthy children [4]. The restrictions on the cereals mentioned can compromise the nutritional status of celiac children and make the implementation of the recommendations for a varied and balanced diet difficult sometimes.

On the other hand, celiac patients tend to consume refined gluten-free cereal products, which do not have the same nutritional composition as their unrefined analogues.

Studies aimed at analyzing the nutritional composition of GFP have reported differences in calories, macronutrients, fiber, sodium, salt and cholesterol content between some gluten-free rendered and gluten-containing foodstuffs [5]. Moreover, whereas wheat flour products are usually enriched, gluten-free cereal products are not, and therefore they often do not contain the same levels of micronutrients, such as thiamine, riboflavin, niacin, folate, vitamin D, calcium and iron [6, 7].

In this sense, people with celiac disease following GFDs develop complications such as anemia, which is related to a lack of iron and folic acid, or osteoporosis, associated with a lack of calcium and vitamin D, among others [8, 9]. In the following sections we explain the most important outcomes from studies on celiac adults and children, especially results obtained when their nutritional status and dietary intakes have been assessed.

6.2 GFD in Adult Celiac Patients: Nutritional Status and Dietary Habits

Studies of adult celiac patients show that nutritional deficiencies (low vitamin and mineral plasma levels) due to the malabsorption caused by intestinal atrophy are common. It has been known for a long time that newly diagnosed celiac patients suffer from malnutrition, but what is not so widely analyzed is whether this situation persists when these patients start following a GFD, which is, as mentioned above, the unique treatment for celiac disease.

Non-treated celiac patients regularly develop diseases such as anemia (caused by iron deficiency), retinopathy (vitamin A deficiency), systemic and peripheral neuropathy (vitamins B12 and E), complications of pregnancy (iron or folic acid), dental disease, limited joint motility, osteopenia, and osteoporosis [8]. In fact, there have been several studies of newly diagnosed celiac adults that point out iron and copper deficiencies, as well as vitamins A and D, with the E and B groups (folate, B12 and B6) as the most common ones [10, 11]. Moreover, when the nutritional status of these patients was analyzed with anthropometric data, adult celiac patients generally showed lower body weight, BMI, fat mass (FM) and fat-free mass (FFM) than control subjects [12–16]. This makes an evaluation of the nutritional status and metabolic aspects of celiac patients essential at the time of diagnosis in order to detect possible malnutrition.

It could be thought that the situation at diagnosis would be recovered when patients begin a GFD; indeed, it has been shown that when celiac patients begin one, intestinal atrophy improves and there is a remission of symptoms. Thus, vitamin and mineral absorption should be improved in the intestinal villi. Nevertheless, there has been recent evidence that nutritional deficiencies are not completely normalized after GFD follow-up [12, 13, 17] and Capristo et al. [18]. For instance, the metabolic variables of celiac patients who had a marked improvement in the duodenal mucosa after following a GFD were compared with those of recently diagnosed patients and the control population. As a result, they found that hemoglobin,

hematocrit, iron, and transferrin serum concentrations were lower in all celiac patients (treated and newly diagnosed). Hallert et al. [19] measured total plasma homocysteine levels (as the metabolic marker of folate), and vitamins B6 and B12 in 30 adults with celiac disease who had followed a GFD for 8–12 years. At this period, a third of the subjects showed higher plasma homocysteine levels and lower B6 and folate content; thus, authors concluded that vitamin status should be regularly reviewed in the follow-up of adult celiac patients.

Even though anthropometric data in patients going on a GFD improve when compared to those from a baseline, they are no better than those of the control population. In a study by Smecuol et al. [20], 25 newly diagnosed patients were recruited, presenting low percentages of fat, lean tissue, and bone compartments. However, after 37 months of GFD, body weight, BMI, fat mass and bone mass had significantly increased. Along these lines, Ukkola et al. [21] observed that patients who were underweight at diagnosis increased their body weight after 1 year of GFD, and that those who were overweight or obese lost weight. By contrast, when Churruca et al. [22] analyzed anthropometric measurements of a cohort of adult celiac women and compared them to those of Spanish control women, most of them showed lower body weight and fat mass than the women in the control group.

In another study carried out by Bardella et al. [23], similar results were observed: lower values of body weight, FM, lean mass and bone mass of celiac adult patients compared to those of the control subjects. These results as a whole suggest that even if an improvement of the nutritional status is achieved with GFD, it still does not reach the situation found in the control population. There can be two explanations for this: on the one hand, the pathological situation that going on a GFD still provokes a low bioavailability of nutrients, and on the other, these patients are more concerned about their dietary habits and hence lower weight, BMI, and fat mass values. In fact, it is remarkable that not all authors comparing data from baseline with those after following GFD show an amelioration of the nutritional status. For instance, Capristo et al. [17], observed that anthropometric data on patients going on a GFD were as bad as those of the newly diagnosed, which were, altogether, worse than those observed in the control population. They therefore concluded that GFD does not improve the nutritional status of adult celiac patients. A summary of the studies demonstrating biochemical and anthropometric characteristics of newly diagnosed celiac patients and those starting a GFD are shown in Table 6.2.

In view of all this, following a GFD does not seem to fulfill all the nutritional requirements of adult celiac patients. In fact, it has been shown that the nutritional composition of GFD is unbalanced in terms of energy requirements, macronutrient distribution, and micronutrient intake. For instance, when Churruca et al. [22] analyzed the eating patterns and diet composition of 54 adult celiac women, they found that their energy intake was slightly lower than the dietary reference intakes. Moreover, excessive protein intake and over-consumption of fat was observed. Carbohydrate and fiber consumption was below the recommended levels, and vitamin D, iron, and iodine had a low percentage of compliance to the recommendations.

Other researchers have also assessed the nutritional composition of GFD followed by adult celiac patients, and similar results have been obtained: a lower

Table 6.2 Biochemical and anthropometric data of celiac adult subjects at diagnosis and after following a GFD

Untreated celiac disease patients				Celiac patients following a GFD				
Author	*n*	Anthrophometric measurements	Biochemical data	Author	*n*	Anthrophometric measurements	Biochemical data	Dietary intakes
Hallert et al. (1981) [52]	48	NA	Low folate serum concentration	Smecuol et al. (1997) [20]	25	BW increase FM increase Lean mass unchanged *(No differences between patients with strict and partial adherence to GFD)*	No differences in serum albumin or hemoglobin between patients with strict and partial adherence to GFD	Lower energy intake of patients with strict adherence than patients with partial adherence
Stene-Larsen et al. (1988) [53]	3	NA	B12 malabsorption	Kaemppanien et al. (1998)	40	BMI increase	Normal folate, vitamin B-12, vitamin A, hemoglobin, ferritin, iron, and zinc	No changes in intakes of energy, carbohydrate, protein, or fat. Decreased intakes of fiber and thiamine
Kemppainen et al. (1995) [54]	40	No differences between 2 celiac groups	Low Hb, ferritin, iron, B12	Ukkola et al. (2012) [21]	698	Underweight increased weight; obese lost weight	NA	NA
Kemppainen et al. (1998) [55]	40	Acceptable: normal BW, normal FM	Low ferritin and folate	Capristo et al. (2009) [18]	18	low BMI, WH ratio, FFM, FM, EE	Low Hb, hematocrit, iron, transferrin	No differences in energy intake Low protein intake High CH and fat intake

Alwitny et al. (2000) [56]	1	NA	Low A vitamin	Churruca et al. (2015) [22]	54	Normal BW, BMI, FM	NA	Less energy intake tan DRI High protein and fat intake, low CH Low fiber Low D, E, folate, Ca, Fe, I, Se B12 ok Low cereal and vegetable intake, High meat intake
Dahele et al. (2001) [57]	39	NA	Low B12, folate vitamins	Bardella et al. (2000) [23]	71	Low weight, BMI, FM, LM NS in bone mass	NA	Low energy consumption High fat intake, low CH
Dickey et al. (2002) [58]	159	NA	Low B12 vitamin	Martin et al. (2013) [24]	88	NA	NA	High fat intake Low CH and fiber intake Low B1, B2, B6, AF, Mg, Fe intake
Hozyasz et al. (2003) [59]	18	NA	Low E vitamin	Hallert et al. (2002) [19]	30 (8–12 years on GFD)	BW increase	Low piridoxal, low folate B12 ok	Low folate intake Low B12 intake Underestimation of energy intake
Harper et al. (2007) [60]	405	NA	Low Fe, B12 vitamins					
Dickey et al. (2008) [61]	100	NA	Low folate levels Tendency to lower B12 levels					

(continued)

Table 6.2 (continued)

Untreated celiac disease patients				Celiac patients following a GFD				
Author	*n*	Anthrophometric measurements	Biochemical data	Author	*n*	Anthrophometric measurements	Biochemical data	Dietary intakes
Henri-Bhargava et al. (2008) [62]	1	NA	E vitamin, copper					
Bergamaschi et al. (2008) [63]	150	NA	Iron, vitamins					
Lerner et al. (2013) [64]	22	NA	D vitamin					
Wierdsma et al. (2013) [10]	80	Low BMI in 7.5% of participants High BMI in 29%	Low serum concentration of B6, B12, folate, D, A, Hb, ferritin					
Smecuol et al. (1997) [20]	25	Low FM, low LM, low bone mass	NA					
Capristo et al. (2009) [18]	16	Low BMI, Waist-hip ratio, FFM, FM, EE	Low Hb, albumin, total protein, hematocrit, iron, transferrin					

NA not analyzed

Table 6.3 Nutrition errors in GFD of adult people, children and adolescents [18, 22–24, 28, 29, 34, 36–41, 43, 45]

	Adults		Children and adolescents	
	Deficiencies	Excesses	Deficiencies	Excesses
Energy	Energy		Energy	
Macronutrients	Complex carbohydrates	Total protein Total fat	Complex carbohydrates Unsaturated fatty acids	Simple carbohydrates Total protein Total fat Saturated fatty acids
Fiber	Fiber		Fiber	
Micronutrients Vitamins	D, E, Folic Acid, B1, B2, B6		D vitamin C vitamin Thiamine	
Minerals	Mg, Fe, Ca, I, Se		Calcium Magnesium Selenium Iron	

consumption of energy than control subjects, a high percentage of fat, and a low percentage of CH and protein [23, 24]. Capristo et al. [18] saw that although patients had similar energy intake to that of the control patients, the macronutrient distribution was unbalanced: a high percentage of fat and CH and low consumption of protein. The enhanced dietary consumption of fats accompanied by a reduction in protein content could be related to the GFP and, concretely, to their formulations (Table 6.3).

Commonly, lipid-rich ingredients, such as animal or vegetable oils and emulsifiers (mono- and diglycerides of fatty acids) are added in order to improve the palatability and texture of GF foodstuffs [25–27]. Moreover, these products have a lower protein content due to the use of starches, flours from low-protein cereals such as rice or corn, or the use of gums or enzymes in their elaboration [25]. The discrepancies observed in CH intake could be due to the differences seen between the composition of GFP, which have higher amounts than their analogues [5, 25], and the reported low intake of non-starch carbohydrates among people following a GFD [25].

Data on fiber intake in people on a GFD also show that the requirements for this nutrient are not fulfilled [22, 24] (Table 6.3). In order to argue this fact, two causes have to be taken into consideration: first, the gluten-containing cereals provide nearly a third of all the recommended dietary fiber [4], and second, refined gluten-free cereal products' composition supplies less fiber than their not-refined analogues because they are not as enriched or fortified [5, 28]. Regarding the micronutrient intake in GFD, Martin et al. indicated that the vitamin B1, B2, B6, folic acid, magnesium and iron intake were lower among adult celiac patients than those observed among the general population [22, 24]. These nutritional deficiencies can be due to the exclusion of gluten-containing grains and foods from the GFD, which are a major source of micronutrients [2, 3], and to the consumption in a GFP, which often does not contain the same levels of micronutrients as their counterparts – e.g., thiamine, riboflavin, niacin, folate, vitamin D, calcium, or iron [6, 28, 29].

Data on the food consumption indicate as well that people following GFDs do not adhere to adequate eating plans. In the study carried out by Churruca et al. [22], the group observed that whereas recommendations for dairy products and fruit intake were followed, vegetable consumption was not enough for the vast majority of adult celiac women. Moreover, more than three-quarters of the participants consumed excessive amounts of meat. It must be kept in mind that the observed eating patterns and energy and nutrient intakes of celiac women from that study did not differ very much from those of the women in the control group; despite this, the authors concluded that some considerations, such as reducing fat and protein consumption and increasing fiber intake, should be taken into account.

6.3 GFDs in Celiac Children and Adolescents: Nutritional Status and Dietary Habits

Among children, CD is one of the most common food-related chronic diseases. The incidence of CD in European children is about 1%. However, it is increasing continuously [30, 31] and there is a significant percentage of patients still undiagnosed [32]; indeed, a Swedish screening study put the figure at 3% in children aged 12–14 [33].

As mentioned, a GFD must ensure the absence of gluten in the diet, but it must also be nutritionally balanced, covering all energy and nutrient requirements. This is even more important in the case of children and adolescents, whose diets must also ensure adequate growth and development to prevent deficiencies and promote healthy lives. Celiac children usually have lower weight, height, and BMIs than age- and sex-matched controls, leading to less overweight and obesity and more underweight children. Nevertheless, some authors have reported that following a GFD may contribute to undesirable weight gain due to the imbalance in its composition [28, 34, 35].

Several studies have found unbalanced dietary intakes [28, 29] due to a reduced energy intake [36], low intakes of complex carbohydrates and fibers [34, 37, 38], and subsequent higher protein [36] and fat intakes [34, 36, 38, 39]. With regard to lipids, the intake of fatty acids is also unbalanced, and celiac children and adolescents show a high consumption of saturated fats and low consumption of unsaturated fats [38, 40] (Table 6.3).

These dietary habits are similar to those observed in non-celiac children and adolescents [38, 40]. Nevertheless, different results have been reported when comparing the GFDs of celiac children and adolescents to that of the general population. Zuccoti et al. [41] reported that although both celiac and non-celiac children showed higher protein, fat, and sugar intake than was recommended in Italy, celiac children presented higher energy and carbohydrate intakes and lower fat intakes, without differences in protein-derived energy. In a study conducted on celiac children in Spain to assess whether there was any change in the diet with gluten prior to diagnosis and the gluten-free diet one year after diagnosis, an increase in the ingestion of monounsaturated

fatty acid and a decrease of saturated ones in GFDs was observed [42]. In contrast, Babio et al. [43] observed higher consumption of added sugar, total fat, and foods rich in protein, such as meat, fish, and eggs, and lower amounts of foods rich in starch in celiacs than in the control group in people aged 10–23.

With regard to fiber, its intake in celiac children is below the recommendation, as occurs in the general population [34, 38, 43]. Some researchers have not observed differences in fiber content in celiac and non-celiac children's diets [38, 43], but Mariani and colleagues showed a lower consumption of carbohydrates, and in particular a low fiber content, in the diets of celiac adolescents as compared to healthy adolescents [34]; this could be explained, as mentioned above, by the lack of cereals rich in fiber and the presence of refined cereals in the GFD [4, 28].

GFDs also lead to micronutrient deficiencies in celiac children; these deficiencies include vitamins D, A, K, and E, as well as folic acid, vitamin B12, zinc, iron, and calcium- related ones [38, 41, 43–45] (Table 6.3). Most of them are first associated with untreated celiac disease due to malabsorption caused by the atrophy of intestinal villi. However, it has been reported that among celiac adults and children on GFDs, some micronutrient deficiencies may persist as well [7, 28, 29]. Concerning vitamins, several researchers have observed insufficient intakes of vitamins D [38] and C [40] as well as thiamine [40] in celiac children. Similar results have been observed regarding minerals, such as calcium [41], magnesium [38, 41], selenium [38] and iron [41]. Therefore, as proposed for adults, the serum levels of some of these micronutrients should be checked in all children with celiac disease who follow a GFD [44]. Although many researchers have found diminished micronutrient intake among celiac children, it must be pointed out that Ohlund et al. [38] observed an increase in the intake of iron and calcium in celiac with respect to participants in the control group.

As to the dietary habits of celiac children and adolescents, the consumption of food groups shows a low adherence to pyramid guidelines. Celiac children consume a very low amount of cereals as a way to avoid gluten. Babio et al. [43] found that celiac patients consume less starch (pasta, bread, and pastries) and more protein-rich foods (meat, fish, eggs) than the non-celiac population. Moreover, celiac children do not fulfill the recommendations for foods rich in fiber, such as legumes, vegetables, and fruits. Thus, as proposed for adults, reducing protein consumption and increasing fiber intake should be also considered [22].

6.4 Strategies and New Proposals for Improving the Nutritional Status of Celiac Patients and Making GFDs More Balanced

Generally speaking, all the studies previously mentioned show that celiac patients on GFDs do not fulfill energy recommendations, that macronutrient distribution is unbalanced, and that the consumption of certain micronutrients and fibers is inadequate. Considering that the GFD has to be maintained for a lifetime, it will be

Table 6.4 Natural dietary sources that may help fulfill the requirements of vitamins and minerals that are commonly lacking in celiac patients

Nutrient	Sources
Iron	Meats (beef, liver), fish (sardines, clams, oysters), beans (white, lentil, chickpea), grains (quinoa, buckwheat, teff), dark green leafy vegetables, seeds and nuts
Calcium	Dairy products (milk, yogurt, cheese), fortified milk substitutes and juices Fish (sardines, salmon with bones, trout, perch), dark green leafy vegetables (spinach, dandelion and turnip greens, kale), beans, grains (quinoa, buckwheat, millet)
B Complex vitamins	Grains (quinoa, buckwheat, millet, brown rice, amaranth) dark green leafy vegetables, legumes, meats and dairy products
Fiber	Beans (navy, kidney, split pea, lentils, black beans, pinto beans, chickpeas) sweet potato with skin, grains (quinoa, buckwheat, millet, brown rice, amaranth, flax seed meal, rice bran) raw fruits and vegetables; stewed prunes, dried figs, nuts

necessary to make efforts and design new strategies to be followed by celiac patients in order to improve diet quality and their nutritional status. Some of these strategies could include the following features:

(a) The consumption of cereals other than rice and corn that are free of gluten per se should be encouraged [28]. This is the situation with quinoa and amaranth, which are good sources of vitamins (riboflavin, folic acid, vitamin C and vitamin E). Moreover, the appropriate consumption of other foods (apart from cereals), such as, fish, meat, and mainly fruits and vegetables, should be potentiated, due to their containing the vitamins and minerals that celiac patients lack [29]. Table 6.4 shows a few strategies that can be followed with natural products in order to fulfill micronutrient recommendations.

(b) GF products' fortification: Despite the traditional, yet unusual fortification in order to avoid potential contamination, it seems that gluten-free products are now being fortified [7]. This fortification should be one of the main objectives of the food industry's GFPs' specialized manufacturing.

(c) The use of supplements in a GFD: It has been shown that the regular use of supplements can improve the nutritional deficiencies that were observed. For instance, B-vitamin supplements have been shown to be effective in reducing homocysteine levels in patients with celiac disease, and they have been proposed as a possible tool to be considered for disease management [46].

(d) Nutrition education for celiac patients: Education on nutrition should become part of the therapeutic pathway, above all in childhood [28], and specific dietetic recommendations have to be developed for people on GFDs. In fact, dietetic and celiac associations have published nutritional guides that alert patients to the potential nutritional deficiencies resulting from a nutritionally unbalanced GFD [1]. Moreover, specific tools to evaluate and design gluten-free diets are needed because those available on the market do not contain the nutritional composition of gluten-free products.

(e) Increasing the adherence to GFDs among celiac patients: Adherence to the diet has been described as a crucial factor for the achievement of total correction of body composition, the minimization of symptoms, and the healing of intestinal mucosa. Prospective studies of newly diagnosed celiac adults with dietary assessments after 1 and 5 years, have shown that those patients who strictly complied with GFDs were the ones whose intestinal lesions and body composition became normal more quickly [20, 47]. However, patients with no adherence to the diet or, as the authors classified, those with regular gluten intake episodes, did not improve.

(f) Reducing the incidence of gluten transgressions: Some patients who strictly adhere to GFDs still suffer from digestive pain and discomfort; this can be due to the ingestion of traces of gluten, which is known as gluten transgression. Consuming gluten traces even for one day leads to an accumulation of small amounts that can result in the appearance of symptoms. Along these lines, it has been found that exposure to less than 10 mg/day is unlikely to cause histological changes in the intestinal mucosa, and that exposures to 50 mg/day or more is likely to do so [48–50]. For this reason, the acceptable gluten threshold for foodstuffs has progressively declined over the last few years, to 20 mg/kg or ppm. Understanding that total gluten intake must be limited to less than 50 mg/day, it would be possible to consume a large amount of these foods on a daily basis. Higher ingestions, either by a high rate of transgression or low adherence to the GFD could explain the lack of the symptoms' disappearance or of the nutritional status improvement among celiac patients following GFD.

Unintentional transgressions are also usual in celiac patients who "eat out," due to the fact that kitchen staff could have limited knowledge of the disease and often are not qualified enough to produce GF meals, so celiac patients usually avoid eating in restaurants. Therefore, it is necessary to improve the understanding of CD in food services and to ensure safe food consumption for celiac sufferers [51].

(g) A regular follow-up of celiac patients on a GFD: These regular tests should include measuring serum antibodies, body composition, and a detailed dietary history, as well as checking for symptoms of nutritional deficiencies, the presence of malignancies, and other autoimmune diseases [8].

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