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Recent progress in analytical method development to ensure the safety of gluten-free foods for celiac disease patients

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ABSTRACT

As laid down by the Codex Alimentarius, products bearing a gluten-free label must not contain gluten levels above 20 mg/kg to be safe for consumption by celiac disease patients. Analytical methods to detect gluten from wheat, rye and barley need to be sufficiently sensitive, specific, suitable for routine analyses and validated by collaborative studies. With continuous progress in the field of gluten analysis, the aim of this paper is to provide an up-to-date overview of legislation regarding gluten-free products worldwide, as well as immunochemical, proteomics-based, genomics-based and other methods designed to analyse gluten traces. While ELISA test kits and PCR are still most widely used in quality control, liquid chromatography tandem mass spectrometry (LC-MS/MS) is gaining more and more importance by providing unprecedented insights into gluten. Several other methods such as immunosensors, other sensors and microarrays are being developed. The pro's and con's of the different methods are discussed as well as the remaining challenges, including the need for improved extraction procedures, comprehensive reference materials and independent reference methods.

1. Introduction

The consumption of foods made from wheat, rye or barley may cause adverse immune reactions in predisposed individuals who suffer from wheat allergy, non-celiac gluten sensitivity (NCGS) or celiac disease (CD) (Dale et al., 2019). With a seroprevalence of 1.4% of the population, CD is one of the most frequent hypersensitivities worldwide (Singh et al., 2018). It is caused by a combination of genetic (human leukocyte antigens HLA-DQ2 and -DQ8) and environmental (gluten consumption) factors together with a third, still unknown factor that initiates the loss of immunotolerance to gluten (Scherf et al., 2020). Once triggered, CD results in inflammation of the upper small intestine with infiltration of intraepithelial lymphocytes and partial to total villous atrophy that eventually leads to decreased absorption of essential nutrients. While a wheat-reduced diet may be sufficient to alleviate symptoms of NCGS (Dieterich et al., 2019), CD patients need to follow a strict lifelong gluten-free diet with a maximal intake of 20 mg of gluten per day (Catassi et al., 2007). Nevertheless, a gluten-free diet is also recommended for patients suffering from NCGS or wheat allergy as it improves clinical and psychological symptoms (Dieterich et al., 2019). The term "gluten" comprises the storage proteins of wheat (gliadins and glutenins), rye (secalins) and barley (hordeins) that, unlike albumins and globulins, are insoluble in water and 0.5 mol/L NaCl. Gluten is composed of prolamins that are mostly monomeric alcohol-soluble (i.e., 40–70% aqueous ethanol or 2-propanol) proteins accounting for 40–55% of gluten and of glutelins that are polymeric insoluble proteins accounting for 45–50% of gluten depending of the cereal source (Fig. 1). Therefore, a gluten-free diet essentially relies on avoidance of these gluten-containing cereals by consuming naturally gluten-free foods such as vegetables, pulses, fruits and animal-based products. Dietary gluten-free foods intended for CD patients must not contain more than 20 mg/kg of gluten according to international legislation (Codex Standard 118-1979, 2015). This is exactly where reliable analytical methods for gluten detection are needed to ensure the safety of such foods for CD patients. Naturally gluten-free grains such as maize, rice and pseudocereals may become contaminated with gluten due to comingling already on the field, during transportation or later over the course of food processing. In addition, gluten is widely used in the food industry as a thickener, emulsifier or flavor enhancer and may be present in foods where it is not obvious. That is why accurate detection of gluten traces in supposedly gluten-free foods is essential to protect CD patients from inadvertent gluten intake and associated health risks (Syage et al.,

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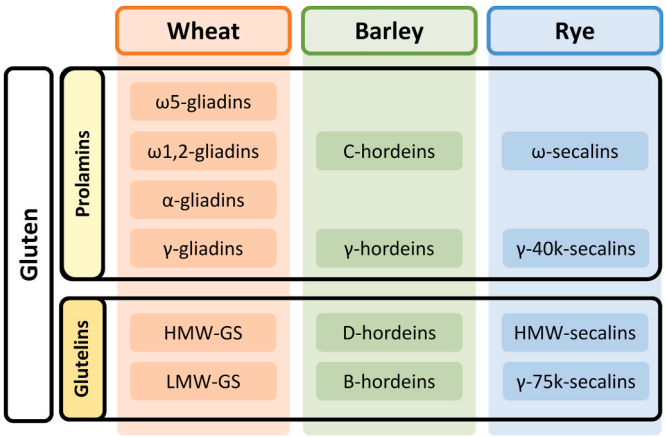

Fig. 1. Overview of gluten protein types. Gluten protein types from wheat including ω5-gliadins, ω1,2-gliadins, α-gliadins, γ-gliadins and high- (HMW-GS) and low-molecular-weight glutenin subunits (LMW-GS), barley including C-hordeins, γ-hordeins, B-hordeins and D-hordeins and rye including ω-secalins, γ-75k-secalins, γ-40k-secalins and high-molecular-weight (HMW)-secalins.

2018). With continuous progress in the field of gluten analysis since our last review (Scherf and Poms, 2016), the aim of this paper is to provide an up-to-date overview of legislation regarding gluten-free products and immunochemical, proteomics-based, genomics-based and other methods designed to analyse gluten traces. We specifically focused on new developments from 2016 onwards, while keeping the essential information from earlier studies.

2. Labelling of gluten-free foods

2.1. Codex Alimentarius legislation

According to the general labelling provisions, it is mandatory to declare gluten-containing cereals, i.e., wheat, rye, barley, oats, spelt or their hybridized strains and products of these, as part of the ingredients list on the label of prepacked foods (Codex Standard 1-1985, 2018; Codex Standard 146-1985, 2009). Legislation regarding gluten-free products is specified in Codex Standard 118–1979, where gluten-free foods are defined as dietary foods "consisting of or made only from one or more ingredients which do not contain wheat (i.e. all *Triticum* species, such as durum wheat, spelt, and khorasan wheat, which is also marketed under different trademarks such as KAMUT), rye, barley, oats or their 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" (Table 1). Foods consisting of one or more ingredients from gluten-containing cereals can be specially processed to remove gluten and achieve a gluten level not exceeding 20 mg/kg. The consumption of oats not contaminated with wheat, rye or barley is generally well-tolerated and considered to be safe for the vast majority of CD patients at moderate amounts (20–25 g/day for children, 50–70 g/day for adults) (Pinto-Sánchez et al., 2017) and therefore national regulations on the allowance of oats can be made.

Regarding methods of analysis and sampling, Codex Standard 118–1979 states that quantitative gluten determination in foods "shall be based on an immunologic method or other method", as long as it is at least equally sensitive and specific. The method should specifically detect the immunogenic protein fractions, be validated and calibrated against a certified reference material and have a detection limit of 10 mg/kg of gluten or less. The enzyme-linked immunosorbent assay (ELISA) R5 Mendez method is laid down as a type I method for gluten determination, which means that it is the only method for establishing the accepted value (Codex Standard 234–1999, 2019).

Table 1
Overview of thresholds for gluten-free claims in different countries or regions, listed in alphabetical order, as well as that of Codex Standard 118-1979 (2015).

Country	Gluten threshold [mg/kg]
Argentina	10
Australia/New Zealand	n.d.
Brazil	n.a.
Canada	20
Chile	5
Colombia	20
Costa Rica	20
El Salvador	10[a]
European Union[b]	20
India	20
Israel	20
Japan	10[c]
Paraguay	20
South Africa	20
United States	20
Codex	20

n.d., not detectable according to state-of-the-art techniques, i.e., below 3 mg/kg of gluten.
n.a., not available, sometimes interpreted as 20 mg/kg, sometimes as not detectable.
[a] Threshold level originally given as 0.05 g of total nitrogen per 100 g based on grain dry matter, but now converted to gluten.
[b] The same thresholds are in place in non-EU member countries that participate in the European Licensing System, including Bosnia Herzegovina, Croatia, Montenegro, Norway, Russia, Serbia, Switzerland, Turkey, Ukraine and the United Kingdom.
[c] Threshold level is given as mg of wheat protein per kg of the food product.

2.2. European Union

The labelling requirements for gluten-free foods, both prepacked and non-prepacked are laid down in Regulation (EU) No. 1169/2011. The indication of cereals containing gluten, namely wheat, rye, barley, oats, spelt, kamut or their hybridized strains, and products thereof, except wheat- or barley-based glucose syrups and maltodextrins, is mandatory. More specific requirements for the provision of information to consumers on the absence or reduced presence of gluten in food are provided in Commission Implementing Regulation (EU) No. 828/2014 and the definitions, labelling requirements and threshold of 20 mg/kg of gluten are equivalent to those of Codex Standard 118–1979. Under the framework of the European Licensing System of the Association of European Celiac Societies (AOECS), manufacturers of gluten-free foods can obtain an annual license with a registration code from the respective national celiac society, provided that the product contains 20 mg/kg of gluten or less and meets further standards for traceability, hazard control, audits and gluten analysis certificates. Products that have been licensed accordingly, may use the well-known and internationally recognized crossed grain symbol on the label (Fig. 2A).

2.3. America

In Canada, section B.24.018 of the Food and Drug Regulations (FDR) prohibits "to label, package, sell or advertise a food in a manner likely to create an impression that it is a gluten-free food if the food contains any gluten protein or modified gluten protein, including any gluten protein fraction". The term gluten is defined in FDR section B.01.010.1(1) as any gluten protein from barley, oats, rye, triticale or wheat and includes hybridized strains as well as modified gluten. The FDR do not refer to any specific threshold, but Health Canada considers 20 mg/kg of gluten and below as a safe level.

In the United States, gluten-free claims are regulated under the Food

Fig. 2. Logos used on the labels of gluten-free products worldwide. (A) Crossed grain symbol for gluten-free products certified within the framework of the European Licensing System (reprinted with permission by Coeliac UK), (B) Trusted mark of the Gluten-Free Certification Program of the Canadian Celiac Association, (C) Trusted mark of the Gluten-Free Certification Program endorsed by Beyond Celiac in the United States, (D) Gluten-Free Manufacturing Program (GFMP) Certified Mark of Trust™ (www.GF-Certified.com) endorsed by the National Celiac Association in the United States, (E) Gluten-Free Certification Organization (GFCO) Global Certification Mark endorsed by the Gluten Intolerance Group in the United States, (F) Official trademark logo of the gluten-free certification program of the Argentinian Celiac Association (Asociación Celíaca Argentina), (G) Official trademark logo of the gluten-free certification program of the Chilean Celiac Society (Corporación de Apoyo al Celíaco).

Allergen Labelling and Consumer Protection Act of 2004 (FALCPA) in title 21 of the Code of Federal Regulations (CFR), part 101.91. Gluten-containing grains are defined as any grain of wheat, rye or barley and crossbred hybrids. Any food bearing the gluten-free claim must not contain any ingredient that is a gluten-containing grain, or ingredients thereof unless it has been processed to remove gluten to a final level of 20 mg/kg of gluten or less. Any unavoidable presence of gluten in inherently gluten-free foods must meet the same level. Both Canada and the US do not have a harmonized licensing system equivalent to that of the AOECS, so that gluten-free labels vary widely in appearance from various pictograms to simple gluten-free statements (Fig. 2B–E). Some associations, such as the National Celiac Association (NCA), the Gluten-Free Certification Organization (GFCO) of the Gluten Intolerance Group and the Gluten-Free Certification Program (GFCP) of Beyond Celiac offer product certifications, but adopt different gluten thresholds such as 5 mg/kg (NCA), 10 mg/kg (GFCO) or 20 mg/kg (GFCP).

In Central and South America, there is a wide range of regulations regarding gluten-free claims, from very strict legislation, e.g., in Argentina and Chile, to no dedicated legislation at all, e.g., in most Central American nations, Bolivia and Ecuador that do not have celiac associations either (Table 1) (Mattioni et al., 2019). In Argentina, the Argentinian Food Code, article n°. 1383, defines gluten-free foods as those containing less than 10 mg/kg of gluten and a logo with the inscription Sin T.A.C.C. (no wheat, oat, barley and rye) was created (Fig. 2F). In Brazil, gluten labelling is regulated by the Brazilian Health Regulatory Agency (ANVISA) under the law n°. 10.674/2003 that requires all food products to be labelled as "contains gluten" or "does not contain gluten". No threshold level has been specified, but it is currently taken to be either 20 mg/kg or below the limit of detection of the most sensitive methods for gluten detection, i.e., currently 3 mg/kg of gluten. A threshold level of 5 mg/kg of gluten is applied in Chile, where gluten-free legislation is found in the Food Sanitary Regulation, Title XXVIII, Paragraph VI, Articles 516 to 518 (Fig. 2G). Some other South and Central American countries have adopted the Codex threshold of 20 mg/kg of gluten (Table 1).

2.4. Africa, Asia, Australia and New Zealand

Awareness of CD in Africa and Asia is only beginning to emerge (Singh et al., 2018) and this is why many African and Asian countries have not adopted national legislation regarding gluten-free products. In the absence of national regulations, many countries, such as, e.g., China, implicitly follow the Codex Standard 118-1979 (2015), that is also applicable in international trade. Some exceptions include South Africa, India and Japan. The South African Foodstuffs, Cosmetics and Disinfectants Act (Act 54 of 1972) – Regulations Relating to the Labelling and Advertising of Foodstuffs (R 146/2010) adopted legislation equivalent to that in Codex Standard 118-1979 (2015). The Indian Food Safety and Standards (Food Products Standards and Food Additives), Regulations, 2011, subregulation 2.14 states that gluten-free food consists of "or is made of one or more ingredients containing rice, millets, ragi, pulses or legumes", shall bear the "gluten free" label and must have gluten levels below 20 mg/kg. Japan has an entirely different approach, because it does not distinguish between gluten (in the context of CD) and wheat (in the context of allergy). According to the Japanese regulations for labelling of food allergenic ingredients, the threshold for wheat is set at 10 mg protein/kg of the food (Akiyama et al., 2011).

Australia and New Zealand regulate claims in relation to gluten content in the Food Standards Code – Standard 2.9.5. A gluten-free claim may only be made if the food contains no detectable gluten and no oats or oat products and no cereals containing gluten that have been malted, or products of such cereals. Based on the most sensitive methods currently available, the gluten level thus needs to be below 3 mg/kg. As analytical methods become increasingly sensitive, this strict definition is bound to make gluten-free claims ever harder which will, most likely unnecessarily, limit the availability of products for CD patients.

3. Extraction of gluten from food matrices

Since complete protein extraction is crucial for proper quantitation, the extraction of gluten from food matrices is the most critical part of gluten analysis. This poses considerable difficulties, because of the complex structure of gluten itself and the composition of the

surrounding food matrix (Fig. 3). Based on the foundations laid by T. B. Osborne, protein classification depends on their solubility, with the grain proteins being classified as follows: water-soluble albumins, salt-soluble globulins, alcohol-soluble prolamins and alkaline- or acid-soluble glutelins (Osborne, 1895). Extraction protocols for gluten (which is composed of prolamins and glutelins) are usually designed on the basis of Osborne's principle.

However, gluten extraction procedures vary depending on the analytical method, the food matrix and whether the sample is processed or not (Table 2). The extraction of non-processed foods such as flours is usually performed using aqueous alcohols such as ethanol or propanol. However, the use of alcohol solutions mainly allows the extraction of the prolamin fraction, which is assumed to be 50% of the total gluten content (Codex Standard 118-1979, 2015). The polymeric structure of glutelins however, can only be extracted by reducing disulphide bonds which are the connecting structures within the protein. Therefore, in order to extract both prolamins and glutelins, in either processed or unprocessed foods, the addition of reducing and/or disaggregating agents such as 2-mercaptoethanol and sodium dodecyl sulphate, respectively, is required (Fallahbaghery et al., 2017).

Furthermore, heating gluten proteins (as in, e.g., cookies, bread and pasta) causes aggregation and the formation of interchain disulphide bonds, which cannot be dissolved with aqueous alcohols or sodium dodecyl sulphate buffer alone (García et al., 2005; Wagner et al., 2011). Commonly used methods use a combination of stepwise extraction, which consists of the initial removal of albumins and globulins using salt solutions, followed by dissolving the prolamins and glutelins with the aid of aqueous alcohols and various reducing agents such as 2-mercaptoethanol, tris(2-carboxyethyl)-phosphine, dithiothreitol (DTT) or dithioerythritol, depending on the method used. The R5 ELISA method recommended by the Codex Standard utilises the so-called "cocktail" extraction solution consisting of 2-mercaptoethanol and guanidine hydrochloride reagents in phosphate buffered saline, which enables the extraction of gliadins from heated food (García et al., 2005).

Previous work has focused on the identification and purification of gluten protein types extracted using a modified Osborne fractionation method for preparative RP-HPLC (Schalk et al., 2017b). The data obtained from proteomic studies showed that this combined strategy of protein purification according to solubility (Osborne fractionation) and hydrophobicity (RP-HPLC) resulted in the incomplete separation of prolamins and glutelins due to the heterogeneity of gluten proteins and their partly polymeric nature (Lexhaller et al., 2019).

Furthermore, the efficiency and reproducibility of five gluten extraction protocols was evaluated by comparing the gluten yield using mass spectrometric peptide detection. The study showed that extraction with 2-propanol (IPA) and DTT using a two-step approach, which includes the re-extraction of the residual pellet with IPA-DTT-Tris buffer, has a comparatively high reproducibility (CV $<$ 15%). However, these results also indicated that multi-step protocols did not achieve effective separation of the gluten fractions (Fallahbaghery et al., 2017; Lexhaller et al., 2019). A further study used different extraction methods and buffers on wheat, rye and barley samples designed for proteomics. The extraction was performed with urea, Tris-HCl and IPA/DTT-based buffers, using the total number of identified proteins as a basis for comparison. Each buffer had a specific influence on the proteins extracted. For example, Tris-HCl, urea and IPA/DTT provided 228 (15.2%), 139 (9.3%) and 31 (2.1%) unique proteins from barley, respectively. The results emphasized the influence of the extraction method on the results, because different protein functional classes were detected depending on the protein extraction protocol. In addition, defatting or precipitation steps prior to extraction or after extraction provided no benefit. However, with regard to proteomics experiments, the enzyme chosen for digestion should be taken into account when comparing results, as it plays an important role in sequence and proteome coverage (Bose et al., 2019).

An alternative extraction solvent has been tested recently on both processed and non-processed food samples using new generation ionic liquids called deep eutectic solvents (DESs) (Lores et al., 2017). A DES is a composition of a hydrogen bond acceptor and a hydrogen bond donor with useful physical and chemical characteristics such as solubility for a wide range of solutes and water non-reactivity (Emami and Shayanfar, 2020). Because of their low toxicity, biodegradability and cost effectiveness, they are often used as alternatives to organic solvents, following the principles of green chemistry.

The extraction performances of two different choline chloride-based DESs were compared with the conventionally used 60% (v/v) ethanol/water solution by Svigelj et al. using a commercially available ELISA assay detecting prolamins. They reported that gluten recoveries from food samples were higher using DESs than those found with 60% ethanol (Svigelj et al., 2017), but the authors did not compare DESs with current ELISA extraction methods, such as the "cocktail". Similar results were demonstrated using DESs, particularly diluted fructose-citric acid in combination with ultrasound-assisted extraction. Comparable amounts were found in gluten-containing foods by replacing ethanol-water extraction combined with reduction using 2-mercaptoethanol by the use of diluted DES and sonication. In this way, the extraction time was reduced and the sensitivity was improved by a factor of ten (Lores et al., 2017). Although these approaches improve performance using ELISA assays, more research should be done in the future to validate the extraction method by using different analytical methods as well as commonly used ELISA extraction methods (García et al., 2005).

4. Gluten reference materials

Reference materials (RMs) are essential for the validation of analytical methods, calibration of instruments, verification of laboratory performance, estimation of uncertainty and quality control. In general, a suitable RM should have several properties such as high purity, good solubility and high stability. In terms of gluten, the Prolamin Working Group (PWG) succeeded in producing a RM by extracting prolamins

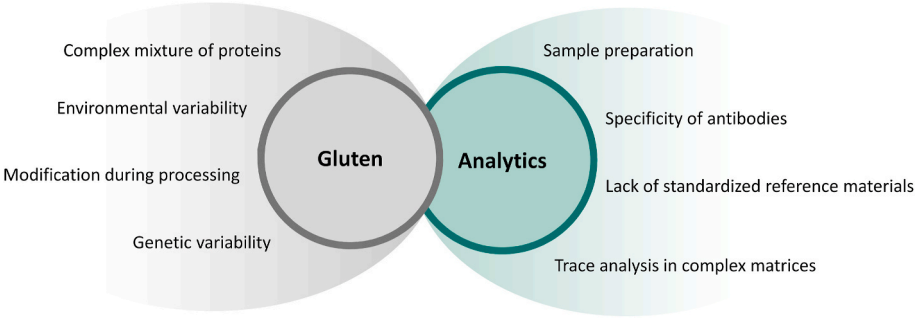

Fig. 3. Challenges of gluten analysis. Overview of different factors influencing gluten analysis related to the complexity of gluten as an analyte and the corresponding analytical methods.

Table 2
Overview of recent methods for gluten analysis designed to detect values of 20 mg/kg of gluten or lower, first sorted by type of method and then by year of publication/ author name.

Analytical approach	Study	Samples	Extraction method	Calibrants	Sensitivity
ELISA, competitive, combining five antibodies	Panda et al. (2017)	Wheat and barley beers; barley beers processed to reduce gluten; soy, teriyaki and Worcestershire sauces; vinegars; sourdough breads	Buffer (105 mmol/L sodium phosphate, 75 mmol/L NaCl, 2.5% skim milk powder, 0.05% Tween 20, pH 7.4)	Wheat gluten	LOQ: 2.6–2.9 μg/mL for majority of antibodies; LOQ (Skerritt): 0.85–0 .95 μg/mL; LOQ (MIoBS): 1.0 μg/mL.
ELISA, seven commercial test kits	Rzychon et al. (2017)	Gluten-free flours spiked with known gluten content; cereals: bakery products; snacks; diet foods; food supplements; meat; confectionery; condiments	Buffer (PBS, pH 7.4 + 0.5% SDS + 2% 2-mercaptoethanol)	Wheat gluten	LOQs of the commercial test kits
ELISA, seven commercial test kits	Scherf (2017)	30 wheat starch samples (14 labelled as gluten-free)	According to each test kit manufacturer	Calibrants available in each test kit	LOQs of the commercial test kits
ELISA, sandwich RIDASCREEN® Total Gluten	Lacorn et al. (2019)	Oat flour, spiked with wheat, rye and barley; Oat flour, unspiked; corn-based snack contaminated with wheat flour; mixture of corn-based processed snacks; rice flour contaminated with gluten, all with known gluten contents	Cocktail provided with the ELISA kit	Gluten extract from a mixture of four different wheat cultivars	LOQ: 5 mg/kg of gluten
Immunosensor	Chekin et al. (2016)	Rice flour; "gluten-free" labelled wheat flour spiked with known gliadin concentrations	60% ethanol; 60% ethanol + 5 mmol/L dithiothreitol + 6% SDS in PBS buffer	Gliadin	LOD: 1.2 ng/mL for gliadin
Immunosensor	Funari et al. (2017)	Rice flour; gluten-free corn flour; commercial standards	Cocktail provided with the ELISA kit, UPEX buffer	Gliadin	LOD: 5 mg/kg for gliadin
Immunosensor	Angelopoulou et al. (2018)	Bovine κ-casein; whole peanut, soy protein; gliadin	10 mmol/L PBS (pH 7.4) + 0.5 g/L NaN_3	Gliadin	LOD: 0.10 μg/mL for gliadin
Immunosensor	Marín-Barroso et al. (2019)	Flour samples (manioc, rice, gluten-free and common wheat flour) spiked with 10 mg/kg of gliadin	60% ethanol	Gliadin	LOD: 0.005 mg/kg for gliadin
Proteomics, LC-MS/MS	Manfredi et al. (2015)	Flours; seeds; pasta; biscuits; cookies; crackers; beverages; breads; breakfast cereals; snacks	50 mmol/L bicarbonate buffer (pH 8.0) + 250 mmol/L 2-mercaptoethanol + 2 mol/L guanidine hydrochloride	Incurred rice bread with and without addition of prolamins	LOQ: 2–18 mg/kg of peptide
Proteomics, LC-MS/MS	Colgrave et al. (2016)	Cereal grains (barley, wheat, rye, oats, millet, maize, rice, green wheat, amaranth, chia, quinoa, sorghum and tef); flours (rye, sorghum, buckwheat, soy, oats and millet); breakfast cereals	55% 2-propanol + 2% dithiothreitol	n.a.	LOD: 0.02–0.7% of barley-based cereal in non-barley breakfast cereals
Proteomics, LC-MS/MS	Liao et al. (2017)	Gluten-free products spiked with known contents of gliadin and hordein	1-butanol; 0.5 mol/L NaCl; 70% ethanol; 50% propanol+ 2% 2-mercaptoethanol + 1% CH_3COOH	Isotope-labelled gliadin and hordein peptides	LOD: 2.5 mg/kg of gliadin
Proteomics, LC-MS/MS	Li et al. (2018)	Distilled vinegar; malt vinegar containing barley and wheat; soy sauce (gluten-free and non-gluten free)	Dilution with 50 mmol/L ammonium bicarbonate	PWG-gliadin	LOQ: 1–5 mg/L equivalent of PWG-gliadin
Proteomics, LC-MS/MS	Schalk et al. (2018a)	Wheat starches	50% 1-propanol + 0.1 mol/L TRIS-HCl (pH 7.5) + 0.06 mol/L dithiothreitol	Isotope-labelled wheat peptide and isolated gluten protein types	LOQ: 0.9–22.2 μg/g of peptide
Proteomics, LC-MS/MS	Schalk et al. (2018b)	Barley-based beer samples and rye-based raw materials for sourdough fermentation	50% 1-propanol + 0.1 mol/L TRIS-HCl (pH 7.5) + 0.06 mol/L dithiothreitol	Isotope-labelled rye or barley peptide and isolated gluten protein types	LOQ: 0.1–4.5 μg/g of peptide
Proteomics, LC-MS/MS	Henrottin et al. (2019)	Wheat; barley; rye; spelt; einkorn; Khorasan wheat (Kamut); oats; rice; millet; buckwheat; linseed; white sesame seed; black sesame seed; tapioca; tiger nut sedge; chestnut.	Tris-HCl + urea buffer	Standard reference materials (milk, egg, peanut butter, soy); allergenic labelled peptides	LOD: 2.5–50 mg/kg of peptide
Proteomics, LC-MS/MS	Pasquali et al. (2019)	Rye cultivars; cereal grains (barley, wheat, rye, oats, millet, maize, rice, green wheat, amaranth, chia, quinoa, sorghum and tef); flours (rye, sorghum, buckwheat, soy, oats and millet); breakfast cereals; snacks	55% 2-propanol + 2% dithiothreitol	n.a.	LOD: 2% rye in non-rye based cereals
Genomics, PCR	Ahmed & Meng (2019)	Whole wheat flour, spaghetti, instant noodles, bread, biscuit, cake, oat flakes; gluten-free potato chips, spaghetti and tomato sauce	DNA extraction according to EN ISO 21571	Targeted DNA mixed with soya and/or corn DNA	LOD: 0.9 mg/kg of gluten in corn flour
Genomics, PCR	García-García et al. (2019)	Wheat, barley, rye, triticale, oats, rice and maize kernels; different commercial food products	DNA clean-up kit	Wheat, rye and barley mixture in maize flour, untreated and heat-treated	LOD: 1–5 mg/kg of gluten in maize flour
Sensor using aptamers	Malvano et al. (2017)	Beer; gluten-free beer; gluten-free toasted bread; rice; corn flour	Cocktail solution	PWG-gliadin	LOD: 5 mg/kg of gluten
		Wheat and barley	Cocktail solution	PWG-gliadin	

(*continued on next page*)

Table 2 (*continued*)

Analytical approach	Study	Samples	Extraction method	Calibrants	Sensitivity
Sensor using aptamers and G12 and barley antibodies	White et al. (2018)				LOD: 0.1–1.0 μg/mL of wheat or barley gluten
Sensor using magnetic molecularly imprinted polymers (MMIP)	Limthin et al. (2019)	Rice flour, glutinous rice flour, tapioca starch, wheat flour, corn starch and gluten-free corn starch	0.1 mol/L PBS	Gluten-MMIP	LOD: 8.5 mg/kg of gluten-MMIP

LOD, limit of detection; LOQ, limit of quantitation; MIoBS, Morinaga Institute of Biological Sciences; n.a., not available; PBS, phosphate-buffered saline; PCR, polymerase-chain reaction; PWG, Prolamin Working Group; SDS, sodium dodecyl sulphate; UPEX, universal prolamin extraction solution.

from a selection of the most common European wheat cultivars (van Eckert et al., 2006). Although it has not been approved by the Institute for Reference Materials and Measurements of the European Commission (IRMM), it is still used for calibration such as in the current Codex type I method, the R5 ELISA. So far there is no better characterized RM available for gluten analysis. However, PWG-gliadin does not represent the total gluten content and is based on wheat prolamins only.

In addition to targeting different epitopes depending on the antibody used, different ELISAs do not always give the same result, because different RM are used for calibration (Rzychon et al., 2017). As the gluten proteins of wheat, compared to rye or barley show different reactivity to the antibodies used in common ELISAs (Lexhaller et al., 2016), the use of a wheat-based RM leads to over- or underestimation of gluten levels, which could be partly prevented by choosing suitable RMs (Huang et al., 2016). Most ELISA antibodies mainly target the alcohol-soluble prolamin fraction. The typical assumption that prolamin makes up 50% of the gluten content is not the case for all cereals, as the distribution of prolamins and glutelins varies depending on the cereal species, cultivar and environmental factors (Hajas et al., 2018). Several types of RM such as recombinant proteins, flours or isolated gluten protein types (GPTs) have been proposed for gluten quantitation using ELISA, HPLC and LC-MS/MS. Recombinant proteins are mainly used in diagnostic assays for the detection of specific immunoglobulins in patients' sera. A number of recombinant wheat proteins were raised against immunoglobulins such as a wheat alpha-amylase inhibitor (Sánchez et al. 2018) or γ-gliadins (Srinivasan et al. 2015). Recently, a recombinant phage single-domain antibody fragment was used in an indirect ELISA to detect gluten in various foods. The antibody fragments were cultured against wheat-, rye- and barley-based foods and the results agreed well with the commonly used monoclonal antibody R5 (García-García et al., 2020). However, recombinant proteins for gluten are still being developed and they represent one single protein each, which is disadvantageous since gluten is a complex protein mixture.

Flours or isolated GPTs are considered to be more suitable as RM than recombinant proteins, because they are more easily accessible, derived from the natural sources and are therefore as similar as possible to the food samples to be analysed. However, the protein composition and content is influenced to a large, but usually unknown extent by both genetic and environmental factors, which should be taken into account when preparing RM from natural sources. In recent years, the influence of the type (flour or isolate) of RM and degree of genetic and environmental variability has been studied. Different natural sources have been investigated such as flours or GPTs, both either based on single cultivars or mixtures of several cultivars of wheat, rye or barley (Schall et al., 2020). To identify suitable cultivars as a basis for RM production, the variation in protein composition of a set of 23 common wheat cultivars grown around the world was determined. According to the results obtained by profound analytical characterization of the flours, five cultivars were found to be suitable for the set RM criteria, which were based on qualitative and quantitative characteristics, e.g., origin and availability of the cultivars, a crude protein content between 12.1 and 15.1%, a typical gluten composition with a gliadin/glutenin ratio between 2.1 and 3.1 and adequate gliadin recovery assessed with two different ELISA test kits (Hajas et al., 2018). Subsequently, genetic variability and the influence of the harvest year on the protein composition was investigated by characterizing these five selected wheat cultivars. According to the ELISA results, the flour mixture of the five cultivars seemed to be the preferable choice to significantly reduce the genetic and environmental effects in comparison to flours of single cultivars (Schall et al., 2020).

Further, GPTs isolated from the flours have been proposed for use as well-defined RMs. GPTs of specific species, such as barley prolamins, proved to be more suitable for R5 ELISA quantitation of barley-based or -contaminated foods, compared to wheat-based RMs, due to the differences in protein composition and antibody specificity to different grain species (Huang et al., 2017). GPTs from flour mixtures of four commonly used wheat, rye and barley cultivars each (ω5-gliadins, ω1,2-gliadins, α-gliadins, γ-gliadins and high- and low-molecular-weight glutenin subunits from wheat, ω-secalins, γ-75k-secalins, γ-40k-secalins and high-molecular-weight secalins from rye, and C-hordeins, γ-hordeins, B-hordeins and D-hordeins from barley) were reproducibly isolated in high purity. It has been shown that they were suitable for calibration of several quantitative methods, including targeted LC-MS/MS and ELISA (Schalk et al., 2017b). The isolated GPTs were fully characterized by discovery-driven mass spectrometry (Lexhaller et al., 2019).

The recent developments emphasized the complexity of gluten due to its heterogeneity and the challenges in finding a suitable RM. There are many factors to consider, in order to establish a suitable RM for gluten analysis, for example, genetic and environmental factors and the different forms of RMs (recombinant proteins, flours and GPTs either from single cultivars or mixtures of several cultivars). Moreover, the solubility and stability of the RM play an important role in the production process and in the consideration as a suitable RM.

5. Immunochemical methods

One of the main strategies used to detect and quantitate allergens are immunochemical methods. In these methods, specific antibodies are used to detect the substance (allergen) of interest. The method known as ELISA (enzyme-linked immunosorbent assay) is actually recommended by the Codex Alimentarius to analyse the presence of gluten in food matrices. Both competitive and sandwich ELISA test kits are used to analyse the presence of gluten in foods (Halbmayr-Jech et al., 2015). The competitive ELISA is suitable to detect small antigens with only one epitope as found in processed foods, while the sandwich ELISA is suitable for intact proteins with at least two epitopes. ELISA is the most widely used method to quantitate gluten in foods, because of its specificity, sensitivity, and suitability for routine analysis and it does not require expensive specialized equipment (Panda et al., 2017). However, ELISA has disadvantages, such as the possibility to identify only certain types of gluten proteins and the possible underestimation of the total gluten content, which leads to divergent results between different ELISA test kits in different types of gluten-based foods (e.g. flour, beer, sauces, bread).

Multiplex ELISAs have been developed to be able to detect different GPTs (e.g., gliadins, deamidated gliadins and glutenins) in the same test using nine different gluten antibodies, each one reacting with its specific epitope. In a study by Panda et al., many products were tested and the results indicated that this type of approach has the potential to define

the protein profile in different processing methods. The limits of detection (LODs) of gluten varied from 0.85 to 2.9 μg/mL (Panda et al., 2017).

Other studies report the lack of compatibility between the results of the quantitation amongst different ELISA kits and different food products (Martínez-Esteso et al., 2017; Rzychon et al., 2017; Scherf, 2017). These studies concluded that results from different test kits were not correlated and that a RM is necessary to ensure better comparability of the results from different test kits.

Due to the aforementioned limitations, new ELISA methods are being developed. A collaborative study was carried out to assess the performance of a new ELISA test kit, called RIDASCREEN® Total Gluten that is based in four monoclonal antibodies, to quantitate wheat, rye and barley gluten in oats. The results of the study confirmed that the method fulfills the criteria determined by the AOAC International (Boison et al., 2018), showing that it is accurate to determine gluten from different grains in oats and oat products (Lacorn et al., 2019).

Associated to immunochemical techniques are lateral flow assays (LFAs), affordable, simple and fast paper-based devices used in many areas to detect the presence or absence of an analyte, such as gluten (Koczula and Gallotta, 2016). Related to gluten detection, the currently available LFAs are able to detect the same antigens as many ELISA tests, but in a faster and more accessible way (Scherf and Poms, 2016).

Other strategies utilizing biosensors or immunosensors have proven to be potential analytical tools regarding gluten detection in foods, with the advantages of speed, ease, sensitivity, specificity and low costs (Hosu et al., 2018). In this case, the antibodies are the bioreceptors used amongst different biosensors. They can be used for the detection of different food allergens simultaneously, even if they come from different matrices. An optical biosensor was developed to detect gliadins in food with an LOD of 0.1 μg/mL (Angelopoulou et al., 2018). Also, an interferometric sensor chip was developed for competitive protein immunoassays that also had an LOD of 0.1 μg/mL for gliadins. It is important to highlight that these LODs correspond to those of ELISA, but sensors are more convenient since they can detect various allergens in a single assay (Jones et al., 2020).

Other assays are focused on the specific detection of gluten-related proteins. A voltammetric immunoassay was reported to detect gliadin contents in the order of nanograms. This assay seems to be appropriate to evaluate the real absence of gluten in foods bearing a gluten-free label (Chekin et al., 2016). An electrochemical immunosensor modified with carbon nanofibers and coupled to a paper platform detected gliadins in different types of flour on a scale of 0.005 mg/kg, 300 times superior to ELISA detection limits (Marín-Barroso et al., 2019). Funari et al. (2017) developed an immunosensor based on quartz crystal microbalances that resulted in a very reliable device with an LOD of about 4 mg/kg and high sensitivity (Funari et al., 2017). These recently developed techniques could be used as easy and cheap strategies to determine gliadins in food samples. Meanwhile, further tests are necessary to prove the reproducibility of the results and assess the overall performance of these sensors.

6. Proteomics-based methods

A promising and powerful non-immunochemical alternative for the detection of gluten in foodstuffs is the proteomics-based approach employing mass spectrometry (MS), which is usually combined with the previous separation using high-performance liquid chromatography (HPLC). The setup for targeted gluten peptide quantitation is a HPLC connected to a triple quadrupole mass analyser (QQQ) using electrospray ionisation (ESI) as ion source. Proteomics-based methods are based on the MS identification of specific gluten peptides. The analysis requires sample preparation that consists of gluten extraction followed by enzymatic digestion resulting in a peptide mixture containing specific marker peptides. These marker peptides can either be immunogenic peptide sequences or peptides specific to individual cereal species (Alves et al., 2018, 2019). Proteomics can therefore be used for two approaches, one is the identification and characterization of cereals and the other is protein and gluten quantitation. A typical proteomics workflow which combines the identification (shotgun) and quantitation of marker peptides with untargeted experiments and targeted experiments, respectively, is shown in Fig. 4.

To identify suitable and specific gluten marker peptides, untargeted MS/MS experiments need to be conducted to generate and characterize a proteomic profile of the digested samples (Colgrave et al., 2017). Databases and bioinformatics tools are used to identify unique gluten marker peptides, which include both sequences that are known to be immunogenic and sequences that are specific to the grain species (Colgrave et al., 2016; Martínez-Esteso et al., 2016). The marker peptides detected using untargeted experiments are used to perform targeted peptide analysis, focusing on the identification of pre-selected peptides, such as immunogenic peptides. One example is the immunodominant 33-mer peptide from α-gliadins that was investigated in 40 modern and old common wheat and spelt cultivars. The results confirmed the presence of the 33-mer in all hexaploid wheat cultivars, making it an important target peptide for future food analyses (Schalk et al., 2017a). The identification of rye-specific peptide markers was established by a comprehensive proteomic analysis of 20 different rye cultivars. Pasquali et al. identified a panel of rye-specific peptide markers which were assessed in several breakfast cereals, snack foods and pseudocereal flours. Rye traces were found in one sample that did not have rye declared on the label (Pasquali et al., 2019).

In order to ensure food safety, the gluten content must be determined. In several studies, labelled internal peptide standards were used to determine the contents of selected peptides. However, as the legal requirement is to declare gluten contents in mg/kg of the product, the quantitation of the peptides alone is not sufficient. A simple conversion of the peptide content to total gluten content is not possible due to the lack of standardised RM. Moreover, the complexity of gluten, the unpredictable amount of total gluten peptides and the partial modification during food processing, leads to differences in the gluten composition. Nonetheless, for quantitation purposes, several approaches have been proposed using external calibration procedures by spiking peptides or peptide mixtures into gluten-free or gluten-containing matrices (Manfredi et al., 2015; van den Broeck et al., 2015).

Schalk et al. presented a new approach where specific wheat, barley and rye marker peptides were quantitated using well-characterized reference GPTs from a mixture of cereal cultivars. The peptide concentration in the respective GPTs was the basis to convert the peptide yields into protein contents. Applied to the quantitation of gluten in raw and processed food samples and compared with ELISA and HPLC methods, this approach led to medium to strong correlations between the results of the different methods. This shows that this procedure still has shortcomings, especially since it requires a great effort in terms of instrumentation, time and expertise (Schalk et al., 2018a, 2018b).

Additionally, many factors strongly influence gluten analysis with MS, such as the selection of enzymes for sample preparation, the choice of the instrumental setup and the discrimination of peptides that have been post-translationally modified. Nonetheless, LC-MS/MS methods offer great potential for the quantitation of gluten in foods due to their selectivity, sensitivity, flexibility and applicability. Therefore, the MS technique is recommended as a complementary method to ELISA. In both cases, ELISA and MS would require a standardised RM and standardised workflow in order to perform a successful and responsible comparison of results. One of the major advantages of proteomics over ELISA is the ability to identify grain-specific gluten marker peptides in either processed foods or non-processed foods. Several reports showed the ability of LC-MS/MS to detect gluten marker peptides of rye, barley and wheat in modified, fermented and hydrolysed foods such as soy sauce, vinegar, beer, wine and cookies in a reproducible manner (Liao et al., 2017; Panda et al., 2017; Li et al., 2018; Schalk et al., 2018b).

Furthermore, Henrottin et al. have recently developed a semi-quantitative multi-allergen and grain-specific UHPLC-MS/MS method

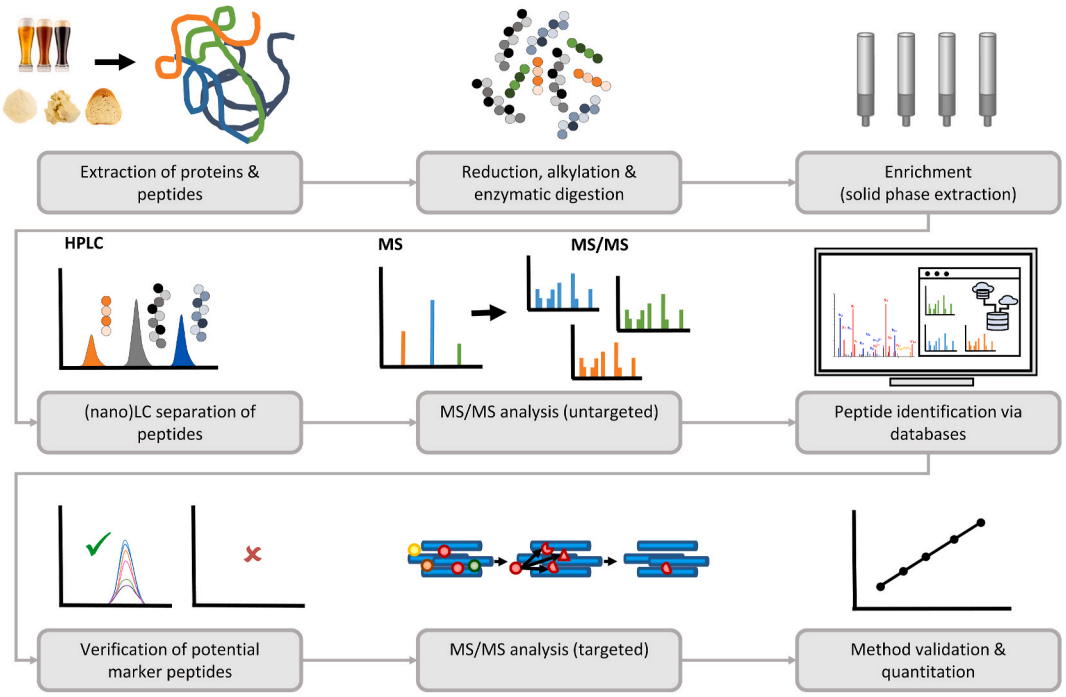

Fig. 4. Proteomics workflow. Schematic proteomics workflow combining untargeted and targeted liquid chromatography tandem mass spectrometry (LC-MS/MS). Proteins are extracted from the matrix, followed by reduction, alkylation and enzymatic digestion, which produces individual peptides. After enrichment, the peptides are separated utilizing liquid chromatography (LC). In order to identify marker peptides, untargeted analysis is performed in combination with peptide identification using databases. Potential marker peptides are verified by tandem mass spectrometry to set up targeted analyses. The marker peptides in the samples are then quantitated using suitable calibration methods.

that allows the detection of seven cereals simultaneously with ten other allergens, which can be used for the analysis of a broad range of food products to ensure food safety (Henrottin et al., 2019). Regardless of the difficulties, proteomics-based methods bring us closer to a better understanding of the complex gluten protein in terms of molecular and structural characterization as well as identification of new immunogenic peptides (Comino et al., 2016).

7. Genomics-based methods

The presence of wheat, rye and barley can be sensitively screened with genome-based methods such as polymerase chain reaction (PCR). The method is based on the amplification and detection of either gluten-encoding or species-specific regions of the genes. Quantitative PCR methods such as quantitative real-time PCR (rt-qPCR) show higher sensitivity and specificity compared to ELISA (García-García et al., 2019). This is due to the ability to rapidly amplify and detect specific DNA fragments at low concentrations. The amplification of a specific gene region is only possible when suitable primers are used. Different gene regions of the DNA are suitable as targets. The use of mitochondrial DNA as a target, e.g., led to sensitivity improvement of gluten-rich cereals and processed foods. Two DNA regions were selected, one of which is present in wheat, rye and barley whereas the other one is specific for each grain. Thus, very low detection limits of about 0.2 pg DNA could be achieved for DNA purified from wheat, rye or barley (Ahmed and Meng, 2019).

In rt-qPCR, the amount of amplified product genes is monitored in real-time during the exponential phase of the reaction with fluorescent dyes such as SYBR Green or TaqMan. This methodology was currently used by Garcia-Garcia et al. for the screening of gluten-containing cereals, using the ribosomal internal transcribed spacer regions (ITS) of wheat, barley and rye as targets. To evaluate the method, 220 food products were tested with the in-house validated method and compared with the results of the R5 ELISA. The validation was based on serial dilutions of reference wheat-barley-rye mixtures, which were separated into three treatment groups, one untreated and two heat-treated (160 °C for 13 min and 200 °C for 20 min). The theoretical concentrations of the target species in the samples were calculated using a semi-logarithmic correlation. The results showed good comparability with ELISA results and an overall practical limit of detection of 10–50 mg/kg of wheat, rye and barley, depending on the applied processing treatment (García-García et al., 2019). Comparable detection limits of 50 mg/kg of wheat, rye and barley in reference mixtures, targeting gliadin sequences were reported previously (Garrido-Maestu et al., 2018).

However, despite the low DNA detection limits, PCR methods show their limitations in highly hydrolysed and processed food samples, which are subject to DNA degradation. In addition, the food matrix affects the results which should be considered when developing rt-qPCR assays (Martín-Fernández et al., 2016). It should be noted that DNA-based analysis is an indirect method compared to immunochemical or MS detection of the gluten proteins directly. Therefore, genomics-based methods can only draw limited conclusions about the true gluten protein content in supposedly gluten-free products. Nonetheless, genome-based analyses are necessary and well known for screening and confirmatory tests for the presence of certain cereal species, which can be used to complement other analytical methods. For instance, new target genes coding for certain gluten protein types were identified by combining genome-based studies and proteomics using bioinformatics approaches (Wang et al., 2017).

8. Further methods

In recent years, different and new methods for the analysis and detection of gluten have been developed based on a wide variety of approaches such as binding assays with aptamers or electrochemical methods. Aptamers have been used as alternatives to antibodies for the selective determination of gluten in foods. They are short single-stranded oligonucleotide sequences (DNA or RNA) or peptide

sequences designed to bind to a specific target molecule or ligand. Since they target specific biological structures, they are also called "chemical antibodies". The production of aptamers is based on the *in vitro* selection, also referred to as systematic evolution of ligands by exponential enrichment (SELEX) (Miranda-Castro et al., 2020). Compared to antibodies, aptamers can be produced faster and show higher thermal and chemical stability. Furthermore, they can be easily modified with functional groups, which can easily be incorporated into aptamer-based assays. A fast and reliable label-free impedimetric aptasensor for gliadin detection was reported, showing a detection limit of 5 mg/kg and comparable results with R5 ELISA assays (Malvano et al., 2017).

A set of sensors for gluten detection was created using immunological assays with floating gate transistors (FGT) biosensing technology. For sensing biomacromolecules such as DNA and proteins, this technology is based on a potentiometric transducer, which combines electronics and microfluidics. Wheat and barley grains were tested with three fabricated sensors, which were functionalized with aptamer-33mer, wheat G12 antibody and barley antibody. The tested sensors were able to detect gluten below the gluten-free limit of 20 mg/kg. The prototype sensor showed several advantages over the commercial ELISA kits such as reduced analysis time of about 45 min, lower material costs and the ability to differentiate between different gluten sources such as wheat and barley (White et al., 2018).

Another potential approach to detect gluten in flours using surface modified nanoparticles in combination with electrochemical determination was presented recently (Limthin et al., 2019). Therefore, a gluten-sensitive receptor was designed using the molecular imprinted polymers (MIP) technique, which allows the formation of a complex polymeric cavity with functional monomers using a template, which is gluten in this case. In this study, magnetic MIPs were used to enable the analytic detection of an electrochemical signal by an external magnetic field. Different flour samples were tested for the gluten-MMIP resulting a limit of detection of 8.5 mg/kg (Limthin et al., 2019).

9. Future perspectives and conclusions

There are clear discrepancies in legislation concerning the labelling of gluten-free products in the different countries or regions as well as the respective prerequisites. According to the Codex Alimentarius, it is mandatory to declare gluten-containing cereals as part of the ingredients list in prepacked foods. Gluten-free claims should follow the Codex Standard threshold of 20 mg/kg, but some countries only regulate gluten-free labelling based on the use of ingredients containing gluten or not, without respecting a threshold. On the other hand, some countries and regions (i.e., European Union) possess a licensing system including traceability and quality control certificates, providing specific and useful information to guarantee product safety for CD patients. While official legislation in the USA and Canada also states the gluten-free labelling threshold of 20 mg/kg, gluten-free certifications are provided by different associations that adopt different levels varying from 5 to 20 mg/kg. Australia and New Zealand have implemented the lowest gluten level (3 mg/kg) related to gluten-free claims. This strict approach is being discussed as analytical methods for gluten trace detection and quantitation become more and more sensitive, because it will become increasingly difficult for manufacturers to make a gluten-free claims. This is bound to limit the availability of products for CD patients, most likely unnecessarily.

The ELISA R5 Mendez method still remains the only method recommended by the Codex for gluten analysis to establish the accepted value. With other ELISA test kits being available on the market using different antibodies, this unique position of the ELISA R5 Mendez method is being challenged. However, comprehensive collaborative studies involving at least ten laboratories, well-defined wheat-, rye- and/or barley-contaminated samples and at least two different gluten ELISAs are missing that allow a clear answer as to whether different gluten ELISAs classify the same products of various matrices as gluten-free or not. Despite the great suitability for routine analysis, the gluten content determined by ELISA may be underestimated especially in samples that contain heated or partially hydrolysed gluten.

Different approaches have been proposed to overcome the drawbacks of gluten extraction and quantitation methods, regardless of whether ELISA, LC-MS/MS or other methods are used. The various strategies for gluten protein extraction show that the selection of protocols can drive the enrichment in different functional classes of proteins, affecting the biological interpretation. Therefore, it appears that multiple strategies of protein extraction and proteolytic digestion will lead to improved protein identification, since no single method appears to be universally applicable to all classes of proteins, cereals and processed food products. There is some advancement in the use of sustainable protocols, but it still remains unclear if alternative solvents such as DES are consistent as a medium for gluten extraction followed by characterization based on MS approaches.

The need of an appropriate RM is a unanimous requirement to improve methods of extraction, validation and analysis of gluten from wheat, rye and barley. To date, blends of cultivars seem to be preferred for RM compared to single flours or recombinant proteins, because they take the stability of protein composition, genetic and environmental effects into account. For long-term stability, providing protein isolates seems to be the best choice, because the PWG-gliadin RM has shown excellent stability over a period of almost 20 years so far.

Genomics- and especially proteomics-based methods have substantially increased our understanding of gluten and cereal proteins and possible modifications. LC-MS/MS in its versatile forms is the most promising technique not only for the quantitation of gluten trace levels, but also for studying factors affecting gluten composition and immunoreactivity. However, the equipment still remains a huge investment in terms of costs and expertise, that limits its widespread adoption in routine settings. One important point for LC-MS/MS methods is still the calculation of gluten contents based on the concentrations of marker peptides determined. Although different approaches have been published recently, all of these had specific drawbacks and also require appropriate RM.

Altogether, all methods discussed in this review are complementary to each other, because all of them have specific advantages and disadvantages. The choice of the preferred method depends on the food product to be analysed, the analytical question, the sensitivity and specificity required and time and cost considerations. With continuous progress in analytical method development, more efficient and simple sample extraction, preparation and measurements procedures will be offered that allow the analysis of large numbers of samples in short periods of time. As more and more methods emerge, method validation, proficiency testing and appropriate calibration will become increasingly important together with recommendations from legislative and standardisation bodies.

CRediT authorship contribution statement

Majlinda Xhaferaj: Writing - original draft, Visualization. **Thais O. Alves:** Writing - review & editing. **Mariana S.L. Ferreira:** Writing - review & editing. **Katharina Anne Scherf:** Writing - review & editing.

Declaration of competing interest

None.

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