

Commercial ELISA Measurement of Allergens and Gluten: What We Can Learn from Case Studies

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During the last decade, results from ELISA test kits have often been criticized as being flawed. Therefore, it may appear counterintuitive that ELISAs are used for most food allergen and gluten analytical needs. One reason, in addition to the nonavailability of comparable alternative methods, is the fact that the methods used underwent a long validation and learning period in the market. This publication presents several case studies on interference, cross-reactivity issues, calibrators, fragmented allergens and gluten, matrix influences, and misunderstood intended-use statements. Afterward, the relevant validation parameter LOD, LOQ, selectivity, and precision are discussed. Finally, a comprehensive list of practical recommendations for ELISA test kit users is presented.

Quantifying a known measurand in a pure buffer solution with no interfering components is an easy task for every test kit developer. In reality, a problem called “matrix” is always part of every food sample, environmental surface, or cleaning-in-place (CIP) water. Although other measurands, like antibiotics or mycotoxins, are often analyzed in a limited number of well-characterized and often non-processed food matrixes, the number of food matrixes in the case of allergen and gluten measurements seems to be countless. One reason for this variety is the fact that not only raw materials are of interest, but also processed and final products, due to the high risk of cross-contamination with allergens and gluten during food manufacturing. Furthermore, every food manufacturer has its own recipe with individual processing types and conditions (e.g., times and temperatures). Even hydrolyzation steps during processing of food are used. Another aspect is that more and more novel ingredients will be used in the food industry, enlarging the number of matrixes that require testing. Modern food production nearly always ends up with products with a high level of complexity in which allergens or gluten are sometimes only a minor or residual component. Also, environmental surfaces, such as, e.g., stainless steel or ceramic tiles, are quite challenging because the measurand may interact with the surface via several physical mechanisms. In this case, one of the main analytical goals is to resolve this binding. Furthermore, as described later on, it is quite complicated to

mimic surface contamination and, at present, it is impossible to validate such surfaces in a collaborative test in an acceptable way.

For all the reasons mentioned above, it seems reasonable that a method developer only starts with a limited number of representative and important matrixes during the initial validation of a method to quantify allergens or gluten in food. Hence, the choice of representative food is also dependent on the location of the headquarters of the test kit manufacturer and may not always be the best choice in other geographical regions due to dietary preferences. For any matrixes that have not undergone validation, a laboratory seeking to analyze such matrixes should undertake a verification study to demonstrate the fitness-for-purpose of the method in an accredited laboratory according to ISO 17025 (1). Central information that needs to be considered before starting the practical work are (1) the composition of the matrix and (2) the chemical–physical form in which the allergen is present. If the nature of the calibrator material and the measurand in the sample are not comparable (e.g., different levels of processing or fragmentation), results will be biased. If the sample contains commodities that are known to cross-react or interfere in the measurement system, results will also show a bias. Also very important is the homogeneity of a sample. If the homogeneity of the sample is not guaranteed or even unknown, results will be imprecise and measurement uncertainty may increase beyond an acceptable level. Sampling issues are currently under discussion at AOAC INTERNATIONAL, Codex Alimentarius, the International Organization for Standardization, and Eurachem. In the next section, several case studies are provided to explain these central questions. In a subsequent section, several relevant validation parameters are discussed.

Case Studies

Interferences

In 2010–2011, traces of peanut were detected by ELISA in chicken tikka masala meals. Later on, it was suspected that almond was detected in cumin samples. Although in the first case peanut was confirmed in the meals, in the second case it was found that mahaleb cherry, not almond, was present (2). Later, it will be discussed whether this latter analysis was flawed or not.

In both cases, test kit developers were confronted with requests from customers on validation data for allergens and gluten in spices. About 20 different spices were analyzed, and the results showed a reasonable number of different spices with reduced recovery due to interfering substances in the samples. Until that point, spices had been expected to be less important matrixes because their amount in final meals is normally not higher than 1 or 2%. It was speculated that the interferences were

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due to essential oils and polyphenols in the spices; therefore, it was not surprising to observe that the addition of skim-milk powder or casein significantly improved low recoveries for most of the spices. Nevertheless, a few of the spices still showed recoveries that were not acceptable when tested as pure commodities. In order to mimic ready meals, these spices were mixed with a noninterfering rice meal at a 10% level. Checking for recovery revealed acceptable values between 70 and 130%. However, in the cases in which analysis of pure spices is required (e.g., for certificates of spice suppliers), the operator should be aware that spices can pose a real challenge for a successful ELISA (and equally for other nonimmunochemical detection methods). False-positive results are very rare, but false-negative results may occur due to low recoveries. It is thus recommended that spiking experiments be performed for spice analyses.

It should be noted that spices often show positive results for allergens or gluten. In almost all cases, these positive results are due to a real cross-contamination with these allergens or gluten. Spices are often mixed with other materials for economic reasons (e.g., food fraud) or to improve the appearance of the spices (e.g., more intense color). The best way to confirm an ELISA result independently is to use a technology other than ELISA, such as an MS method. PCR, on the other hand, only gives an indication that the allergen is present.

Assumed Cross-Reactivity

The R5 antibody for gluten measurement shows no cross-reactivity toward soy proteins. Nevertheless, it was published and advertised word-of-mouth that R5-based gluten ELISA shows cross-reactivity toward soy proteins and that, therefore, soy-containing matrixes should not be analyzed for gluten using this monoclonal antibody (3, 4). A possible explanation for this assumption is a misunderstanding based on the fact that the analysis of soy-containing nonprocessed food needs the addition of skim-milk powder if extracted with 60% ethanol. This was clearly stated for the last decade in the test kit instructions for use. If the addition of skim-milk powder is omitted, soy-containing samples can produce a positive result when extracted with 60% ethanol only due to the development of high background in the ELISA. Using so-called cocktail extraction for processed food or food with unknown processing status does not require the addition of skim-milk powder to soy-containing matrixes to any degree. To avoid confusion when analyzing food with an unknown processing status, the test kit manufacturer of the R5-based gluten ELISA opted some years ago to delete the extraction method with 60% ethanol from the test kit description. Studies performed in the laboratory of Mendez and coworkers also showed no cross-reactivity to soy a decade ago (5). Therefore, reports by Sousa et al. (3) and Buckenhüskes (4) on positive cross-reactivity testing were not confirmed by other peer-reviewed publications. Additionally, during FAPAS proficiency testing rounds, soy-containing materials were tested by a total of 310 participants between 2012 and 2015, and no false-positive results were obtained.

Cross-Reactivities to Near-Neighbors

The plant kingdom contains important edible plants that are closely related genetically to each other. Good examples are the

Leguminosae family (soy, beans, peanuts, lentils, and peas) or the *Prunus* genus, with important stone fruits, such as almonds, apricots, peaches, plums, cherries, and nectarines. Every legume or stone fruit can be subdivided into several species and varieties. Western legislation only demands the labeling of varieties of soy, peanut, and almond. In contrast, Japanese allergen-labeling laws recommend that peach also be labeled in food when present. To highlight the relevance of this, it should be noted that eight cases of anaphylactic shock after consumption of peaches were reported in Japan between 2001 and 2002 (6).

To illustrate a typical evaluation of potential cross-reactivity, a panel of 18 phylogenetically near-neighbors were tested in an ELISA specific for soy (7). To avoid any confusion in terminology, only botanical names are presented herewith. No cross-reactivities were observed for *Lupinus angustifolius*, *L. albus*, and *L. luteus*. In contrast, cross-reactivities were observed against *Pisum sativum* (dried and fresh seeds), *Vicia pannonica*, *Lens culinaris*, *Arachis hypogea* (roasted and raw), *Cicer arietinum*, *Trigonella foenum-graecum*, *Trifolium pratense*, *Phaseolus vulgaris*, *P. lunatus*, *V. faba*, *P. coccineus*, *Vigna radiata*, and *V. angularis*. These cross-reactivities are not surprising because soy is closely related to other members of the *Leguminosae* family. However, although from a regulatory point of view, these assay results could be considered undesirable due to cross-reactivities, they may still be relevant to those patients who are sensitive to soy and suffer from allergies to other *Leguminosae* members (8).

In the case of almonds, ELISA methods show cross-reactivities against phylogenetically near-neighbors, such as apricot stones or mahaleb cherry (*Prunus mahaleb*), which is used as a spice due to its fragrant smell and taste comparable with bitter almonds. This example shows the dilemma of planning the extent of cross-reactivity testing when a possible cross-reactant is unknown (e.g., not used in one geographical area), when it is only mentioned with a few sequences in databases (2), and/or when it is not labeled in spice mixtures. The usage of apricot stones is well known because they form the basis for persipan, a common cheaper alternative to marzipan. In contrast, the existence of mahaleb cherry in food production was widely unknown in Western countries before the mahaleb cherry incident. Therefore, method developers routinely checked apricot stones for cross-reactivity in almond ELISA, but not mahaleb cherry. Nevertheless, it may turn out that this cross-reactivity is also beneficial from a clinical standpoint. Due to the close relationship, it would not be surprising if extracts of mahaleb tested positive in sera from patients that are sensitized against almonds.

One could argue that a monoclonal antibody with higher specificity would solve this problem, but it should be kept in mind that narrowing specificity may result in a loss of varieties that are regulated by legislation and could otherwise be detected using the same assay. In the above-mentioned examples, some almond or soy varieties may not be recognized by monoclonal antibodies with a specificity that is too narrow. Only a comprehensive knowledge of all existing varieties and possible near-neighbors will allow the design of a monoclonal antibody that fulfills all requirements. The questions are whether all relevant varieties are really known and whether they are accessible at the moment. As a consequence, Walker et al. (2) recommended strengthening activities in this area. In detail, they asked for (1) a coordinated international program for the production of reference materials and calibrants and (2) an

international program to widen the scope of proteomics and genomics bioinformatics for genera that contain the major allergens. Furthermore, they asked for the initiation of a coordinated international program that leads to reference methods that are traceable to SI units. Hence, a suitable epitope that only exists on varieties of interest is subject to food-processing susceptibility and may be impossible to identify.

Is There Equivalency for Standard Reference Materials as Calibrators?

Calibrator materials for allergen ELISA test kits are often based on complex food commodities, e.g., skim-milk powder or whole-egg powder. Standardization of these materials seems to be a good starting point in reducing bias between different test kits and methods. Reference material producers have been offering these commodities for decades and certify them for selected measurands, e.g., fatty acids or trace elements. Because of the reputation of these suppliers, researchers and method developers of allergen ELISAs started to use their materials directly as calibrators or use them to calibrate their in-house calibration materials, although this was not their originally intended use. In 2010, Lacorn and Immer (9) published data on a comparison of two whole-egg powder reference materials (NIST SRM 8415 and NIST SRM 8445) in a commercial ELISA test kit, RIDASCREEN FAST Egg. It was found that both materials reacted differently by a factor of 10. A possible explanation for this remarkable difference is the fact that NIST SRM 8415 was γ -irradiated with ^{60}Co (20 kGy dose) and contained silica aluminates. In comparison with a commercially available egg-white powder preparation, it turned out that the γ -irradiated material reacted significantly differently than the other two.

The material was not comparable with nonirradiated egg materials. In 2015, Goma and Boye (10) proved that irradiation with 0.75 and 10 kGy egg-incurred food material did not alter antibody detection by several commercial ELISA test kits. In contrast to the National Institute of Standards and Technology Standard Reference Material, the whole egg-incurred food material adsorbed the energy of the radiation so that the radiation dose of the egg constituents was far lower than for the pure egg material that was additionally radiated at a higher dose. It can be concluded that radiation of the pure egg commodity was the reason for the 10-fold lower ELISA response.

Gluten-Containing Food: Unknown Level of Fragmentation

One important food compound that is the basis for many gluten-free bakery goods is gluten-free wheat starch, which is made from wheat flour. For production, starch is cleaned up by the very thorough cold-water washing-out of gluten, or gluten is additionally fragmented by enzymes into peptides. In the latter case, remaining gluten fragments are potentially too small to be detected by sandwich ELISA systems in a quantitative way due to the fact that only one epitope remains in the peptide. In this case, the use of a competitive ELISA assay format is strongly advisable that is also able to detect very small fragments of proteins. However, competitive assays usually have to use less stringent extraction buffers, which may lead to incomplete

extraction in heat-treated materials. This may also apply to starch that has been strongly heated to dryness after washing out gluten proteins. Comparing results from sandwich and competitive analyses will indicate whether the sample was extracted with water only or additionally treated with enzymes and whether the starch was strongly heated for drying.

When the Calibrator Is Different from the Measurand

In 2003, the AOAC Research Institute (RI) initiated a collaborative test for peanut ELISA systems using different commercial test kits. AOAC-RI asked the test kit manufacturers to use the peanut butter material, NIST SRM 2387 (11). The intended use of this material was not as food allergens. It was certified as a calibrator for the determination of fatty acids, vitamins, elements, amino acids, and aflatoxins. Until today, FAPAS proficiency tests have shown remarkably low bias between different test kits, which have been successfully evaluated and use the AOAC-requested calibration material (12). It was quite interesting to see that the use of defatted peanut flour during the European research projects on the prevalence, cost, and basis of food allergy across Europe, EUROPREVALL, and integrated approaches to food allergen and allergy risk management, iFAAM, resulted in significantly different results compared with peanut butter (Mills, E. N. C., University of Manchester, Manchester, UK, personal communication, 2017).

Following Intended-Use Statements for Test Kits

In 2010, Kerkaert et al. (13) published their results on lysozyme detection and quantification in cheese using a commercial ELISA for the quantification of egg proteins in noodles, salad dressing, cookie mixtures, bread mixtures, and ice cream. The cross-reactivity of lysozyme in this ELISA system was reported to be <10% by the manufacturer. Nevertheless, Kerkaert et al. (13) used the egg ELISA and recalibrated it for lysozyme with a quantification range of 1000 up to 100 000 mg/kg. The problem is that the authors never changed the extraction method, which was developed for egg-white traces not lysozyme in quite high amounts alone. Therefore, their publication, "Detection of Hen's Egg White Lysozyme in Food: Comparison Between a Sensitive HPLC and a Commercial ELISA Method," analyzes an ELISA method that has never been developed, intended, or stated to be applicable for the detection of lysozyme alone. Before publication, Kerkaert et al. (13) should have verified that their modified ELISA method was fit-for-purpose. Meanwhile, the manufacturer of the ELISA test kit developed a specific lysozyme test kit for quantification in wine and cheese. This will hopefully circumvent more equivocal experiments as a result of misunderstood intended-use statements.

Matrix Components Alter or Destroy the Measurand

During an AOAC validation study of the R5 lateral-flow device (LFD) for the determination of gluten in CIP waters, the manufacturer checked several chemically different cleansing reagents. One of them contained alkaline hypochlorite at a quite high concentration. In contrast with all other tested cleansing reagents, the results were completely negative even at quite high gluten concentrations. When gluten was spiked after neutralizing the alkaline hypochlorite solution, results were positive, as

expected (neutralization prevents the otherwise very strong oxidative effect of the hypochlorite solution). Obviously, hypochlorite altered or destroyed gluten to the extent that antibody recognition was no longer possible. Whether gluten in this altered or destroyed form is still toxic to celiac patients remains to be elucidated. Another explanation is that hypochlorite directly interacts with the LFD system, e.g., altering R5 antibody-binding sites. In any case, the manufacturer updated the instructions for use for this LFD to inform customers that hypochlorite-containing cleansing agents cannot be analyzed with this method.

Matrix Components Cross-Linked with the Measurand

Users of ELISA test kits are often confronted with apparently complicated sample extraction procedures that demand special extraction buffers and additives in addition to harsh extraction conditions, such as boiling in a water bath. However, these complicated extraction methods can improve recoveries from incurred materials. Although easy extraction methods usually work well for spiked processed samples, they fail to show good recoveries for incurred processed samples. In the case of milk, β -lactoglobulin is the most reactive protein for the potential of cross-linkage and aggregation (14). Its extraction requires reductive conditions and chaotropic salts in addition to extraction temperatures of 100°C to prevent cross-linkage and aggregation, which lead to reduced detection. The extraction of casein is less challenging.

This is not only relevant to β -lactoglobulin ELISA kits, but also to total milk ELISA kits, which detect caseins and β -lactoglobulin. In order to obtain a reliable result, it is necessary to extract both proteins with comparable efficiency. In the food industry, whey powder, caseinates, and dried skim-milk powder are added to food. All these contaminants need to be detected; therefore, rigorous extraction is necessary.

Validation

The level of relevant validation depends on the intended use claimed by the method provider and the desired use by a single customer. A method provider should assess all validations necessary to support all claimed intended uses. However, the desired use by a single customer may deviate from the stated and validated intended uses. For example, a customer may use a quantitative method in a qualitative way only. Although the method provider validated recoveries at different levels, the customer will be more interested in discrimination between negative and positive results at his/her chosen threshold. A customer may also want to use the assay for a different matrix not validated by the method provider.

In order to enable a customer to check the validation data assessed by the method provider for this special use, it is necessary for both to have the same understanding of the validation parameters. Herewith, we describe the most important validation parameters for allergen ELISA methods that are often a source of misunderstanding and misinterpretation.

Performance Characteristics

LOD and LOQ.—In addition to the simplicity of the concept, the term LOD is beset with problems. To form an idea about the magnitude of an LOD, one needs concentration numbers for

blank samples in a concentration area in which reliable numbers do not exist. To obtain these concentrations, the measurement signal (absorbance, in the case of an ELISA) has to be converted into a concentration. Within the measurement range, this is performed by using a nonlinear calibration graph. The easiest way to apparently convert measurement signals into concentrations outside the measurement range is, of course, to extrapolate the calibration graph to very low concentrations. Depending on the mathematical function underlying the calibration graph, this may lead to very inaccurate results. From our experience, the curve fitting used to cover the measurement range should not be used to calculate numbers below the LOQ. Instead, a curve fitting that uses second- or third-order polynomials of low-concentration standards, including the zero standard, is a more appropriate method for the estimation of numbers near zero. If a user of a test kit wants to verify the LOD values stated in the validation reports of the method developer, the user should repeat exactly the curve-fitting algorithm stated in the report. Alternatively, a user may establish his/her own matrix-dependent LOD value and a curve fitting of his/her own choice.

In early versions of internationally accepted validation guidelines, the LOQ was calculated from LOD measurements. In light of the statements in the previous paragraph, it is obvious that this calculation is beset with high uncertainty, and the calculated value is often too high. A much better and much more robust way to calculate an LOQ is to spike or incur blank samples repeatedly at an assumed LOQ level and check whether recovery and precision are within an acceptable range. The LOQ is predominantly matrix-dependent; however, other factors, like operator and equipment used, can introduce additional variability. This means, as a consequence, that a user of a test kit should verify the results that are stated in the validation report from the method developer, as required by ISO 17025. Typically, the LOQ is set at a concentration in which the RSD is approximately 10% or less and the recovery is between 70 and 130% if spiked samples are used. In the case of incurred matrixes, recoveries between 50 and 150% are acceptable (15).

This directly implies that at values lower than the LOQ, the CV is rising and the uncertainty of the results is increasing. Therefore, only qualitative results should be stated for these extrapolated concentrations that are below the LOQ. One possibility is to report “positive but below the LOQ.” If this low residual concentration implies a remaining risk for consumers, a food manufacturer confronted with these values should perform an analysis on the remaining risk. If an estimated concentration is at or below the LOD, the report should state “not detected.”

Cross-reactivity and interferences.—In the case of cross-reactivity and interference testing, it is common practice to test food matrixes of interest and accompanying substances that do not contain the analyte. We should remember that this approach contains gaps. These gaps are clearly related to a lack of knowledge: e.g., as in mahaleb cherry, for which it was not known that mahaleb existed in the recipe and the bioinformatics only delivered a few sequences. This was clearly identified by Walker et al. (2) who asked for a program to strengthen genomic and proteomic databases in Europe by including a global multidisciplinary consortium coordinated by the Directorate-General Health and Food Safety of the European Commission. Test kit manufacturers and method developers can use this information to plan validation schemes based on

state-of-the art knowledge. It should be clear that this knowledge is continually growing and that consumer security depends on the quality of the completeness of these databases. In addition to the availability of data from these sources, the availability of characterized commodities for practical testing is crucial. It would be useful to not only have access to databases, but also have access to pure materials.

Traceability.—Every measurement is accompanied by measurement uncertainty. Therefore, it is necessary to characterize systematic errors (bias) and random errors (precision) for each method. Although characterization of precision is a matter of workload and man-power, bias estimation requires (certified) reference materials. As discussed earlier (9, 12), certified reference materials, as defined in *International Vocabulary in Metrology* (16), are presently not available, and it is questionable whether they will become available in the future.

Nevertheless, results from allergen measurements should have a certain degree of traceability to somewhat defined references. All that are involved in allergen measurement would be content with a “relative reference material” or so to say an “anchor point.” These anchor points were discussed and set up by a group of experts under the auspices of the MoniQA Association (17). At present, materials for milk quantification are available. They consist of a commercially available skim-milk powder with certified protein content. This material reflects milk contaminations that occur in the food industry in a production setting. This material was used to incur cookie dough at two different levels that were baked, dried, and milled. The resulting concentrations were 3.54 and 17.7 mg/kg milk protein, respectively. These values are derived from the amount of skim-milk powder that was used to incur the cookie dough. A blank cookie material was also produced. Other allergens and gluten materials will follow.

Validation of the Measurand on Surfaces

Under practical conditions, surfaces are contaminated with particles of allergens or gluten, e.g., dust on a stainless steel working bench. In contrast, contamination of surfaces in a laboratory for validation purposes will often result in a film of the measurand(s) on the surface due to the fact that solutions of the measurand are used for contamination. At present, there is no way to mimic surface contamination in a realistic way. However, it should be noted that a film of previously solubilized allergens or glutens could be more adhesive than real contamination with particles. Recently, there has been considerable speculation on how to perform collaborative tests of surfaces. There are three possibilities: (1) contamination of all surface materials at a central point (e.g., the laboratory of the organizer of the collaborative test) and delivery of blind-coded surfaces to all participants, (2) delivery of blind-coded spiking solutions to each participant and contamination of each single surface by each participant, and (3) contamination of surfaces by each participant using blind-coded powders (e.g., rice starch) that contain the measurand as the particles. The first approach is subject to possible cross-contamination or loss of material during transport. The second possibility is used for microbiology collaborative tests; however, it has never been validated before performing a collaborative test with allergens. The last possibility seems to be the most complicated approach because

measurement uncertainties due to variations in surface preparation will be much higher than the uncertainty derived from the analytical method alone. Furthermore, the material used for contamination would need to have the correct concentration of allergen (e.g., it is unrealistic to contaminate a 10×10 cm surface with 1 g particles) and would need to be extremely homogenous due to the low amount of sample applied to each surface. So far, the second possibility is considered the most practical method, but the limitations of this approach should be kept in mind.

Recommendations for Users of Commercial ELISA Kits

From the long practical experience of the authors, a comprehensive overview of the most important recommendations when using a commercial ELISA kit for allergen and gluten measurement is given in Table 1.

Table 1. Reliability of the analytical results for the food allergens

Analytical step
Study the test kit insert and validation reports
• Check the intended use for the matrixes and applications • Check the extraction strategy • Identify the limitations of the ELISA • Check for cross-reactivities
Check each test run
• Negative result for the negative control sample • Results are within range for the positive control sample (control sample chart) • Consult the compliance criteria for the calibration curves • CV of <10% for technical replicates within the measurement range
Verify the homogeneity of unknown samples
• Analyze five independent sample aliquots • Check whether cross-contamination in the laboratory is probable
Evaluate sample composition
• Verify that food ingredients do not result in false-positive or -negative results • Analyze ingredients separately if the composed sample tested unexpectedly positive or negative • It may be necessary to spike zero matrixes/ingredients
Complex food matrixes might cross-link to the analyte
• Simulate food processing by incurring the samples • Verify recovery from the processed samples • Validate the LOD/LOQ for unknown matrixes
Ensure clean production conditions
• Swab the surfaces of shared production lines • Analyze the cleansing water
Use other methods for confirmation/indication
• ELISA • LFD • PCR • LC-tandem MS
Consider any remaining risks
• The calibrator and analyte extracted from the matrix should be comparable • If the analyte is fragmented, then a competitive ELISA should be used

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