

FOOD COMPOSITION AND ADDITIVES

Validation Procedures for Quantitative Gluten ELISA Methods: AOAC Allergen Community Guidance and Best Practices

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The food allergen analytical community is endeavoring to create harmonized guidelines for the validation of food allergen ELISA methodologies to help protect food-sensitive individuals and promote consumer confidence. This document provides additional guidance to existing method validation publications for quantitative food allergen ELISA methods. The gluten-specific criterion provided in this document is divided into sections for information required by the method developer about the assay and information for the implementation of the multilaboratory validation study. Many of these recommendations and guidance are built upon the widely accepted Codex Alimentarius definitions and recommendations for gluten-free foods. The information in this document can be used as the basis of a harmonized validation protocol for any ELISA method for gluten, whether proprietary or nonproprietary, that will be submitted to AOAC and/or regulatory authorities or other bodies for status recognition. Future work is planned for the implementation of this guidance document for the validation of gluten methods and the creation of gluten reference materials.

is estimated to affect approximately 1% of the population in developed countries (1), and scientific data indicate that its prevalence is on the rise (2). This intolerance to gluten is characterized by damage to the intestinal mucosa, resulting in malabsorption of nutrients (carbohydrates, protein, and fat), vitamins, and minerals. Celiac disease manifests as a complex array of symptoms, from classical intestinal irregularities to extraintestinal manifestations, such as iron-deficiency anemia, fatigue, osteoporosis, and peripheral neuropathy and ataxia (3, 4). Fortunately for most people, withdrawal of gluten-containing foods from the diet will reverse this damage and a significant recovery of the intestinal mucosa can be achieved (5). With the only prescription being avoidance of gluten in the diet, clear labeling of products to the presence of these proteins is essential for individuals to mitigate the risk and improve the quality of life for themselves and their families.

In order to help sensitive consumers identify products where gluten is absent, the Codex Alimentarius revised the Standard 118-1979 in 2008, which defines gluten and the requirements for use of a “gluten-free” statement (6). The standard states that below the level of 20 mg gluten/kg of food, a product can be labeled “gluten-free.” Although many countries do not have regulated thresholds for gluten-free labeling, many follow this Codex Standard. For example, Canadian food regulatory authorities have proposed a threshold value that aligns with the Codex Alimentarius recommendation of 20 ppm of gluten protein (<http://www.hc-sc.gc.ca/fn-an/securit/allerg/cel-coe/gluten-position-eng.php>) and the United States has recently done the same (<http://www.gpo.gov/fdsys/pkg/FR-2013-08-05/pdf/2013-18813.pdf>). Others like Australia consider gluten-free products those where gluten is non-detectable by current analytical methods and Brazil mandates the labeling of all food products either “gluten-free” or “contains gluten,” in spite of

Celiac disease occurs in genetically predisposed individuals, causing intolerance to storage proteins (gluten) of wheat, rye, barley, and possibly oats. It

not defining threshold levels or measures to ensure compliance. In the EU, Commission Regulation (EC) No. 41/2009, which is in part based on the Codex Standard, has been adopted (<http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2009:016:0003:0005:EN:PDF>). All jurisdictions will require methods to confidently detect and quantify gluten in a variety of food matrixes in order to assess the validity of these “gluten-free” statements. In this regard, internationally accepted validation protocols will be important.

Gluten as it relates to celiac disease is defined in the Codex Alimentarius (6). From a legal point of view, “gluten is defined as a protein fraction from wheat, rye, barley, oats or their crossbred varieties and derivatives thereof, to which some persons are intolerant and that is insoluble in water and 0.5 mol/L NaCl. Prolamins are defined as the fraction from gluten that can be extracted by 40–70% of ethanol. The prolamin from wheat is gliadin, from rye is secalin, from barley hordein, and from oats avenin. The prolamin content of gluten is generally taken as 50%.” This definition shows that gluten is not an individual protein but a complex mixture of different, more or less related, proteins.

Numerous documents in the literature have addressed method validation in general and some address validation of ELISA methods for small molecules (7, 8). Recently, a document to address the specifics of food allergen analysis, including reference materials, spiking methods, and choice of matrix, has been published with an emphasis on milk and egg allergens (9). Methods for detecting the components of gluten have been available for approximately 20 years, and most of these methods use ELISA-based techniques to detect specific proteins of gluten in food matrixes (10, 11). The detection of gluten by ELISA is a unique analytical procedure characterized by the binding of specific antigens to antibodies. The specificity and sensitivity of commercial ELISA methods for gluten quantitation vary as they use different antibodies, which target different soluble protein types, rather than a specific protein or different epitopes on specific protein fractions. This mixture of target proteins will have diverse structural and chemical properties specific to the food matrix, and the ability of an ELISA method to detect gluten proteins in a test sample will be determined by a combination of the efficiency of these interactions and how well the proteins are extracted from the matrix. The fact that gluten-sensitive individuals react differently to soluble protein constituents of gluten further complicates the choice of targets (12). Furthermore, most food products are heat-treated in some fashion, which can have a significant influence on the solubility and extractability of the target proteins, as well as on the ability of the antibody or antibodies used in the ELISA to recognize them (13).

Considering all of these factors, there is a need to harmonize the validation of gluten methods in order to obtain confidence that a method is suitable for its intended purpose. Providing harmonized validation protocols for gluten will enable industry to use a validated method for their gluten control programs, labeling claims, and regulatory compliance. Important steps toward harmonization will require harmonized validation protocols, a commonly accepted gluten reference material, and at least one independent reference method to verify the routine methods. With all these measures in place, the harmonization of validation procedures for gluten ELISA methods will provide a

level of confidence and acceptance of the results obtained from different methods.

This document is designed to accompany the AOAC Guidelines for *Collaborative Study Procedures to Validate Characteristics of a Method of Analysis* (14) and to provide specific guidance to the validation of quantitative ELISA-based methods for gluten. This protocol was designed to meet or exceed the minimum requirements set forth in Appendix D of the AOAC guidelines; it was developed with input from a wide range of experts in the area of gluten analysis, including the AOAC Food Allergens Community, the AOAC Gluten Working Group, and the Working Group on Prolamin Analysis and Toxicity. A recent publication provided some guidance for the validation of the performance characteristics of quantitative ELISA methods for food allergens (9) and was a guide for the development of this guidance document. This publication is intended to guide the collection of appropriate validation data for gluten ELISA methods that could be suitable for submission to AOAC INTERNATIONAL or regulatory bodies for scrutiny and recognition.

Required Information for Gluten ELISA Methods

Definition of Gluten

This document will provide guidance on the validation of ELISA methods for the quantification of gluten proteins in foods and raw materials. In this regard, it is essential to establish a practical definition of gluten so that subsequent sections and guidance can be developed. Many jurisdictions have adopted or are moving toward adopting the Codex recommendations to standardize gluten-free foods intended for people with celiac disease. Considering these efforts and the objective of this guidance document, the Codex definition of gluten will be used here, and is defined as a protein fraction from wheat, rye, barley, oats or their crossbred varieties and derivatives thereof, to which some persons are intolerant and that is insoluble in water and 0.5 M NaCl (6). Additional information has been provided about the consumption of oats in a gluten-free diet. Based on current science, pure oats can be tolerated by the vast majority of people with celiac disease (15). Therefore, the allowance for uncontaminated oats (not contaminated with wheat, rye, or barley) in the dietary management of celiac disease and the need for methods to distinguish pure oats must be determined at the national level.

Antibodies

ELISA methods for gluten are based on the interactions of antibodies with certain gluten proteins or segments of gluten proteins, and some information should be supplied on these interactions. It should be known whether the antibody is mono- or polyclonal and if it targets a peptide sequence, a single protein, or multiple proteins. It should be known if the protein used to develop the antibodies was fractionated, modified, or synthesized in some way.

Information must also be supplied on the antibody's specificity to gluten-containing cereals, such as common wheat, barley, rye, and oats. Data on the cross-reactivity to other related grains, including but not limited to spelt wheat, khorasan wheat (Kamut™), triticale, durum wheat, einkorn wheat, and

Table 1. Items typically used in the manufacture of gluten-free products or products thought to be gluten-free^a

Almond flour	Coffee	Guar gum	Potato flour/starch	Tapioca flour/starch
Amaranth flour	Corn starch/meal	Hazelnut flour	Quinoa flour	Tea
Arrowroot	Dried fruits	Lentil flour	Romano bean flour	White bean flour
Black bean flour	Egg powder	Lima bean flour	Sesame flour	White rice flour
Brown rice flour	Fava bean flour	Meats	Sorghum flour	Xanthan gum
Buckwheat flour	Flax seed flour/meal	Milk powder	Soya flour	Yellow pea flour
Chestnut flour	Garfava flour	Millet flour	Spices	
Coconut flour	Green pea flour	Oat flour	Sweet rice flour	

^a These items should be tested for cross-reactivity as part of gluten ELISA development.

emmer wheat, may also be included. All of the cross-reactivity data needs to be expressed in relative terms or normalized to the response of common wheat because this will be the most important cereal used and calibrated against.

Cross-Reactivity

A positive response to a sample that does not contain any gluten is referred to as cross-reactivity, and the extent of cross-reactivity should be reported. ELISA developers must test their gluten methods for cross-reactivity in a selection of foods and ingredients, particularly those used in the production of gluten-free products. There is no recommended number of items that should be tested for cross-reactivity, but the more items tested the better the confidence in the assay. In addition, the testing should be on the products as they would normally be consumed (raw or cooked), and initially based on full-strength extracts, i.e., the extract obtained by applying the extraction procedure as prescribed by the manufacturer of the test kit. If a positive result is obtained, then dilutions should be performed to characterize the extent of cross-reactivity. Although there are a large variety of potential matrixes, a minimum list of food commodities that should be included in cross-reactivity testing for gluten is provided in Table 1.

Calibrators

The calibrators used to generate the calibration curve are a very important component of the quantitative ELISA and will be used to calculate the level of gluten in a sample, which will ultimately be used in making decisions. Ideally, the gluten community would accept a well-defined and characterized material for calibrating their assays or a material to reference their calibrators against. Gluten is a complex mixture of many proteins that have differing solubility, and the calibration standards must clearly define the target protein or fraction used to determine the level of gluten. Although there is some evidence to support the claim that both the prolamin and glutelin fractions of gluten are immuno-stimulatory in people with celiac disease (12), historically it has been the prolamin fraction that is extracted and used to indirectly measure total gluten content in a sample. It is known that the ratio of prolamin to glutelin in total gluten can be different depending on the cereal, and if the soluble fraction is being used, then it must be known how this calibrator relates to total gluten for the specific cereal (16).

A number of methods have been used to separate and

characterize the different fractions of gluten and it should be known how the material used for the calibrator was characterized (17–19). Assay developers will need to specify what material they use as a calibrator: extract from flour, extract from purified gluten, purified protein fraction (prolamin or glutelin), or a peptide. If the assay is being validated for fermented or hydrolyzed food matrixes (e.g., beer, yogurt, soy sauce) the calibrator must be fit-for-purpose in regard to these types of hydrolyzed sources of gluten. The calibrators must represent the appropriate gluten fragment composition, with regard to the degree of hydrolysis, abundance, and length of the fragments for hydrolyzed gluten. All source information about the calibrator, whether for intact or hydrolyzed gluten, needs to be identified. This will include the grain(s) used, the cultivar(s), the commercial supplier, and the methods used to generate the calibrator(s). Another important component in any ELISA is the extraction buffer. The kit developer will need to describe any consequences in the analysis if there is a difference between the preparation of the calibrator(s) and the extraction of gluten using a proprietary extraction buffer.

Matrixes

Ideally, methods would be able to analyze all matrixes with equal reliability, but a method that is fit-for-purpose in one, or even several, matrixes may not be applicable in others. Gluten ELISA methods can be susceptible to matrix effects and have diminished performance due to interferences in some matrixes. This could be due to tannins in a sample or a certain degree of hydrolysis in some processes. Table 2 suggests matrixes that should be tested when a gluten ELISA method is being developed. The method developer should clearly identify which matrixes the method is fit-for-purpose on the basis of their in-house data, and identify any matrixes that diminish the method performance. Although incurred materials are preferred for validation studies (vide infra), a preliminary investigation before a multilaboratory study using spiked samples (direct spiking of gluten into the matrix) is acceptable due to the large number of materials tested. In these preliminary studies, it is important to determine whether the matrix has an influence on the measurement and, if so, the nature and magnitude of the effect.

LOQ, LOD, and Limit of Applicability (LLA)

Before conducting an interlaboratory study for gluten analysis, the LOD and the LOQ need to be determined. The

Table 2. Candidate food matrixes that could be tested when performing a multilaboratory validation study

Beer	Cereals	Energy/cereal bars	Oats	Sauce
Bread	Chips	Ice cream	Pasta	Soups
Breakfast cereals	Coated meat (baked)	Meat burger	Pies	Veggie burger
Cakes	Coated meat (fried)	Muffins	Salad dressing	Wine

LOD is defined as the lowest concentration of gluten that can be distinguished from a true blank. The LOD should be estimated by a statistical analysis of the calibration data according to the ISO standards for linear (20) and nonlinear data (21), with a default error probability of 0.05 for false positive (α) and false negative (β). The LOQ, on the other hand, is the lowest level of gluten in a sample that can be consistently quantified at a specific level of precision. Due to matrix, processing, and manufacturing variability, a kit developer may want to define the LLA rather than the LOQ. This will represent a level below which the method developer does not support or recommend the use of the method. Guidelines for single-laboratory method validations are available to assist determination of these parameters and any sources of possible variation (8). In order to obtain robust estimates for LOD/LOQ/LLA, it is recommended that data be collected in single-laboratory studies from at least three analysts over a minimum of 3 different days and preferably using at least two different instruments.

Ruggedness and Lot-to-Lot Variability

The determination of the ruggedness, or robustness, of an assay is a measure of its capacity to remain unaffected by small variations in procedural parameters. These types of experiments are investigated during method development and are reported in the assay documentation. Some parameters important to the end-user and final assay results will be the variation in reagent volumes, reagent concentrations (those prepared by end-user), extraction time and temperature, and incubation time and temperature. It is recommended that deviations for time and volume be investigated at ± 5 to 10%, and incubation temperatures tried at ± 3 to 5°C. Once the experimental variation

that provides consistent results is known, the limits of these parameters must be included in the assay documentation. Other parameters that are important and must be tested and reported are the shelf life and stability of all reagents and components in the test kit, as well as their storage parameters. An expiration date for each of these components of the test kit, as well as of the kit itself, should be clearly indicated. A small number of test kits from each lot should be set aside for comparison with future lots to determine if any characteristics of the assay have changed. For example, a positive control sample, such as an incurred test sample or spiked sample, should be analyzed with each new lot to be sure that consistent results are achieved. Information on the lot-to-lot variability should be provided by the kit manufacturer as part of the data submission package.

Interlaboratory Validation Study

Key Elements for Laboratories and Samples

The key elements for an interlaboratory validation study have already been described for food allergen ELISA and will only be briefly detailed here in order to make specific recommendations for gluten analysis (9). It is important to obtain enough statistically relevant information from an interlaboratory study; a minimum of eight laboratories contributing usable data is required, but it is recommended that more laboratories are recruited so that enough usable data are available for the study. It is also recommended that no more than one-fourth of the total number of laboratories contributing data be from the same organization.

The initial interlaboratory validation study must evaluate the method for a minimum of two matrixes. Each matrix set must contain a blank and have four concentration levels, one level

Table 3. Theoretical raw data randomly generated in a collaborative study for a gluten ELISA with a stated LOQ of 5.0 mg/kg and an upper range of 100 mg/kg^a

Lab	0 mg/kg		2.5 mg/kg		10 mg/kg		40 mg/kg		80 mg/kg	
	Sample A	Sample B	Sample A	Sample B	Sample A	Sample B	Sample A	Sample B	Sample A	Sample B
1	-0.68	-0.19	2.84	3.07	8.11	11.63	46.99	36.94	73.52	72.18
2	-0.87	0.68	2.87	2.66	10.78	11.74	33.61	40.40	69.71	90.86
3	-0.66	-0.27	3.10	3.18	12.93	10.02	32.64	38.66	80.48	95.33
4	-0.62	-0.08	2.06	2.29	9.82	10.10	50.11	42.87	97.07	76.01
5	-0.60	-0.30	2.92	2.64	12.13	12.18	50.10	44.08	100.50	79.08
6	-0.82	-0.67	3.25	2.84	8.37	10.35	32.52	51.95	80.81	82.14
7	-0.68	0.44	2.12	2.18	10.74	9.36	47.49	49.72	65.96	81.96
8	0.52	-0.10	3.05	2.02	11.67	8.22	34.85	48.08	74.31	102.03
9	-0.09	0.05	2.88	1.95	9.04	9.80	33.22	48.27	76.06	89.94
10	-0.78	-0.53	2.28	2.41	10.68	11.25	36.36	34.37	84.29	94.59

^a The study involved 10 laboratories each analyzing a blank and four concentration levels in duplicate (A and B) for a total of 10 samples.

Table 4. Calculated results for the hypothetical interlaboratory validation study in Table 3

	0 mg/kg	2.5 mg/kg	10 mg/kg	40 mg/kg	80 mg/kg
Total No. of laboratories	10	10	10	10	10
Total replicates	20	20	20	20	20
Overall mean	0.31	2.63	10.45	41.66	83.34
Repeatability SD (s_r)	0.50	0.34	1.42	7.10	12.03
Reproducibility SD (s_R)	0.46	0.43	1.38	7.09	10.74
Repeatability RSD (RSD_r)	158.7	13.0	13.6	17.1	14.4
Reproducibility RSD (RSD_R)	147.5	16.4	13.2	17.0	12.9

of which should be below the LOQ or LLA concentration. One of the concentration levels should be at the lower end of the calibration curve, below two times the LOQ or LLA stated for the method. The remaining nonzero levels should be evenly distributed throughout the range of the calibration curve. For example, if a single-laboratory investigation (*vive supra*) determines the LOQ of the assay to be 5 mg/kg and the upper limit to be 100 mg/kg, then the recommended levels for the interlaboratory study would be 0, 2.5, 10, 40, and 80 mg/kg in each of the two matrixes chosen from Table 2. All samples will be tested as blind duplicates, so that each laboratory in the study will analyze 20 individual test portions (2 matrixes $\times$ 5 levels $\times$ 2 blind duplicates).

Data Analysis for Interlaboratory Studies

The ISO standards (22), AOAC *Official Methods of Analysis* (7), and a guidance document for the validation of food allergen ELISA methods (9) outline how to analyze the data from an interlaboratory study. In general, each matrix/level combination should be treated as a separate experiment and analyzed as such. Results obtained from blank samples should not be censored in any way. It is very important to evaluate the correct mean and distribution of the blank sample results in order to have an unbiased estimate for LOD and LOQ. As such, negative values should be treated unchanged and not censored to zero. Likewise, any thresholds, such as LOQ or LLA limits, should not be applied to collaborative study data sets. Participating laboratories should be instructed to report all results as calculated, and to not report as “ND” or “<LOQ.”

The initial step in the workflow is to remove any outliers by sequential use of the Cochran and Grubbs tests, as indicated in AOAC *Official Methods of Analysis*, Appendix D (14). The mean, accuracy (if applicable), repeatability (S_r), reproducibility (S_R), RSD of repeatability (RSD_r), and RSD of reproducibility (RSD_R) should be calculated and reported. If the variance is found to be constant and normally distributed, then the LOD and LOQ can be estimated as 3.3 times and 10 times the SD of the distribution of blank results, respectively. If the variance is not constant with concentration, then a more accurate method of estimating the LOD and LOQ can be used (9). Table 3 gives an example of data generated in a hypothetical collaborative study. The levels were chosen based on the LOQ (5 mg/kg) and the upper limit of the calibration curve (100 mg/kg) determined from a pre-interlaboratory study. From these raw data, all the necessary information can be calculated in order to determine

the LOD and LOQ for the assay in this hypothetical matrix (Table 4). Figure 1 shows that the reproducibility (S_R) in this study increases with the mean concentration, and an advanced formula is recommended to better estimate the LOD and LOQ. This formula uses the slope (0.131), the intercept (0.400), and the overall mean for the zero level (0.313) to calculate the LOD = $(\bar{x}(0) + 3.3 \times \text{intercept}) / (1 - [1.65 \times \text{slope}])$, LOD = 2.20 mg/kg, and LOQ = $3 \times \text{LOD} = 6.61 \text{ mg/kg}$.

This information can now be used to construct an operational curve for this assay and matrix combination. An example of such an operational curve is given in Figure 2, from which the probability of obtaining a result higher than the LOQ can be determined based on the concentration in the sample. For example, if the concentration of gluten in the sample was 10 mg/kg, there would be a 97.5% probability that the sample measurement could be higher than the estimated LOQ of 6.61 mg/kg.

Gluten-Specific Criteria

Reference Materials

The term “gluten” is used differently depending on the field of research. Historically, gluten is a highly complex mixture of proteins defined by their solubility. In 1907, T.B. Osborne defined gluten as being the protein fraction of the wheat kernel is not soluble in water or dilute salts, and this definition has carried through to this day. In terms of celiac disease, the Codex Alimentarius Standard for Foods for Special Dietary Use for Persons Intolerant to Gluten (6), defines gluten as “a protein

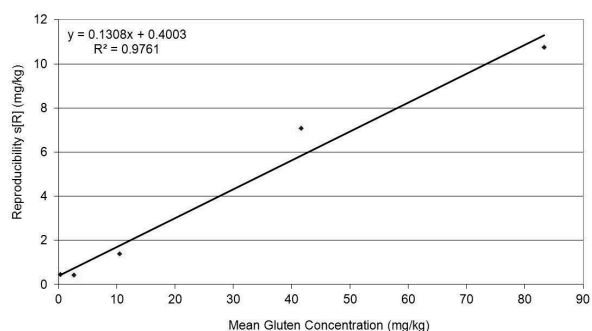


Figure 1. Plot of reproducibility versus the global mean observed gluten concentration for the hypothetical interlaboratory study.

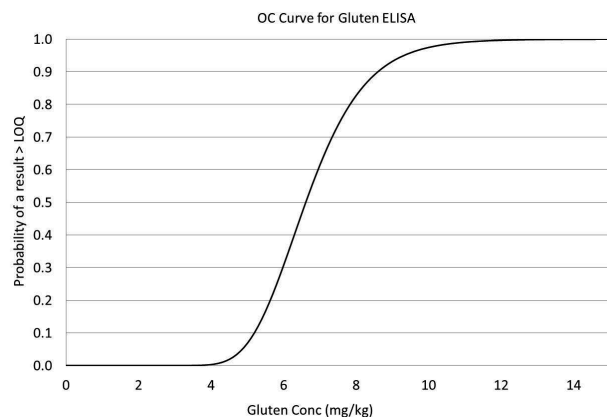


Figure 2. Operational curve calculated from the results of the theoretical interlaboratory validation study.

fraction from wheat, rye, barley, oats, or their crossbred varieties and derivatives thereof, to which some persons are intolerant and that is insoluble in water and 0.5 mol/L NaCl.” Within the total gluten fraction, Osborne also defined two further protein subclasses referred to as the prolamins, which are soluble in aqueous alcohols, and glutelins, which are soluble in dilute acids, alkali, or in the presence of reducing agents. Although a good deal of clinical work has defined epitopes or fractions of the prolamins and glutelin proteins as contributing to the onset or exacerbation of gluten intolerance (celiac disease), the effects of all fractions are currently unknown.

To obtain the level of gluten in a sample, an assay will have a set of calibrators to generate a standard curve to which a response obtained on a sample can be related. These calibrators will be correlated to a gluten reference material in order to convert to a level of gluten in the sample. These reference materials may also be used to develop incurred materials or for spiking into blank food matrixes during a validation study. It would be recommended that a commercially available, well-characterized reference material be used for these purposes, but these materials are sometimes not available. To date the best characterized reference material is the so-called Prolamin Working Group-gliadin (19), which can be obtained from the Working Group on Prolamin Analysis and Toxicity (<http://www.wgpat.com/index.html>). Although this material is well-characterized, it represents only one fraction of total gluten and total cereal proteins. Currently, most commercial gluten ELISA methods detect the prolamins fraction. As the science around celiac disease is evolving, efforts should be made to develop and characterize an improved reference material containing all gluten protein fractions based on the Codex definition of gluten. This reference material would be of particular importance if ELISA methods able to detect both prolamins and glutelins become available.

Using an extracted gluten source instead of one minimally processed (e.g., cereal flour) can be controversial. Both materials are produced from a natural source and are subject to batch-to-batch variation, requiring a full characterization for every new batch. The extracted material might lack individual protein components present in a minimally processed sample. However, with respect to good laboratory practice, it would be desirable to have the reference material available in a soluble form to

facilitate routine work. This soluble form could be achieved for an extracted gluten source but not for a native sample, which would require additional extraction and centrifugation steps before use. Obviously, the production of a common reference material for partially hydrolyzed gluten can be produced only from an extracted gluten source. Finally, a commonly accepted reference material should also be suitable for clinical studies to link toxicity of the material to a concentration.

For isolating the gluten fraction from wheat, the following protocol may be useful. Wheat flour is mixed to a dough, which is then washed with NaCl solution to remove starch and soluble proteins, leaving a gluten mass that can be dried and weighed after being tested for the absence of residual starch. Different organizations provide standard protocols for isolating wet and dry gluten, either by hand-washing or automatically by a Glutomatic machine (23–30). It has been suggested to remove the excess NaCl washing solution, which affects the final weight of the gluten, by additional washing with water. For the development of a gluten reference material this would not be recommended due to the potential loss of the ω -gliadin fraction. If only the weight of the gluten has to be determined, drying by heating can be carried out, but if the material is intended to be used as a reference, it should be dried by lyophilization. Because of the differences in gluten-containing cereals that are toxic to an individual with celiac disease, it will be necessary to characterize separate reference materials for wheat, barley, and rye.

Spiking Methods

In a validation study, the best source of information for the detection and quantification of the level of gluten will come from incurred samples. These samples will have a known amount of gluten incorporated into the product and will undergo processes similar to those used commercially. When incurred samples are included in a validation study, it is important that information be included about these materials. This information should include how the materials were prepared, including the recipe and preparation conditions, how they were characterized, homogeneity experiments, and analytical methodologies used. There are numerous matrixes, and it may be difficult to obtain incurred materials for gluten validation studies; therefore, spiked samples will be considered an acceptable way to prepare materials to generate performance data in a specific matrix. The preferred method of spiking will involve the fortification of a large batch of matrix with a high level of gluten followed by serial dilution with the blank matrix material. As with the incurred materials, the particulars of the preparation should be described in the validation, as well as the results of homogeneity studies. In some matrixes, burgers and sausages, for example, it will be difficult to prepare consistent serial dilutions from a master sample, and direct spiking of a gluten reference material may be required to determine how the matrix will affect the result.

Food Matrixes

The matrixes being analyzed can have a large impact on the performance of an ELISA method. When validating an ELISA method for gluten, it is advisable to determine how the kit performs with those matrixes that are important foods for

individuals following a gluten-free diet. Some of these foods are shown in Table 2, and represent potential products that could have cross-contaminated starting materials. This list is not exhaustive and there may be examples more appropriate for a specific jurisdiction.

Conclusions

Intolerance to gluten (celiac disease) requires the strict avoidance of gluten sources from wheat, barley, rye, or their crossbred species. Codex Alimentarius recommends that for a food to be labeled gluten-free, it must not contain more than 20 mg gluten/kg of food. The food allergen analytical community is endeavouring to create harmonized guidelines for food allergen ELISA methodologies to help protect food-sensitive consumers and promote consumer confidence. The guidance described here reflects a consensus reached, through input and collaboration, from various allergen analytical experts, and contains specific recommendations and requirements for the validation of ELISA methods for the detection and quantification of gluten proteins in food matrixes. Future work is planned for the implementation of this guidance document for the validation of gluten methods and the creation of gluten reference materials.

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