Krisztina Takács[1], Erika Koppányné Szabó[1], Anna Jánosi[1]

Received: April 2018 – Accepted: September 2018

Advances in the development of gluten detection and quantitative determination methods

Keywords: celiac disease, gluten, ELISA, PCR, ω-gliadin antibody, R5 antibody, monoclonal G12 antibody

1. Summary

The detection of gluten in foods is an essential, extremely important test for the protection of celiac patients. It is important to pay close attention to a safe, gluten-free diet and to the control of gluten-free foods, and for this, a reliable measurement method should be available.

Gluten and gluten-containing cereal flours are widely used in the food industry. They may be present in several food products in which lay consumers would not expect it. It can be a flavor enhancer or texturizer in various food products (e.g., meats or confectionery), and on the other hand, a gluten-free food may get contaminated accidentally with celiac active cereals during harvesting, transport, storage or processing.

There are several methods for the detection and analysis of gluten (including microscopy, electrophoresis, chromatography, immunology or DNA-based methods, etc.). However, the quantitative detection of gluten has to be primarily protein-based, that is, an immunological method (R5-ELISA) according to CODEX STAN 118-1979 [1]. If there is a method with the same sensitivity and specificity as the immunological method for the quantitative analysis of raw and processed, heat-treated foods, it also could be a possible way of analysis.

Experts involved in this topic continuously strive to develop detection methods that are more sensitive and more specific than current ones. Initially, they concentrated on the development of antibodies that recognize gliadin, then the focus shifted to the development of antibodies that recognize the T-cell stimulating epitopes of gliadin (that trigger celiac disease). As an alternative and supplementary method, the most accepted technique is the DNA-based PCR detection, which can predict the risk of presence of proteins that may be expressed.

This article presents the advances in gluten detection and quantitative determination methods, the difficulties of detection, and legal regulations related to these analytical tests.

2. Introduction

Cereals have played an important role in the diet of humans since ancient times. The consumption of cereals accounts for nearly one half of our total energy intake and, in addition, their nutrient content is also extremely important: they are our the most important sources of carbohydrates, vitamins, minerals, dietary fibers and proteins. Of cereals, wheat, rye, barley, oats, rice and corn are most frequently consumed.

However, gluten proteins of the cereals wheat, rye, barley and oats may trigger adverse, abnormal reactions in individuals sensitive to them, cereal allergies on the one hand, and celiac disease on the other hand. In the background of these two sensitivities lie different immunological processes. Celiac disease, also known as flour sensitivity or gluten sensitivity, is a genetics-based autoimmune disease (in terms of its mechanism, a T-cell immuno-mediated process), while cereal allergy is a process

[1] National Agricultural Research and Innovation Centre, Food Science Research Institute, Department of Biology

immune-mediated by IgE antibodies. Food allergies affect 0.2 to 0.5% of the population, while the incidence of celiac disease is 0.1 to 1.6%. However, according to some authors, the latter might have a higher incidence **[2].**

While in the case of celiac disease, only the gluten proteins (prolamins and glutelins) of wheat, rye, barley, oats and their crossbred varieties (e.g., triticale) play a role in triggering the disease, in the case of cereal allergies, in addition to gluten, mainly the proteins of the albumin and globulin fractions are responsible (α-amylase inhibitors (CM3, 0.53), non-specific lipid transfer proteins) **[3].** In most celiac patients, oats do not trigger the autoimmune reaction, and so oats that are otherwise not contaminated with gluten can be consumed by those who made sure that their body tolerates oats well **[4], [5], [6].**

In the case of celiac disease, a lack of proper treatment (that is, adhering to a gluten-free diet) results in a loss of small intestine villi and, in addition, several other symptoms and complications may appear outside the gastrointestinal tract. Flour sensitivity cannot be cured, it accompanies the entire life of the patient, however, by avoiding the factor that triggers the disease and maintaining the proper diet, it can be treated perfectly (the abnormal process stops and the intestinal tract regenerates, and the symptoms disappear).

Allergic symptoms have a wide range: Wheat Dependent, Exercise Induced Anaphylaxis (WDEIA), Oral Allergy Syndrome (OAS), redness, itching might develop. Sensitization can occur through the respiratory system („baker's asthma") or the gastrointestinal tract. The allergy may also be temporary, it might disappear over time. Cereals that trigger the symptoms should be avoided in the diet (e.g., wheat in the case of a wheat allergy).

It should be noted that there is a new gluten-dependent disorder, non-celiac gluten sensitivity (NCGS) with clinical symptoms similar to those of celiac disease when consuming gluten-containing cereals. Harmful food components that trigger the symptoms have not yet been identified, but it is assumed that the gliadin sequences involved in the development of the disease differ from those participating in celiac disease, and non-gluten proteins (e.g., α-amlyase, trypsin inhibitors, wheat germ agglutinin), as well as fermentable oligo-, di- and monosaccharides and polyols also play a role in pathological processes (e.g., bloating, diarrhea) **[7], [8], [9].**

3. Definition of gluten

Gluten is a protein fraction of wheat, rye, barley, oats, their crossbred varieties and derivatives, which is insoluble in water or in 0.5 M NaCl solution, and to which certain individuals are intolerant. Gluten alcohol is composed of soluble prolamins (monomer) and acid/alkali soluble glutelins (polymer) which are soluble in alcohols under reducing conditions as well, in a ratio of approximately 1:1. Gluten proteins make up 80% of the total protein content of the grain. The rest of the proteins are albumins (12%) and globulins (8%). Prolamin proteins are called gliadins in the case of wheat, secalins in the case of rye, hordeins in the case of barley and avenins in the case of oats **[1].** The term gluten includes several hundreds of proteins.

Gliadins are heterogeneous protein mixtures, primarily monomeric proteins containing ca. 40 components. Based on their electromobility, they can be classified at acidic pH values as α, β, γ and ω-gliadins, or based on their N-terminal amino acid sequence, type α/β (44-60%, 28-35 kDa), γ (31-46%, 31-35 kDa) and ω (10-20%, ω1,2-gliadins: 39-44 kDa, ω5-gliadins: 49-55 kDa) gliadins. There are only slight differences in the sequence of the given types. α/β- and γ-type gliadins are rich in sulfur, while ω-type gliadins are poor in sulfur and contain neither cysteine nor disulfide bonds. α-Gliadins contain 6 conservative cysteine moieties and 3 intrachain crosslinks. γ-Gliadins contain 8 conservative cysteine moieties and 4 intrachain bonds.

Glutenins are large protein aggregates consisting of 600 to 800 amino acids and containing great amounts of glycine, glutamine and proline. Their structure is stabilized via intermolecular disulfide bonds. In addition, intramolecular bonds may also form. Glutenins can be classified either as LMW-GS or HMW-GS (Low/High Molecular Weight - Glutenin Subunit). Based on their electromobility (30-70 kDa **[17]**), LMW-GS proteins can be classified as B, C (similar to a and γ-gliadins) or D (similar to ω-gliadins) types. Exactly because of their similarity to gliadins, LMW-glutenins are recognized by numerous antibodies produced against gliadin **[12].** HMW-GS proteins (67-88 kDa **[17]**, 90-120 kDa **[16]**) account for 10% of wheat reserve proteins and have two types, x and y **[13].**

4. Knowledge of celiac disease and the gluten peptides participating in it from a gluten detection point of view

There are several factors involved in the pathogenesis of celiac disease:

- Environmental effects (viruses),
- Bacteria (Proteobacteria/Firmicutes),
- Breast feeding,
- Genetic predisposition (autoimmunity genes, genes that regulate the operation of the immune system: HLA DQ2 (90% of patients) and HLA DQ8 (10% of patients) haplotype carriers), and a disorder of immunological factors (innate and adaptive immune response) **[7], [10].**

Gluten proteins are hard to digest, their degradation is incomplete: they are broken down to long gluten peptides of at least 9 amino acids by gastrointestinal enzymes (stomach endopeptidases: pepsin, trypsin, chymotrypsin, elastase, carbopeptidases, and then brush border exopeptidases).

Gluten proteins have similar amino acid sequences and they often contain recurring sections with proline (about 15%) and glutamine (about 35%) moieties. This high proline-glutamine content makes them resistant to proteolysis, and so long gluten fragments can survive in the upper section of the small intestine, and are converted into immunogenic peptides that trigger T-cell response **[11].**

Immunogenic gluten peptides are presented to T lymphocytes by antigen presenting cells possessing HLA DQ2 and HLA DQ8 molecules. This stimulates T cell response and triggers pathological immune reactions (toxic gluten peptides) that cause tissue mucosal damage in the small intestine **[10], [11], [12].**

More than 50 types of gluten peptides (immunogens that can trigger an immune response and are toxic, causing damage to the intestinal epithelium) are involved in the development of the disease. In the case of adults, α- and ω-gliadins, while in the case of children, LMW glutenins and γ-gliadins are immunodominant (the latter two are less common in adults). In their publication, Ciccocioppo et al. presented 9 peptide sections as toxic, as well as 36 immunogenic epitopes found among certain peptide sections of α- and γ-gliadin and glutenin, 10 of which were immunodominant (i.e., strongly immunogenic) **[12].** Since it is the amino acid content, the proline moiety arrangement and the specific deamidation of tTG are important in T-cell recognition, computer methods can be used for the mapping of harmful sequences **[13].**

All the celiac active gluten fragments **[15]** listed (reported) before the submission of our article are contained in the Allergen Online database **[14]**, but experts are still searching for new toxic peptides/ sequences **[16].**

5. Taxonomy – relationship between cereals – cross-reactions between grain proteins

Rye (*Secale cereale* species), barley (*Hordeum vulgare* species) and triticale are grasses similar to wheat (*Triticum aestivum* species) from a taxonomy point of view (all members of the Poaceae family/ Pooideae subfamily/Triticeae tribe), so they express peptides (gluten proteins) of similar structure that are toxic to celiac patients.

Cross-reactions are likely to occur between proteins of closely related cereal species. However, cross-reactions may also occur between the proteins of cereals that are further away from each other in terms of degree of kinship, such as oats (Poaceae family/Pooideae subfamily/Avenae tribe/Avena sativa species), maize (Poaceae family/Panicoideae subfamily/Andropogoneae tribe/Zea mays species) and rice (Poaceae family/Bambusoideae subfamily/ Oryzeae tribe/ Oryza sativa species) **[18].**

Immunological cross-reactivity may be detected between cereals causing celiac disease and the prolamin proteins of the above-mentioned cereals (e.g., oat avenin, sorghum kafirin, rice oryzenin), but their toxicity as recognized by T-cells has not been confirmed **[19]**. The safety of maize consumption is also questionable, because studies have shown that zeins (maize prolamins) may be able to trigger an inflammatory response in some celiac patients when getting into contact with the mucous membrane. There is indeed a high degree of similarity between zeins and peptides causing celiac disease, but their integrity after gastrointestinal proteolysis is unknown. Pathological response of celiac patients to maize prolamin is rare, but may occur **[18], [20], [21]**.

6. Gluten as a food ingredient with mandatory labeling

The indication and traceability of allergens on food labels is mandatory in accordance with EU regulations. The list of allergenic ingredients with a labeling requirement is contained in Directive 2003/89/EC of the European Parliament and of the Council **[23]** amending directive 2000/13/EC **[22]** and Commission Directive 2006/142/EC Annex IIIa **[24]**, including gluten-containing cereals and products made from them as well. Exceptions to the labeling requirement are:

a) wheat glucose syrup, including dextrose;

b) maltodextrin made from wheat;

c) barley glucose syrup;

d) cereals used for the production of alcoholic distillates, such as ethyl alcohol of agricultural origin.

Mandatory preventive measures are currently based on absolute and permanent avoidance of harmful foodstuffs, their labeling and information of consumers of allergenic risks are regulated by the following decrees:

- Joint FVM-EüM-GKM decree 167/2004 (XI. 29.) **[26]**, joint FVM-EüM-GKM decree 38/2005 (IV. 27.) **[27]**, joint FVM-EüM-SZMM decree 86/2007 (VIII. 17.) **[28]** amending joint FVM-ESzCsM-GKM decree 19/2004 (II. 26.) **[25]**,
- Regulation (EU) No 1169/2011 of the European Parliament and of the Council, Annex II (mandatory from December 13, 2014) **[29]**,
- VM decree 62/2011 (VI. 30.) **[30]**,
- FM decree 36/2014 (XII. 17.) (mandatory from July 1, 2015 **[31].**

In the European Union, the directions of Commission Implementing Regulation (EU) No 828/2014 **[32]** have to be followed. According to mandatory Regulation (EU) No 609/2013 on the labeling of foodstuffs intended for people with celiac disease **[33]**, as of July 20, 2016, the category of foodstuffs intended for particular nutritional uses ceased to exist and so this resulted in a change in products intended for gluten-sensitive people. Accordingly, starting from July 20, 2016, manufacturers and distributors do not have to report to the authority – in Hungary, to the National Institute of Pharmacy and Nutrition (OGYÉI) – if they intend to market foods for gluten-sensitive people („*gluten-free*" or „*very low gluten*" foods). At the same time, a new regulation also comes into force regarding statements about gluten-free or reduced gluten content foods in accordance with Regulation 828/2014/EU **[32]**. Essential elements of the previous act, Commission Regulation (EC) No 41/2009 **[34]** are maintained, while adding a few points.

1. The statement 'gluten-free' may only be made where the food as sold to the final consumer contains no more than 20 mg/kg gluten. This term can also be used in the case of products naturally free of gluten.
2. The statement 'very low gluten' may only be made where the food, consisting of or containing one or more ingredients made from wheat, rye, barley, oats or their crossbred varieties which 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.
3. The food information may be accompanied by the statements 'suitable for people intolerant to gluten' or 'suitable for coeliacs'.
4. If the food is specially produced to reduce the gluten content of one or more gluten-containing ingredients or to substitute the gluten-containing ingredients with other ingredients naturally free of gluten, then the label may contain the statements 'specifically formulated for people intolerant to gluten' or 'specifically formulated for coeliacs'.
5. The diet of most people intolerant to gluten oats without their health being adversely affected by oat proteins (it should be noted that a smaller proportion of the gluten-sensitive population cannot consume oats either). The contamination of oats with gluten is a serious risk, therefore, the gluten content of oats in 'gluten-free' or 'very low gluten' products has also been regulated by legislators. In foods labeled gluten-free or very low gluten, only oats can be used during the growing, preparation and/or processing of which contact with wheat, rye, barley or their crossbred varieties was specifically avoided, and whose gluten content is no more than 20 mg/kg.

Products labeled 'gluten-free' or 'very low gluten may be consumed by people intolerant to gluten, depending on their tolerance level, regardless of the possible additional statements 'suitable for people intolerant to gluten' or 'suitable for coeliacs'. It is important that the terms 'specifically formulated for people intolerant to gluten' or 'specifically formulated for coeliacs' may also be used on the labels of products containing no more than 20 mg/kg or 100 mg/kg gluten.

On the labels of prepackaged foods it is mandatory to highlight the names of cereals containing gluten, i.e., wheat, rye, barley, oats, spelt, kamut or their hybridized species, in the list of ingredients using a typesetting that clearly separates them from other ingredients (for example, different font, style or background color).

For non-prepackaged foods, providing some kind of information regarding gluten content to consumers is also mandatory, however, because of possible subsequent contamination, the consumption of non-prepackaged foods can only be recommended to people diagnosed with gluten sensitivity while using appropriate caution.

7. The detection of gluten

7.1. Advances in protein-based gluten detection

According to Codex Standard 118-1979 **[1]**, the quantitative determination of gluten in foods and food ingredients must be based on an immunological method (currently the most sensitive method), or another test method that is characterized by at least the same sensitivity and specificity as the method mentioned above must be used. Methods based on the immunological principle use antibodies produced against various prolamin fractions or specific sequences found in prolamin.

The advantage of immunological methods is that they are capable of the reliable, quantitative measurement of gliadin in raw (native) and processed (e.g., heat treated) foods, as opposed to other time-consuming methods requiring expensive instrumentation (microscopy, electrophoresis, immunoblot, HPLC, MS, MALDI-TOF MS, LC-MS/MS, immunosensor, quantitative real-time PCR).

According to CODEX STAN 118-1979 **[1]**, the antibodies used should detect those cereal protein fractions that are toxic to gluten-intolerant people, and may not cross-react with other cereal proteins and other food or food ingredient components. The requirements of reliable gluten detection are adequate sensitivity, specificity, reproducibility, robustness and a validated status. The test methods should be checked by involving several independent laboratories and, if possible, they should be calibrated against a standard. The detection limit should be

10 mg gluten/kg or less. For qualitative analysis, indicating the presence of gluten, the ELISA method based on monoclonal R5 antibodies has been given priority.

7.1.1. Difficulties in detecting gluten

Immunology-based detection is widely used, but there are a number of difficulties in connection with the method **[2]:**

- Legislation with respect to determination is not clear-cut: Measurement of the gluten content is usually based on gliadin determination, even though gluten determination could include toxic components coming from glutenin.
- Gluten is composed of several components and is characterized by an extraordinary complexicity (e.g., the genes of wheat varieties encode at least 50 to 150 gliadins). This variety and lack of knowledge of the amino acid sequence cause problems in the mapping of epitopes **[35]**.
- It is difficult to find/develop antibodies that have the same affinity towards the gluten proteins of wheat, rye or barley. (This is due to differences in protein sequences, and thus to different immunoreactive epitopes) **[36]**.
- There are several commercially available kits based on immunoassay methods for the detection of gliadins/secalins/hordeins, using different antibodies (they are based on monoclonal or polyclonal antibodies), and so their specificities and sensitivities are different. Thus, the above-mentioned prolamin proteins, or a given fraction of them or a gliadin/secalin/hordein subunit, or a sequence (epitope) of it could be detected **[37], [38]**.
- The dissolution of gliadins significant in celiac disease is not strictly regulated either, different extraction methods are recommended in practice, having different prolamin dissolution efficiencies. In the various recommendations, the ethanol concentration to be used is also different (40 to 60%). It is not exactly known either what the effect of alcohol concentration is on the immunochemical and enzymatic reactions that provide the analytical signal. Prolamin dissolution is made more difficult by molecular weight, heterogeneous surface properties, intrachain and interchain covalent bonds and the sensitivity to heat or chemicals.
- Reproducible recording of the calibration curve for gliadin is difficult to accomplish. There are different standard antigens available: Australian Timgalen wheat variety gliadin, RM8418 (a Canadian wheat gliadin), Sigma gliadin (the gliadin fraction form 12 different German wheat varieties), PWG gliadin (the gliadin fraction from the 28 most commonly grown European (mainly French, German and English) wheat varieties, also known as „European wheat gliadin" or „IRMM-480"). These four standards show very similar patterns in 2D electrophoresis. PWG gliadin had the highest gliadin content; RM8418 contained more glutenin, albumin and globulin, but the differences obtained during the analytical tests could not explain the behavior of the standards. What is certain is that if there is only one wheat variety included in the standard, then the differences between the varieties are not taken into account, and this may result in inaccuracies in the result. Currently, the officially recommended reference material is PWG gliadin.
    - In addition, there also exist synthetic peptide standards. In the case of the R-Biopharm competitive R5 ELISA, for example, the (QQPFP) synthetic peptide is used for calibration. However, its disadvantage is that the results obtained using peptide standards indicate peptide concentrations instead of the desired protein concentrations. Since the limit values of gluten-free products refer to the total gluten content and not to the peptide content, it is very difficult to compare peptide concentrations with the total gluten content of the sample. Gessendorfer et al. developed another peptide sequence as a standard by the hydrolysis of a mixture of wheat, barley and rye prolamins **[39]**. This provided a better approximation of total gluten content determination. However, the hydrolysis of proteins is difficult to optimize to avoid any difference between the batches **[13].** Indeed, an acceptably standardized reference material (RM) used for calibration is still lacking **[40]**.
- Modification of proteins (partial or complete hydrolysis, deamidation, transamidation (catalyzed by microbial transglutaminase), degradation, fragmentation, denaturation, aggregation (formation of an insoluble matrix)) decreases the binding affinity for the antibody, thus making gluten detection more difficult. This may happen via technological treatment (in order to improve the functionality or the applicability in various products of gluten: e.g., heat, enzymatic degradation, extrusion), or naturally through the enzymes in the cereal grains.

- The effect of the food matrix (solid, liquid) on the structural changes of gluten is not completely known either, because of an interaction with the antibody, cross-reactivity may occur **[40]**. In the test kits, no control food matrix is available.

It is therefore apparent that many difficulties indicate that the problem of detectability still needs to be addressed and gluten detection methods have to be developed.

There are several research teams in Hungary actively involved in this area:

- The Department of Biology of the Food Science Research Institute of the National Agricultural Research and Innovation Centre (formerly known as the Central Food Research Institute, Budapest) has investigated the applicability of immunoanalytical and DNA-based methods for gluten detection in a model study of raw and processed foods (under the leadership of Dr. Éva Gelencsér) **[41], [42], [43], [44], [45], [46], [47],**
- The Research Group of Cereal Science and Food Quality, Department of Applied Biotechnology and Food Science, Faculty of Chemical Technology and Biotechnology of the Budapest University of Technology and Economics (under the leadership of Dr. Sándor Tömösközi), to facilitate the applicability and validation of the ELISA methodology, has been developing reference materials (containing gluten proteins) that model actual food matrices. With the help of these, determination of the performance characteristics and the comparative analysis of commercially available ELISA methods could be accomplished, as well as the interpretation of the phenomena behind the results. In connection with the topic, the research team of the Department has participated in the work of the Food Allergen Reference Materials Working Group of the MoniQA (Monitoring and Quality Assurance in the Food Supply Chain) Network of Excellence, funded by the Sixth Framework Programme of the European Union **[48], [49], [50], [51] [52], [53].**
- The Applied Genomics Department, Agricultural Institute of the Centre for Agricultural Research, Hungarian Academy of Sciences, Martonvásár (under the leadership of Dr. Angéla Juhász) has been complementing the analyses of cereal proteins causing celiac disease with bioinformatics studies **[54], [55].**

Measurement results can be found in the literature references listed below.

7.1.2. Development of antibodies capable of recognizing gliadin

There has been great progress in the analysis of the gluten content of foods in recent years. Initially, polyclonal antibodies were used for the detection of gliadin subtypes, however, this was not sufficiently specific, e.g., providing a cross-reaction with non-toxic maize.

Later, a more sensitive method, based on monoclonal ω-gliadin antibodies generated in mice (401.21 mAb) was published (Skerrit method **[37]**), aimed specifically at the recognition of the heat stable ω-gliadin fraction, however, to a lesser extent, it also recognizes a and γ-gliadins, as well as LMW and HMW glutenins. ω-Gliadins are cysteine deficient, their lysine content is low, which makes them heat stable. The development of this antibody was a major breakthrough in gluten analysis, as in the case of processed (heat-treated) foods, where the fraction remains unchanged during food processing, it was the first time that gluten could be measured with acceptable specificity and sensitivity using such an antibody. In recommendation 991.19 of the Association of Official Analytical Chemists (AOAC), application of a sandwich-type analytical procedure using this ω-gliadin antibody (401.21 mAb) is recommended, which is suitable for the detection in raw and heat-treated foods. As a standard, gliadin from the Timgalen wheat variety (Australia) was used. Dissolution of gliadins was carried out with 40% ethanol **[13].** The amino acid sequence of the main epitope recognized by the antibody is PQPQPFPQE/ PQQPPFPEE (where: P=Proline, Q=Glutamine, F=Phenylalanine, L=Leucine, E=Glutamic acid) **[56].**

The flaw in the method was that, without reducing agents, the extraction of gliadins by 40% ethanol was only partial, because glutenins did not dissolve. This way, initially, the true amount of total gliadins in the sample was given erroneously by the analytical methodology. Another weakness of the method was that, since the ω-fraction represents a relatively small portion (5-20%) of the total prolamin content, and the amount of the ω-prolamin fraction is different in the various cereals (and it can also change during the development of the cereal), the analytical result varied depending on the relative ω-gliadin content, and was therefore not sufficiently reliable. The weakness of the method was also due to the fact that the antibody bound poorly to barley prolamin (sensitivity was inadequate), therefore, less than 10% of hordeins could be measured. Prolamin content was often underestimated or simply a false negative result was obtained. Gliadins from durum wheat were undermeasured. In triticale and rye, prolamins were overmeasured and there were numerous cross-reactions with different gluten-containing cereals. Since it does not recognize barley and rye prolamins to the same extent, the measurements were often not reproducible. The repeatability of the ω-gliadin

method was 16-22%, and its reproducibility was 24-33%. The method can be used as a semiquantitative procedure in the case of processed meat products and as quantitative procedure in the case of cereals. It is not suitable for the measurement of hydrolyzed gluten **[57].** The method based on the use of ω-gliadin antibodies (401.21 mAb) is still available, but its practical application has been replaced by sandwich R5-ELISA **[13].**

Following the analytical procedure based on the use of ω-gliadin antibodies, a further improvement was achieved by the development of the Mendez method, based on monoclonal R5 antibodies **[38].** The antibody specifically recognizes the thermostable (QQPFP) peptide sequence consisting of 5 amino acids, and also identifies homologous sequences. Thus the sequences (QQQFP), (LQPFP), (QLPFP), (QLPYP), (QLPTF), (QQSFP), (QQTFP), (PQPFP), (QQPYP), (PQPFP) that are strongly homologous to (QQPFP) are also recognized, although with lower reactivity. In the sequences Q=Glutamine, P=Proline, F=Phenylalanine, L=Leucine, S=Serine, T=Threonine, Y=Tyrosine) **[58].** The (QQPFP) peptide occurs in the repeating domains of prolamins (gliadins, hordeins, secalins). It is most commonly found in ω-types. Therefore, this antibody is particularly suitable for the detection of prolamines **[59].**

The R5 antibody produced against the ethanol extract of ω-secalin (rye prolamin) can primarily be used to detect α and γ-type toxic gliadin epitopes (pepscan) with adequate sensitivity. ω-Gliadins with a molecular weight over 75 kDa are weakly detected, and glutenins are recognized in a limited way. The advantage of this method is that all of the wheat gliadin, barley hordein and rye secalin fractions are recognized to the same extent and through the same pepscan epitope portions by the R5 antibody. R5 does not measure maize, rice and oat gluten content, not toxic to celiac patients and, by its nature, does not cross-react with gluten-free cereals.

So ω-gliadin sandwich ELISA was replaced by the application of the sandwich R5-ELISA method, since the latter was capable of measuring barley prolamins (hordeins), and it was not necessary to take into account the differences between the individual grain varieties during its use. R5-ELISA technology has become a Type I method internationally accepted by the Codex Alimentarius Commision, which was used to check the gluten content of raw and heat-treated gluten-free foods. The repeatability of the method was 20% (Ingenasa) and 18% (R-Biopharm), and its reproducibility was 32% (Ingenasa) and 30% (R-Biopharm).

In the case of the sandwich R5 ELISA method, a so-called cocktail solution is used before the 80% ethanol extraction to promote the reduction of prolamins. This is particularly necessary because of the structural change of proteins due to the production technology of foods. In the case of sandwich R5-ELISA, the cocktail solution contains 250 mM 2-mercaptoethanol (reducing agent), which was later supplemented by 2 M guanidine hydrochloride (disaggregating agent) dissolved in PBS buffer (Phosphate Buffered Saline). Gluten recovery was 70-98% with the cocktail solution, while only 30-50% with 60% ethanol **[13], [60].**

The most recently developed reagent combination is the UPEX solution (Universal Prolamin and glutelin EXtractant solution), which is compatible with all gluten analytical methods. Its reducing agent is tris(2-carboxyethyl)phosphine (TCEP), which specifically disrupts disulfide bridges and is less toxic than other reducing agents; its disaggregating agent is N-lauroylsarcosine in PBS. The UPEX solution also exists in another reagent combination. The reducing agents of the latter are 2-mercaptoethanol and TCEP, while the disaggregating agent is guanidine. SDS can also be used as the disaggregating agent.

Another cocktail solution development is UGES (Universal Gluten Extraction Solution), containing a reducing agent, a solubilizing agent (arginine) and an alcoholic antiseptic agent **[2], [61].**

It depends on the food matrix whether other auxiliary steps are required to make the extraction more efficient, or not. For example, in the case of foods containing 10% fat, defatting with n-hexane is recommended. For foods with a high polyphenol content, the addition of fish gelatine and/or polyvinylpyrrolidone or skimmed milk powder may be necessary to prevent the interaction of gluten proteins with polyphenols **[62].**

The use of gliadin standards of different quality was eliminated by the preparation of a so-called European Reference Gliadin Standard (IRMM-480, PWG-gliadin, where IRMM is The Methodology Institute of the European Commision for Reference Materials and Measurements) for the kits, containing the gliadin components of 28 European wheat varieties, by the Working Group on Prolamin Analysis and Toxicity (see the R5 antibody based systems). This reference standard can be used for comparative analyses, as well as an internal standard for calibrations **[63].** In this connection, the question arises whether IRMM-480 represents all wheat varieties on Earth adequately, i.e., it can be used in the standard globally, or it will only play a role in the relevant European regulation after the adoption of the Codex draft.

Weaknesses of the sandwich R5-ELISA method:

- R5 antibodies also recognize other food proteins (not only harmful prolamins, but also soy and lupin). The detectability requirement mainly in ω- and γ-type prolamins is the

presence of the FP dipeptide found in the epitopes. Recognition of this dipeptide may also lead to false results, as the FP dipeptide is present in many other proteins/peptides (e.g., in gluten-free soy) **[58].**

- Sandwich R5-ELISA overestimates hordeins when using a PWG-gliadin standard, thus giving false positive results **[13],** and this is also the case for oats contaminated with barley, even though it uses a hordein standard **[16].**
- Another disadvantage is that it is not suitable for the detection of gluten in fermented and hydrolyzed foods.

In fermented/hydrolyzed foods (beer, soy sauces, vinegars, sourdough breads), gluten could not be measured by either ω-gliadin antibody based or sandwich R5-ELISA systems. During the hydrolysis, small peptides lack the two antibody-binding epitope portions, the presence of which is indispensable for the measurement in the sandwich system.

In order to detect fragmented (hydrolyzed) gluten, the Codex Alimentarius Commision has proposed a transition to the not yet validated competitive R5 ELISA method **[16].** Competitive system ELISA is used to measure protein fragments where only one epitope (immunopathogenic part) is available **[36].**

In this case, extraction is carried out using ethanol, because competitive R5 ELISA is not compatible with cocktail extraction. The reason for this is that prolamins can only be extracted almost completely with ethanol from foods containing native proteins. After heat treatment, when proteins are denatured, ethanol can no longer extract all prolamin fractions. Ideally, in the case of heat-treated foods, a cocktail solution should be used for prolamin extraction, so using a 60 to 80% ethanol extraction for the competitive R5-ELISA test, it is not possible to determine the gluten content of heat-treated and hydrolyzed foods with sufficient accuracy. Only the amount of hydrolyzed and not, or only slightly heat-treated gluten can be determined by this method.

When analyzing hydrolyzed foods, for example, beer, gluten values 1.9 to 17 times higher were obtained using competitive R5-ELISA than in the case of the sandwich R5-ELISA technique. On the other hand, in a study of breakfast cereals, higher gluten content values were obtained by sandwich R5-ELISA than in the case of the competitive R5-ELISA method. The difference can be explained by the heat treatment of the food samples **[13].** Using the competitive R5-ELISA test, more gluten can be measured in wheat (by 26%), rye (by 49%) and barley (by 82%), than by the sandwich R5-ELISA technique **[40].**

In competitive R5 ELISA, a new extraction solution is the application of the UPEX solution, which can be used for both hydrolyzed and heat-treated foods. Rossel et al. used PWG gliadin digested with pepsin, trypsin or chymotrypsin as a standard **[2].**

7.1.3. Development of antibodies that recognize the T-cell stimulating (celiac disease inducing) epitopes of gliadin

The analytical techniques based on ω-gliadin or R5 monoclonal antibodies are not clinically validated methods and therefore can only be used as indicators of the toxicity of foods for celiac patients. At the same time, the goal of researchers is for the designed (produced) antibodies to recognize the T-cell stimulating (celiac disease inducing) epitopes of the immunodominant gliadin among the protein sequences of gliadin **[64].**

In recent years, several different celiac T-cell stimulating gliadin epitopes have been identified that were clustered in the proline-rich region of the protein. Of the epitopes identified, particularly significant are those of α-gliadin, which are recognized by the T-cells of the small intestine in most celiac patients.

The monoclonal PN3 antibody **[65], [66]** is an antibody produced to recognize a 19 amino acid sequence, the epitope of the *in vivo* toxic α-gliadin in the 31-49 position (LG**QQQPFP**PQQPYPQPQPF). The synthetic peptide specific antibody produced specifically for the main recognition epitope sequence (QQQPFP) reacts with α- and γ-gliadins strongly, while ω-gliadins react weakly. In addition, it reacts with LMW glutenins, rye secalins and barley hordeins, but does not react with HMW glutenins, oat avenins or maize zeins. However, Bermundo Redondo et al. assume that the antibody should react with oat avenins, since they contain the QQQPF peptide segment **[67].** The disadvantage of the PN3 antibody based sandwich ELISA developed by Ellis et al. is that toxic prolamins (rye, barley, oats) are not uniformly detected by the antibody **[66],** and because of the sandwich nature of the method, hydrolyzed forms are not recognized.

The monoclonal α-20 antibody is an antibody developed for the recognition of a T-cell stimulating α-gliadin epitope. The specific peptide sequence, i.e., the minimal recognition epitope sequence is as follows: (PFRPQQPYPQP), (where P=Proline, F=Phenylalanine, R=Arginine, Q=Glutamine, Y=Tyrosine). It is suitable for the recognition of gliadins, secalins and hordeins, but we have limited knowledge regarding its reactivity, especially in the case of the glutelin fraction.

The α-20 antibodies are used in the product of the company EuroProxima, the Gluten-Tec ELISA competitive system. For extraction, 60% ethanol dissolution or dithiothreitol (DTT) reducing agent and dissolution with 60% ethanol containing iodoacetamide is recommended. By multiplying

the determined 89 µg/kg peptide equivalent value corresponding to the limit of quantification (LOQ) by a conversion factor of 100 and a duplication factor of 2 (the amount of gluten was deduced from the gliadin content of the sample), a value of 17.8 was obtained, which, when rounded, corresponds to roughly 18 mg/kg gluten.

According to the experiments of Scherf et al., the calibration recorded against the synthetic peptide sequence (GPFRPQQPYPQPB) was reproducible, but calculating with the peptide to protein conversion factor, their results were not uniformly acceptable **[62].**

Particularly interesting is a 33-mer section (56-88) of α-gliadin after gastrointestinal digestion. This fragment was found to be highly resistant to luminal proteases and the brush border enzymes of the small intestine due to its high proline content (13 of the 33 amino acids are proline moieties) **[10],** but the resilience of this fragment does not provide a completely satisfactory explanation for understanding the total pathogenesis of celiac disease. These peptides remain intact, cross mucous membranes and trigger celiac symptoms. They are excreted in urine and faeces. It should be noted that kits for the determination of the gluten content of urine and samples of faecal origin are commercially available. Later, several T-cell activating peptides were mapped, but still the 33-mer section of α-gliadin is most often used as an immunogenic peptide model **[13].**

The 56-88 AS sequence of α-gliadin (33-mer: (LQLQPFP-**QPQLPY**-P-**QPQLPY**-P-**QPQLPY**-PQPQPF), where L=Leucine, Q=Glutamine, P=Proline, F=Phenylalanine, Y=Tyrosine) contains 6 T-cell stimulating immunotoxic epitopes, which is why it is one of the major celiac inducing peptide segments. Two antibodies have been developed for the detection of this toxic peptide segment consisting of 33 amino acids **[68], [69].** The monoclonal G12 antibody is an antibody produced for the (QPQLPY) hexapeptide sequence found in this segment specifically in wheat. The (QPQLPY) peptide sequence is repeated three times in this 33-mer peptide segment. In addition to the (QPQLPY) sequence, this antibody also recognizes the (QPQ(L/Q)P(Y/F/Q), (QPQLPL), (QPELPY) peptides with similar structure in wheat, rye, barley and some oat varieties. For example, the sequence (QPQQPY) is typical in rye, while (QPQLPF) is in barley. In addition, these sequences can be detected not only in α-, but also in ω-, γ-, and β-prolamins, as well as in glutenins. Its advantage is that it detects avenins, but its affinity for oats is limited and this reactivity is proportional to the potential immunotoxicity of the different oat varieties. In the case of hydrolyzed foods, it is suitable for the measurement of gliadin concentrations above 0.5 mg/kg. Naturally, the antibody cannot detect all immunogenic gluten peptides (their number exceeds 1,000), but it can react with 80-95% of them. The R5 ELISA system can only detect 25% of immunogenic gluten peptides **[13].**

Tests made to date for the detection of gluten have focused on the recognition of gliadin proteins or their epitopes, and not on the recognition of the specifically T-cell stimulating epitopes of gliadin. Thus, neither the ω-gliadin antibody, nor the R5 antibody fully recognizes the T-cell stimulating epitopes of immundominant gliadin **[64].** The development of the new generation G12 antibody has been a milestone in gluten detection, because it recognizes selectively the pathogenic (immunotoxic) section of the gliadin molecule, i.e., the 33-mer peptide sequence responsible for triggering the autoimmune response in celiac patients **[68].** While the former detection systems were not specific for the detection of oat gluten content, the G12 antibody is specific for the possibly toxic amino acid sequence also found in oats. This is a much more selective and $6*10^4$ times more sensitive analytical method compared to other available techniques. So far, there is little information available regarding the practical applicability and reliability of the G12 antibody based method, but it is, in any case, promising. In the research of Török et al., the sensitivities and reliabilities of the methods based on R5 and G12 antibodies are tested by the parallel application of these methods **[51].**

The monoclonal A1 antibody is also an antibody suitable for the recognition of one of the wheat heptamer sequences (QLPYPQP) in the 56-88 section of α-gliadin, and it also recognizes other homologous sequences ((Q(L/Q)P(F/Y)P(Q/L)(P/Q)). It is also suitable for the detection of wheat, rye, barley and certain oat varieties. The A1 antibody is more sensitive for gluten detection than the G12 antibody, although the G12 has a higher affinity for the 33-mer amino acid sequence **[13], [69].**

GlutenTox ELISA kits use monoclonal G12 or A1 antibodies in a competitive ELISA system, while in the sandwich system, anti-gliadin (A1)/anti-gliadin (G12), HRP antibodies are used.

The monoclonal CD5 antibody is an antibody produced against a synthetic peptide equivalent to the toxic (T-cell stimulating) 51-75 segment of α-gliadin **[70].**

7.1.4. Methods for the immunological detection of gluten

Immune-based test kits currently available on the market and the chemicals and materials required for the measurement are summarized in ***Table 1***.

7.1.4.2. Execution on an immunochromatographic test membrane

It is used for qualitative and semi-quantitative measurements. It is a fast screening method based on an immunochromatographic principle that shows whether the gluten concentration of the sample exceeds the limit value or not (***Figure 2***). It works on the sandwich

direct principle, using only monoclonal antibodies. It is mainly used during the various technological steps of food production, in quality control tasks or in catering units. The placement of the membranes is achieved in different configurations **[71].**

The membrane is immersed in the protein extract to be examined. The adsorbent zone of the membrane contains colored (substrate) microparticles covered with antigen-specific antibodies. These bind to the target protein (gliadin) in the sample, forming a complex, and thus they move along towards the reaction zone together. Here, at a certain point, the immune complex formed binds to the antigen-specific antibody labeled with a membrane-mobilized enzyme, which is indicated by the discoloration of the microparticle. Correct operation of the test is ensured by a control microparticle.

7.2. Development of other, non-immunological gluten detection methods

7.2.1. Gluten detection procedure based on polymerase chain reaction (PCR)

According to the resolution of the Codex Committee On Nutrition And Foods For Special Dietary Uses (CX/NFSDU), in addition to immunological measurement methods, as an alternative and complementary method, DNA-based qualitative determination is also necessary.

Because of their indirectness, it is hard to develop a PCR method based on the detection of DNA for an allergenic agent, e.g., gluten, as a routine method requiring a lot of optimization. Techniques based on the PCR principle are used primarily in cases where the additive is unlabeled, or an additional test area is the checking of the cleanliness of industrial equipment. However, there is an increasing need for the accuracy, specificity and good reproducibility of the method. The advantage of DNA-based procedures is that DNA can be characterized by a high thermal stability, even individual species or groups of species carrying similar allergens can be detected because of the flexibility of the method, depending on how the base sequences of the key primers participating in the reaction are designed. The analytical technique can also be used on-site, allowing for a test that can be evaluated visually and requiring only a thermostat.

Limitations of gluten analytical methods based on PCR diagnostics:

- Small amount of DNA in the sample to be tested, insufficient sequence length because of the fragmentation of the DNA;
- Effects of technological treatments and the food matrix (partial or complete hydrolysis, enzymatic degradation, complex samples with high carbohydrate or fat content);
- Inhomogeneity of potentially contaminated samples;
- Value of protein-DNA conversion.

The DNA-based determination of the gluten content is an indirect method, since it is not based on the measurement of the protein that triggers the pathological reactions, i.e., gluten, but the DNA sequence encoding it or another DNA fragment characteristic of the cereals that contain the protein damaging the small intestinal mucosa is detected. Therefore, DNA-based methods can only be used for quantitative measurements if it can be demonstrated that the presence of the DNA and the corresponding protein or cereal portion are proportional to each other, their quantities in the sample correlate with each other. The DNA-based procedure can primarily be used to verify the absence of gluten, and in cases where the use of protein tests is somehow limited, e.g., proteins are denatured during food production and become chemically insoluble **[74].**

The DNA-based detection of allergens is supported by a number of genetics studies, since to determine allergen cross-reactions and genetic relationships, as well as more conservative and more variable sequences before selecting the appropriate primer, mapping the genetic bases was indispensable.

Ko et al. **[75]** designed PCR sense and antisense primer pairs that are suitable for the separation of different cereal varieties. For this purpose, a primer pair was designed for the spacer sequence between 5S ribosomal RNA genes and, in addition, RAPD primers (Random Amplified Polymorphic DNA) were used. In the course of their investigations, for the mixtures of cereals, the primer designed for the spacer sequence was found to be effective.

In the method developed by Allmann et al. **[76]**, a TR01/TR02 primer pair was used to multiply a 109 bp (base pair) fragment in a frequently repeating section of the intergenic region between the 25S and 18S loci of the ribosomal RNA gene of wheat. This method was later used for the detection of gluten in emulsifiers, flours, starches, instant soup powders, polenta, curry and other foods **[77].** Köppel et al. **[78]** used this primer pair to check the gluten-free status of muesli samples from different countries.

The primer pair designed by Dahinden et al. **[79]** allowed the simultaneous detection of wheat, rye and barley in foods. The primer designed by them specifically amplifies a non-coding region of the trnL gene of the DNA of only wheat, barley and rye chloroplast. In the course of their experiments, this primer pair was used to study different cereals and starch-containing seeds, as well as heat-treated foods (breads, pasta and infant foods), in the case of which this primer pair was successfully used to detect contamination by toxic cereals. Kuchta **[80]**

has launched an interlaboratory proficiency testing program using a method expanded by the RFLP (Restriction Fragment Length Polymorphism) analysis applied by him. During the proficiency testing, infant formulas were screened using this technique. It was confirmed in the proficiency testing that the method is sufficiently sensitive, reliable and fast, and is suitable for routine use **[81].** Based on the results, a real-time PCR method was developed by Mujico and Méndez **[82]**, for which R5 ELISA testing was performed as a reference method. The experiment showed linear correlation between the prolamin content of the samples used in the measurements and the amount of DNA.

Delano and Schmidt **[83]** used a primer pair designed for the *trnL* (UAA) region of the chloroplast tRNA gene. The characteristic of tRNA genes is that they are much more conservative than protein-coding or ribosomal genes, and so because of their minute differences they are more suitable for the joint characterization of several species. Thus, the use of a single primer pair allowed the detection of different plant DNAs (e.g., rape, maize, potato, soy, rice, peanuts, wheat), because when using the primer pair, the different plant DNAs resulted in fragments of different length. The first real-time PCR method was developed for the detection of rye in raw materials and finished products in 2004 **[84].** Subsequently, Hernandéz et al. **[85]** developed a procedure consisting of four independent real-time PCR measurements. During this, primers and Taq-Man probes were designed for the DNA sequence of barley hordein, for the gos9 DNA sequence of rice, for the DNA sequence of sunflower heliantinin and for the DNA sequence of the acetyl-CoA carboxylase of wheat. The method was tested on heat-treated foods (biscuits, breads) and food samples containing small amounts of DNA (oil, beer).

Terzi et al. used a number of methods and these were compared for the screening of trace amounts of cereals **[86].** Their investigations revealed that all the methods had disadvantages, which primarily manifested during the examination of complex samples. This fact justified further method development, during which a kit for real-time PCR was developed (SureFood® ALLERGEN Gluten real-time PCR). The LOQ of the method is 5 DNA copies or 5 mg/kg of gluten (R-Biopharm).

A new direction of PCR methods could be the so-called isothermal PCR method, making fast tests possible, which can even be performed on the production lines of food manufacturing plants ***(Figure 4)***. For the analyses, for example, the so-called TwistAmp® kit (TwistDx Ltd, UK) can be used, which is available in several versions (e.g., basic, exo, exoRt, nfo). Currently, the method is used primarily for microbiological and virological studies, but it can be readily adapted to the analysis of various food allergens, such as gluten **[87], [88].**

The common characteristic of the different versions is that a DNA recombinase enzyme is used for the PCR reaction, as opposed to the conventional PCR method, which uses a DNA polymerase enzyme. Through the DNA recombinase enzyme, DNA amplification occurs at 37-40 °C, so the sequence of reactions is completed within 30-40 minutes. TwistAmp® kits require special primers that must be at least 30-35 bp long. In the case of the forward primer, biotin labeling of the 5' end is necessary (except for the basic method), and similarly to the PCR-ELISA method, this enables detection using a test strip (nfo kit). In addition, the use of a 50-55 bp probe sequence is also required for the test, during the design of which attention should be paid to the fact that 29-30 bp from the 5' end of the probe tetrahydrofuran should be substituted for a thymine base, because this is where the recombinase enzyme can bind and start the multiplication of the complementary sequence. In addition, care should be taken that the GC ratios (guanine-cytosine ratio) of the primers and the probe sequences are similar.

After DNA amplification using the nfo kit, simple evaluation is performed with the help of the MileniaHybriDetect (MileniaBiotec, Germany) strip. The qualitative result is obtained by dipping the test strip in a tube containing the amplified DNA of the test sample. If no band appears on the strip, amplification has been performed incorrectly, and if there is 1 band, the test sample does not contain gluten in a detectable amount. If two bands appear, the sample contains a detectable amount of gluten. Contrary to conventional PCR methods, this method uses not DNA polymerase, but recombinase enzyme for amplification, and two 10-20 bp primers are insufficient for the assay.

The use of the basic kit is similar to conventional PCR, but the reaction takes place faster. In this case, evaluation is carried out by gel electrophoresis. Similarly to real-time PCR methods, exo and exoRT tests allow for quantitative DNA and RNA determination. The TwistAmp® kit also comes with a portable thermostat that facilitates on-site work and ensures the temperature required for the reaction **[72], [73].**

7. 2. 2. Other novel and innovative gluten detection possibilities

Developments in gluten detection still present a challenge, and novel and innovative methods continuously come to light, the goal of which is to detect the presence of absence of gluten faster and more accurately. These include aptamers, immunomagnetic beads (IMBs) covered with anti-gliadin polyclonal antibodies, the use of protein or peptide microarrays (multiplex lab-on-a-chip devices), multianalyte profiling (xMAP) with magnetic particles containing fluorescent dyes **[62].**

8. Conclusions and declaration

Various allergic or hypersensitivity-based metabolic disorders occur in the human population of our century at an alarming rate. To ensure safe daily living of the persons affected, it is absolutely necessary for food manufacturers and distributors, as well as service providers controlling the sector and authority bodies to possess analytical tools that enable them to check, with great certainty, the composition of foods that intended for people with allergies and intolerance.

Since hypersensitivity reactions (celiac disease, allergy) may occasionally be triggered by very small amounts of undesirable food ingredients, it is important to develop methods that are able to detect and quantify these substances quickly and with adequate accuracy.

With these thoughts in mind, in our review paper we have collected the knowledge which we consider important regarding the analysis of gluten proteins from available literature sources. Since the immunochemical characteristics of the analytical packages (kits) that serve as the test methods for gluten proteins vary considerably in the products developed by the different companies, the fancy names of the individual kits or, occasionally, the names of the companies producing the kits could not be omitted from our manuscript. We hereby declare that, in our work, we have not been in a business relationship with any of the companies involved in the analytics of gluten proteins that would serve the economic interest of said company other than publishing scientific results. By communicating the data summarized in ***Table 1***, we intend to assist everyone during the preparation of test plans in the selection of the measurement principle and measurement method most suitable for the actual test purpose.

9. Acknowledgement

The authors would like to thank the project titled “Gluten-free egg replacement additives providing texture identical to that and plant-based products intended to use them” with registration no. TÉT_15_IL-1-2016-0019, realized through a Hungarian-Israeli scientific and technological cooperation supported by the NKFI Office.

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