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Accuracy of ELISA Detection Methods for Gluten and Reference Materials: A Realistic Assessment

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ABSTRACT: The determination of prolamins by ELISA and subsequent conversion of the resulting concentration to gluten content in food appears to be a comparatively simple and straightforward process with which many laboratories have years-long experience. At the end of the process, a value of gluten, expressed in mg/kg or ppm, is obtained. This value often is the basis for the decision if a product can be labeled gluten-free or not. On the basis of currently available scientific information, the accuracy of the obtained values with commonly used commercial ELISA kits has to be questioned. Although recently several multilaboratory studies have been conducted in an attempt to emphasize and ensure the accuracy of the results, data suggest that it was the precision of these assays, not the accuracy, that was confirmed because some of the underlying assumptions for calculating the gluten content lack scientific data support as well as appropriate reference materials for comparison. This paper discusses the issues of gluten determination and quantification with respect to antibody specificity, extraction procedures, reference materials, and their commutability.

KEYWORDS: *gluten, gluten-free, detection methods, ELISA, validation, reference material, action levels, European Regulation, Codex Alimentarius*

INTRODUCTION

Gluten intolerance, also called celiac disease or celiac sprue, affects about 1% of the population in occidental countries, and this value is thought to be underestimated.[1−3] This disease is chronic, and there is no effective treatment available other than avoiding gluten in the diet. What is gluten? This is a single term that refers to a very complex group of proteins present in cereals and insoluble in water.[4,5] Only gluten from wheat, rye, and barley has been proved to be toxic to celiac patients. Although oats are tolerated by the majority of celiac sufferers, there are a number of studies showing contradicting information about the toxicity of oats.[6−13] Oat toxicity seems to be linked to specific cultivars[13−15] and also to contamination with other cereals (wheat, rye, barley).[16,17] Avoidance of gluten is the only strategy to prevent or minimize the development of symptoms in the celiac population.[18] Gluten proteins are classified differently depending on different criteria, for example, chemical composition or solubility.[19−23] Depending on protein solubility in alcohol or alkaline/acid solutions, gluten proteins are grouped as prolamins or glutelins.[22,23] These two gluten fractions acquire different names depending on the source of gluten. For example, prolamins from wheat are called gliadins, those from rye are named secalins, and hordeins are the prolamins from barley. Glutelins from wheat are called glutenins.

The European Union (EU) has enacted Commission Regulation 41/2009 that defines the term "gluten-free" for labeling purposes allowing affected consumers to identify those products that are safe to consume.[24,25] The European Regulation, which is based on Codex Standard 118, set a limit of 20 mg gluten/kg product as a threshold, below which the gluten-free label in food products is allowed. The existence of action levels requires reliable analytical methodologies to ensure compliance. Assay reliability depends on the availability of suitable (fit for purpose) reference materials.

Unlike reference materials for contaminants, where the target is a single, and in many cases, small molecule, the selection of reference materials for gluten is a challenging task for several reasons: (1) gluten contains numerous large proteins that can be potential targets for detection methods;[4,5] (2) these proteins are physicochemically different, although some of them share a high degree of homology; (3) the expression of these proteins is variable and depends on cereal cultivar and growing conditions; (4) because gluten-containing flours or ingredients are used in food production, their structure and solubility are subject to modifications during food manufacturing, for example, protein aggregation during baking and pasta processing.[4,26,27]

As simple as it sounds, any gluten preparation could be a potential reference material, and there are some companies that claim to be providers of reference materials (RM) or certified reference materials (CRM) for gluten. A proliferation of numerous gluten preparations sold as RM or CRM would not be beneficial to the analytical community, which is looking forward to a common basis for assay calibration and validation. Moreover, these materials need to meet a number of criteria and, because of the complexity of gluten, a consensus on this material is practically a requirement. The material needs to be fit for purpose. The following is a good example to clarify what this means. The NIST whole egg SRM 8415 had been used for the validation of ELISAs for egg allergens until some studies

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showed that it was not a suitable material due to decreased solubility of the egg proteins, which were the assay targets.[28] This material was not produced to be used in egg allergen assays but for the analysis of minerals. In response to this issue, the food allergen analytical community identified and characterized a whole egg material that is deposited in NIST with the code RM 8445, and it is recommended to be used in the validation of egg ELISA kits.[29] Solubility issues of gliadin preparations have also been described.[30,31] The production of a proper material needs to ensure the stability and solubility of the targets of interest. Important criteria for the selection of a reference material for gluten are its purpose, composition, aspects corcerning its production, characterization, and storage conditions. The process needs to undergo an independent evaluation process and should be ideally available from recognized standardization organizations such as NIST or IRMM. The Prolamin Working Group (PWG) gliadin is a well-characterized material produced for use in antibody-based assays.[32] This is a consensus material produced after the careful selection of grains and establishment of more adequate purification and characterization processes. This material was proposed as reference material but it had not gained this status for numerous reasons, including the lack of guaranteed reproducibility and limited commercial availability. The material is available from the acting chair of the PWG.

With the establishments of action levels for gluten, the number of detection methods on the market, mainly antibody-based methods (i.e., ELISA and lateral flow devices), has increased in the past few years. Detailed information on existing and upcoming detection methods for gluten has been covered by recent review papers.[33,34] Quantitative ELISAs differ not only in their design and specificity but also in the results they provide for the same sample. To help deal with this variability and to ensure accurate analytical results, a gluten reference material is urgently needed. This material should cover the needs of as many existing detection methodologies as possible.

BACKGROUND CONSIDERATIONS: FROM PROLAMIN TO GLUTEN

Historically, on the basis of the initial knowledge of gluten toxicity, the prolamin fraction has been reported to be the unique gluten fraction responsible for the toxicity of cereals in celiac patients, and very little attention has been paid to glutelin fraction or gluten as a whole.[35] Consequently, the search for reference materials has been limited to gliadin, with very little consideration to the use of gluten and other cereal sources (rye and barley). Wheat gliadin or gliadin peptides have been used to raise most of the antibodies used in commercial assays such as the monoclonal antibodies Skerritt,[36,37] G12,[27] and α20.[38] The exception is R5 produced from rye secalins.[39] Gliadin has also been used to calibrate and validate ELISAs.[37,40−42] The most pursued material for assay calibration, evaluation, and calibration is the PWG gliadin.[32] However, science has evolved, and additional studies, although still limited in number, are showing that the glutelin fraction may also contribute to the overall toxicity of gluten.[43−46] In addition to this, studies are bringing a more in-depth knowledge about existing and new detection methods for gluten. More current information opens new opportunities for improvement of assays and formulation of new analytical strategies, which also include the selection of more appropriate gluten reference materials.

FIT FOR PURPOSE GLUTEN REFERENCE MATERIALS: ANALYTICAL CONSIDERATIONS

Detection methods for gluten can be used for different purposes, including compliance and enforcement activities. To ensure that the analytical tool is used properly, it is necessary to ensure that the interpretation of regulations is accurate and not misleading. There is a frequent misinterpretation pertaining to the units of the action level, which is set by the Codex Standard and the EU Regulation: the labeling threshold is 20 mg/kg of gluten, not 10 mg/kg prolamin, which is frequently and wrongly set as equal. This misinterpretation occurs because prolamin is measured by most ELISAs, which then needs to be converted into gluten by simply multiplying the prolamin content by a factor of 2. However, reporting results as prolamin content is compliant neither with the existing European Regulation nor with the Codex Standard.

It would be practical to have a reference material for gluten that is commutable (e.g., material used by industry) and had a broad scope, or, in other words, a material that not only covers the needs of one assay (antibody specificity) but also can be used by as many as existing detection methods as possible and also allows development and validation of new technologies that are already showing a relevant potential in the field, such as mass spectrometry. Because most of the commercial detection methods are antibody based, the majority of comments in this paper focuses on ELISA.

Because the reference material would have to be used with already existing commercial detection methods, it is important to understand the characteristics of existing methods, from extraction to results. It is also critical to identify the gaps associated with these methods, which, in some instances, are related to the lack of a suitable reference material. And last but not least, it is necessary to consider new information resulting from recent studies, which are helping the community to better understand and improve the analysis of gluten and ultimately obtain more accurate gluten values. None of this information should be ignored.

Sample Extraction Buffer. Traditionally, aqueous alcohol solutions have been used in gluten detection methods under the consideration that they extract the prolamin fraction only. There are a few comments that need to be made in this respect: (1) Aqueous alcohol solutions can also extract monomeric glutelins mainly in raw ingredients, such as flours;[27,47] (2) Alcoholic solution is effective in extracting prolamins in nonprocessed ingredients (such as flours). However, gluten proteins aggregate due to the formation of disulfide bonds during processing,[26,27] and alcohol solutions are not able to break these bonds, which is necessary to solubilize prolamins, rendering poor protein recoveries.

The need for improved sample extraction methods led to the development of new extraction solutions containing reducing agents such as 2-mercaptoethanol and tris(2-carboxyethyl)-phosphine, which in combination with alcohol extraction are capable of dissolving gluten aggregates.[31,48−50] In addition to improving the solubility of prolamins in processed products, these extraction solutions also reduce disulfide bonds of polymerized glutelins, rendering them also soluble.[4,51] These extraction approaches open new possibilities for detection because food samples contain gluten (not only prolamins but also glutelins). However, a big change in the detection methodology, such as a drastic change in the extraction step, requires the re-evaluation of assays. Now that the new extract

contains additional proteins (glutelins) compared to aqueous alcoholic extracts, there is the need to evaluate the potential binding of the assay antibodies to the glutelin fraction. There is no scientific foundation to simply assume that antibodies do not react with glutelins, considering that some of these proteins share some amino acid sequences with the prolamins.[4] Moreover, the reducing and denaturing agents used with the new extraction solution may affect to some degree the antibodies, which are also proteins; therefore, an assessment of antibody/assay stability is also needed.[52]

Assay Target and Antibody Specificity. The majority of assays commercially available are based on monoclonal antibodies, that is, R5,[53] Skerritt,[36,37] G12,[54] and α20.[38] The specificity of these antibodies is evaluated mainly against wheat gliadins. Some have evaluated antibody specificity more in-depth, focusing only on known toxic peptide fragments present in wheat gliadins with limited considerations to the reactivity of these antibodies to other areas of the same protein, other prolamins, or glutelins of wheat, rye, or barley. There was a demand, especially by the clinical community, suggesting only toxic structures in prolamins, such as the 33-mer from α-gliadin,[55] as the only option for assay specificity, validation, and calibration of gluten detection methods. However, at the time, this community did not have newer evidence showing the potential importance of glutelins in the development of celiac disease, as mentioned above. The lack of scientific evidence demonstrating that assays do not react against the glutelin fraction of gluten introduces a high degree of uncertainty and potential assay inaccuracy. This type of assumption lacks supporting scientific data. Moreover, it should not be forgotten that gluten detection is not limited to wheat. A more complete characterization of assay specificity to all gluten fractions, not only from wheat but also from barley and rye, should be carried out at early development stages to avoid surprises once the assay is already on the market.

Below is a brief description of the main antibodies used in existing commercial assays as well as some of their characteristics.

R5 is a monoclonal antibody raised against rye extracts.[53] The main R5 epitopes have been evaluated by phage display and pepscan studies[56] as a further evaluation of the antibody reactivity was assessed against the amino acid sequences of the N-terminus of wheat α-gliadin.[57] This terminus is known to be toxic to celiac patients.[39] QQPFP, QQQFP, LQPFP, and QLPFP have been identified as the strongest target epitopes and are present not only in α/β-gliadins but also in the γ-type.[57] There is limited information on the reactivity of the antibody against other regions of the same gliadin, other gliadins, or glutenins. Some of the R5 epitopes are also present in wheat LMW glutenins, which are related to α/β- and γ-gliadins not only in molecular weight but also in amino acid composition.[4] Although some studies have shown that R5 does not bind HMW glutenins, they have demonstrated its reactivity to wheat LMW glutenins, which should not be ignored.[27,49,51]

R5 immunoassays have been adopted by Codex Alimentarius as Type I methods for gluten, even though the assay is officially described to detect only prolamin, a fraction of gluten. As discussed below, converting prolamin content into gluten is not straightforward and can lead to assay inaccuracy.

G12 is also a monoclonal antibody, and it has been raised against the synthetic 33-mer toxic α-gliadin peptide, QPQLPY being the most reactive epitope.[54,58] In addition to wheat, rye, and barley prolamins, this antibody has been shown to also bind prolamins from some oat cultivars. A study evaluating the reactivity of G12 to oat cultivars has shown that the antibody binds those that have shown toxicity in in vitro studies.[15] This characteristic of G12 has polarized opinions between those who think that an assay for gluten should detect only gluten from wheat, rye, and barley, but not oats, and those who see this as an opportunity to also protect the small percentage of the population sensitive to oats.

The monoclonal antibody 401.21, better known as the Skerritt antibody, is the oldest of the antibodies mentioned in this paper. This antibody was used in some commercial ELISAs until some disadvantages became known and new developments led to the production and use of other antibodies and newer analytical approaches. The Skerritt antibody was originally reported to be specific to ω-gliadins.[36] However, a few research studies have further evaluated the specificity of this antibody, concluding that it is not only specific to ω-gliadin but also binds strongly to HMW glutenins.[27,51,59,60] This fact needs a serious re-evaluation to the entire Skerritt analytical methodology. There is an important aspect to highlight regarding this discovery: (1) The aqueous alcohol extraction solution used in Skerritt assays is not appropriate because it is not efficient in extracting Skerritt main targets (glutenins). A solution containing a reducing agent would be more suitable. (2) Gliadin preparations, lacking glutenins, should not be used for calibration or validation of the assay Skerritt-based assay because the main target is missed (this is particularly relevant if reducing agents are also used). Obviously, a preparation containing glutelins would be more appropriate. It is a common practice that laboratories compare the performances of different assays.[30] In these studies, samples containing known amounts of gliadin are analyzed by the kits. The assays are run using their respective extraction solutions, that is, cocktail (with reducing and denaturing agents) and aqueous alcohol. Given the facts explained above about the true specificity of the Skerritt antibody, it is not difficult to predict that Skerritt assays do not perform well in this type of study.

Commercial assays are not limited to monoclonal antibodies. There are also ELISA kits using polyclonal antibodies (Morinaga). Antibodies included in this kit are raised against a commercial gliadin preparation. Not much information about this kit is available in the scientific literature. Sharma found that the assay is mostly specific to gliadins.[59]

Cross-Specificity of Commercial Assays to Rye and Barley. There is sufficient evidence that rye and barley are also toxic to celiac patients, but there is limited information about whether the toxicity of rye and barley gluten is equivalent to that of wheat one to one. Comino et al. have found significant differences in the toxicity among wheat and barley cultivars.[14,61] Action levels for gluten-free, 20 ppm, are based on the toxicity of wheat, and there is a nonscientific assumption that the same concentrations of rye and barley are more, less, or equally safe to celiac patients compared to wheat gluten. A question that remains open is whether or not the use of wheat as reference material or assay calibrators is suitable to determine gluten from rye and barley. The gluten composition and the ratio between prolamins and glutelins of rye and barley gluten are different from those of wheat,[31] and it has not been well studied how these differences affect the detection of gluten from different cereals. Kanerva et al.[62] evaluated the ability of R5 assays and one anti-ω-gliadin kit to quantify barley. They used different calibrators, those provided by the kits and a barley material. The study concluded that when using the calibrator from the

kit (from wheat sources), R5 overestimated the concentration of barley and the ω-gliadin kit underestimated it at high concentrations. However, R5 values were closer to the expected values when using the barley hordein standard.

A recent study conducted by the group of Koehler (available online as of November 1, 2012: http://www.wgpat.com.ar/proceeding_24th.html) shows the performance of commercial assays ELISA-Systems (Skerritt), Tepnel (Skerritt), Morinaga (polyclonal antibodies), and R-Biopharm (R5) to purified prolamin and glutelin fractions from wheat, barley, and rye.[63] In addition to antibody specificity, it is important to highlight that each assay was carried out using the extraction solutions recommended by the manufacturers, which vary from aqueous alcohol to two solutions containing different reducing and denaturing agents. Calibration procedures and assay calibrants are also different. Although all of these differences make comparisons practically impossible, the authors set the results for each prolamin fraction (wheat, rye, and barley) analyzed by the R5 kit as reference values for comparison purposes. In other words, all prolamins analyzed by the R5 kit were set to 100%, and the results provided by the other kits are shown as a percentage relative to each prolamin (wheat, barley, and rye) as analyzed by R5. The manner in which the results are reported does not allow evaluating assay responses of rye and barley gluten fractions relative to wheat. Irrespective of how data are presented, it is interesting to note that all of the assays could detect the glutenin fraction. Although the purified glutelins used in the study may have contained residues of prolamin, it would not be surprising to find that some signal is due to the actual cross-specificity of the antibody to the glutelin fraction. As mentioned above, at least in the case of wheat, gliadins and glutenins, LMW glutenins in particular, share some amino acid sequences (such as QQPFP, one of the R5 epitopes). The relative response for wheat glutenins (with respect to prolamins) is over 10% for all ELISA kits tested. More interesting is the case of rye, where the relative response of R5 and Skerritt assays to the glutelin fraction varies between 40 and 55% (about 5% in the case of the assay using polyclonal antibodies).

In the same study the group of Prof. Koehler evaluated different commercially available gliadins and gluten preparations. Of all four assays, results obtained by the R5-based ELISA showed the higher variability for both the gliadin and gluten materials tested. Results varied up to 2 times in the case of gliadins (56.3−100%) and up to almost 3 times in the case of gluten (100−272%). Significantly less variability was shown by the only assay using polyclonal antibodies, where the relative results varied from 97 to 124.6% in the case of gliadin and from 103 to 133.6% for all gluten samples tested. This assay seems to be more appropriate to determine gluten content in foods because it provides similar results regardless of the gluten used. Real food samples contain gluten, not fractions of gluten and not PWG gliadin. Good reference materials are commutable, meaning they are as close to real products as possible. Here, a simple flour would be significantly more suitable than any gluten fraction or PWG gliadin. The accuracy of results provided by R5 assays may be compromised when real food samples are analyzed, considering that the responses of this kit vary significantly among sources of gluten. This is an example of how an assay showing good performance in single- and multilaboratory studies (using PWG gliadin for validation and calibration) may underperform with real samples.

What does this information offer the analytical community regarding existing commercial assays and needs? (1) Wheat, rye, and barley cannot be consider equal. (2) Assays may not necessarily be specific only to the prolamin fraction. (3) Some assays are more sensitive to different sources of gluten and gliadin than others.

Conversion Factor of 2. Another analytical element required by analytical methods specific to prolamins is the conversion factor to convert gliadin (or prolamin) content into gluten concentration to comply with the units established with Codex Standard 118 and the European Regulation. This factor, defined in the Codex Standard only, is set to 2, and it is derived from the understanding that gliadin is half of gluten proteins, the remaining half being the glutenin fraction. This value has been the center of controversy because the ratio of prolamin to glutelin varies among different cereals (wheat, barley, and rye) and within a cereal depending on the cultivar and growing conditions. Wieser and Koehler[64] determined the prolamin and glutelin contents of several cultivars of different cereals and reported a high variability in the ratio prolamin to glutelin. With the exception of starch samples, all of the estimated conversion factors were below 2. The factor ranged from 1.32 to 1.66 for wheat, from 1.12 to 1.16 for rye, and from 1.20 to 1.71 for barley. Taking into consideration this new piece of information, the direct implication of using a factor of 2 is an overestimation of the gluten content in food samples (without considering the effects of food processing in gluten detection).

Another aspect regarding the validity of the conversion factor has to do with the specificity of the assay. The factor is only applicable in those cases when the assay is specific exclusively to prolamins. Results are overestimated in assays that also detect glutelins. As we have seen earlier in this paper, some of the assays are specific to glutelins, such as Skerritt ELISAs and also R5 (glutelins from rye and possibly to LMW glutenins).

■ ASSAY VALIDATION IN CONTEXT

The analytical community needs to be critical with existing validation reports and studies showing comparative evaluation of ELISA kits for gluten. Assays such as R5 ELISAs have been validated in several multilaboratory studies.[40,41] This assay has been built, calibrated, and validated around the PWG gliadin, and therefore it is expected to provide good performance results. However, the assay has not been developed for real-life conditions where foods contain the whole gluten (not only gliadins). This assay was presented as the only alternative to the Skerritt assay in the late 1990s. In the absence of other commercial ELISAs, the R5 ELISA and the PWG gliadin were adopted as reference method and gliadin material with basically no opposition and no questions. The majority of new ELISA developments have been validated using the PWG gliadin, and their performance is, by default, compared to the R5 method. It seems that in these comparative studies only results matching those of R5 are considered to be acceptable. As already mentioned, an assay validated in multiple multilaboratory studies and always under the same conditions does not necessarily guarantee a good performance in the commercial product samples, where analytical conditions (matrices and gluten sources) are so variable. With regard to these samples, it is not uncommon that results are reproducible but not accurate, and this is not only the case for gluten, as it also happens with detection methods for other analytes such as food allergens. As we have explained above, there are still questions regarding the accuracy of the Codex type I R5 method. The fact that newer

assays provide results different from R5 may not necessarily indicate that they are unreliable. With the number of unknowns linked to ELISA methods for gluten, it is difficult to say which assay is more accurate or a better performer.

REFERENCE MATERIAL FOR HYDROLYZED GLUTEN

Some food products contain hydrolyzed gluten proteins, such as beers. The selection of proper reference materials for hydrolyzed gluten is even more complicated than reference material for intact gluten proteins. There are additional factors to consider in addition to the variability of gluten sources and gluten composition. The peptide profile of hydrolyzed gluten proteins depends on the type of hydrolyzation, extent of hydrolysis (which may lead to either long or very short peptide fragments or a mix), and enzyme cleavage points. Moreover, the difficulties of converting hydrolyzed peptide concentration in true gluten content make the quantification of gluten in food samples quite challenging.

Competitive ELISA is more suitable than sandwich ELISA for the detection of hydrolyzed gluten because it requires only one antibody binding epitope instead of two, allowing the detection of smaller peptide fragments. Examples of competitive assays are α-20 and R5 ELISAs. Some commercial assays, such as G12 ELISA, have been evaluated to determine hydrolyzed gluten in hydrolyzed products such as beer.[48,65−69] The assay format is not only an important factor in the analysis of hydrolyzed gluten, but calibrator materials also play a relevant role. A commercial competitive ELISA based on R5 was developed to determine gluten content in hydrolyzed products.[69] This first generation of competitive R5 used gliadin peptides as standard and peptide equivalents as a unit of concentration, which was meaningless in terms of providing gluten content as required by the Codex Standard and EU Regulation. In the second generation of the R5 competitive assay, the calibrator has been changed recently to a more realistic approach using a hydrolyzed material composed of a mix in equal parts of hydrolyzed PWG gliadin amd purified prolamins from rye and barley.[68,70] In this case it is possible to report results as the concentration of gluten. Although reports have shown that this assay is more robust than the previous competitive assay and more suitable than the counterpart sandwich ELISA for samples containing hydrolyzed gluten, the accuracy of the assays is still questionable for the reasons mention above, that is, food samples containing gluten hydrolyzed with different enzymes and to different degrees. Another question to ask with respect to celiac patients is: what is the fragment size (or class) recognized by the antibodies that still triggers an effect in celiac patients? Would antibodies in competitive assays also recognize smaller fragments that no longer trigger celiac disease but still contribute to the ELISA signal, thereby preventing the labeling of suitable products as gluten-free?

DECISIONS FOR REFERENCE MATERIAL

It is clear that the selection of a reference material for gluten is everything but an easy task. As has been discussed here, there are many aspects that still need consideration and additional research.

The composition of a suitable reference material for gluten analysis should be in line with regulatory requirements or Codex Standard. Many argue that known toxic peptides from the prolamin fraction should be the only target of analytical methods for gluten, and therefore gliadin or the prolamin fraction would be the most appropriate reference material. They use the Codex Standard as a basis to justify what they believe is the best assay specificity and the most appropriate composition for the reference material. This is only a misuse and a misinterpretation of the Codex Standard. The Standard states that "the antibody used should react with the cereal protein fractions that are toxic for persons intolerant to gluten". This statement specifies as assay targets neither peptides, nor specific proteins, nor prolamins, nor glutelins, but "toxic fractions". The European Regulation does not suggest any detection methodology or mention targets other than gluten as the unit for action levels. The section of the Codex Standard on analytical methodologies does not exclude the use of nontoxic peptides or peptides/proteins of unknown toxicity that are part of a toxic gluten fraction. The Codex Standard does not limit analytical options, leaving open the number of opportunities for analytical strategies regarding assay development and reference materials.

Perfect reference materials for gluten do not exist and it is very likely that they never will. What would be the proper reference material? Probably there is not a single answer to this question.

(1) A material used in detection methods should contain all of the assay targets and should be commutable. As discussed above, gliadin (the prolamin fraction) is not the only target of antibodies currently used by some commercial assays. As mentioned above, the Skerritt antibody is also specific to the glutelin fraction, in addition to ω-gliadin. Skerritt and R5 antibodies have also shown to be specific to the glutelin fraction of rye.[63] Consequently, a material containing gliadin only is not suitable to calibrate and validate this assay; the material should contain glutelin as well.

Because the use of reference materials based on only one gluten fraction, prolamins or glutenins, may be of limited use to those assays specific for that fraction, an alternate approach would be to select and characterize a gluten material, that is, purified gluten or flour with characterized gluten content. Using a material of narrow scope having a single prolamin or glutenin fraction would be useful only to the assays specific for those fractions, leaving the rest of the assays still waiting for proper reference materials. One additional advantage of using purified gluten or flour is that the conversion factor would not be necessary. In addition, the selection of flour material would be a better representation of a food industry ingredient and meet demands for commutability.

(2) There are multiple cereal sources of gluten. Until now the materials used have been based on wheat gliadin. The PGW gliadin is derived from 28 different European wheat cultivars.[32] Materials like this are difficult to reproduce because the composition of gluten varies from season to season depending on weather and soil conditions. However, a material made of a single cultivar is also subject to the same limitations. A material could also include a mixture of wheat, rye, and barley. Although it sounds ideal to have a representation of all celiac toxic grains in a single preparation, it may be of little use considering the differences in response of each assay to the three grains. It would be more practical to have three reference materials, one per grain, that could be used to evaluate assay response to the individual grains and also allow for customized mixtures and evaluation of existing and new assays.

Somehow related to reference materials, and also relevant in testing, is the availability of suitable negative controls. Corn and rice could be among the potential candidates. However, criteria for selection and characterization also need to be developed.

CONCLUSIONS

There is an urgent need for gluten reference materials and more accurate analytical methods. Perfect reference materials for gluten do not exist, and perhaps unique solutions (i.e., a single analytical approach or unique reference materials) are simply not viable. However, decisions will need to be made on the basis of consensus regarding the criteria for selection and characterization of gluten preparations. Broad scope materials such as flours or, alternatively, purified gluten offer all potential gluten targets, not limiting analytical options. Moreover, unlike gluten protein fractions, the use of flours is a more practical approach because it may be more stable than purified proteins and it is a very common ingredient used by the food industry. The selection and characterization of individual flours from wheat, barley, and rye, which could be used independently or mixed, could provide further options for assay characterization.

There is still a long way to go until new references for gluten become available, and there is still room and need for improving the accuracy of detections methods. The knowledge gained in the past few years regarding issues and gaps surrounding the detection of gluten should not be considered as impediments but opportunities to improve current analytical strategies for gluten detection.

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Notes

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