

Analytical Testing as a Tool for the Enforcement of Future Regulatory Thresholds for Food Allergens

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Food allergen labeling regulations have been implemented in several countries since 2006. Currently, experts are still discussing the introduction of thresholds or action levels, which should lead to the reduction of the widespread use of advisory statements (e.g., “may contain”) for the benefit of the allergic consumer. However, the establishment of threshold requires supporting analytical methodologies to enforce and comply with the regulations. This article discusses the possibilities and limitations of existing and emerging methodologies for the purpose of enabling compliance with and enforcement of allergen action levels.

Regulatory Framework

To protect the allergic consumer, regulatory bodies have implemented labeling requirements to identify the offending allergen in packaged food products. In Europe the labeling regulation for allergens (Directive 2000/13/EC) and further amendments (consolidated in 2007/68/EC) include the major eight allergens originally recommended by Codex Alimentarius (milk, egg, peanut, tree nuts, soybean, cereal containing gluten, fish, and crustacean shellfish) as well as celery, mustard, sesame seed, lupine, and molluscs. Although these requirements only affect allergens used as ingredients, the potential for undeclared allergens in foods is gaining attention, as it is a main food allergen management issue and it is not addressed by the directive. Moreover, the directive neither provides action or threshold levels nor specifies the means to be used for identifying and quantifying the allergen. The lack of regulatory thresholds is partly explained by difficulties in determining the clinical threshold due to the limited availability of clinical data from double-blind placebo-controlled food challenge (DBPCFC) tests and the lack of standardized protocols for diagnostic purposes. The symptoms, severity of the allergic reactions, and eliciting dose vary widely not only within the allergic

population but also within an individual, which, in addition to the intrinsic complexity of food and the way it has been processed, contributes to the difficulties in establishing thresholds. Japan is the only worldwide jurisdiction that has adopted regulatory thresholds at 10 mg/kg (ppm) soluble protein. In Australia, the Allergen Bureau (a food industry initiative) has proposed the VITAL (Voluntary Incidental Trace Allergen Labeling) grid, a standardized allergen risk assessment tool for food producers that provides action levels (inclusion or exclusion of the allergen from the ingredient list or use advisory labeling), but this document has no legal relevance (1).

Although regulatory agencies have provided options for establishing thresholds, they take a more passive position on defining the actual value. Food allergen labeling regulations were implemented mainly to protect the allergic consumer. Among other consequences, the lack of thresholds leads the industry to adopt risk management measures based on uncertainty rather than health risk to address incidental contaminations, not addressed legally or via guidelines, including the proliferation of advisory labeling (e.g., “may contain”), which is justified in a few situations only. The assessment of risk, which should be the responsibility of the industry, is therefore imposed on the consumers, who see their choices significantly reduced. The allergic consumer usually reacts in two different ways to advisory labeling. On one hand, the cautious consumer avoids any product bearing advisory statements, thereby significantly reducing food choices and quality of life due to increased anxiety. On the other hand, the less anxious consumer takes the risk of consuming the product assuming that the likelihood of the product to contain the allergen in amounts triggering a reaction is very low. In general allergic consumers, their families, and surrounding environment (e.g., schools, friends) live under a constant situation of alert and anxiety ultimately affecting their day-to-day lives.

Testing is a main tool for enforcement activities and a main component of the industry’s allergen control programs to ensure compliance. However, given the lack of thresholds, is it possible to enforce regulatory thresholds by using available analytical options as part of the activities aimed to ensure product safety? How do test results correlate with clinical responses? This paper addresses the problematic and it

describes the advantages and limitations of available and emerging analytical opportunities for industry compliance and enforcement of regulatory thresholds when established.

Thresholds in the Context of Clinical and Analytical Variability

Unlike analytical chemistry, testing based on biological systems is subject to a higher degree of variability. Responses concerning the immune system, such as food allergies, are highly variable because they involve complex mechanisms, some of which are still not fully understood. The variability of clinical assays for the diagnosis of food allergies and analytical assays for detection of food allergens have been widely discussed in many forums, and experts try to find an approach to harmonize and standardize the development of testing and validation protocols (2–4). Analytical variability and accuracy are highly scrutinized and criticized because of their impact in regulatory enforcement and compliance activities. However, clinical variability is also a fact and, to some extent, unpredictable. The variability in both analytical and clinical testing depends on the food type, degree of processing, and food composition. Moreover, clinical variability also depends on the physiological state of the individual's gastrointestinal system, frequency of allergic episodes, the concomitance of other pathologies, and the effects of other food components in the development of an allergic reaction. For these reasons threshold values of clinical significance are difficult to establish, although several approaches have been proposed (5, 6).

The questions are: How can analytical techniques be used to support the enforcement of threshold values of clinical relevance? How do analytical results correlate with allergic episodes? Before these questions are addressed, it is necessary to find a common element between clinical and analytical testing, a standardized and well-characterized reference material that could be used to compare and correlate results from both analytical and clinical testing and evaluate their clinical significance. Ideally, this reference material should be well characterized, stable under storage conditions, and widely available to testing laboratories; it has to be relevant to the testing procedures and its homogeneity between batches must be guaranteed. The criteria for the selection of a proper standard reference material, its characterization, and further validation have been the main issues that remain to be solved and agreed upon. Why are standardization and characterization of reference materials needed? There are several reasons with main impact in analytical and clinical reproducibility and variability: There are a wide number of allergenic food materials that can be used and that may differ in origin (strain, breed, or variety of a particular allergenic food, geographical production area, soil composition) that determine the allergen composition in terms of type and quantity. The way the allergenic material is processed affects the availability and the chemical-physical characteristics of the allergen. For example different types of peanut flours can be used for testing, such as light, medium, or dark roasted

peanut flour, that may have been obtained by either dry- or oil-roasting. In addition, these materials may be used differently in different tests. Oral provocation tests usually use the full material as is. However, other in vivo tests, such as skin prick and in vitro testing, require the use of extracts, in which the allergens are extracted from the matrix usually by using an aqueous solution. Because the recovery of the protein is likely to be incomplete during extract preparation, especially in processed foods, is the unrecovered allergen still allergenic?

Traditionally, reference materials used in diagnostic testing or to validate analytical methods are usually spiked food matrixes with no further treatment. However, because most of the foods consumed are processed in some way, recent discussions are addressing the possibility of using incurred materials (instead of spiked matrixes) for testing purposes. Incurred materials are produced by adding a known amount of allergenic reference material to the food before it is processed (under industrial conditions). The allergen is therefore processed along with the other food ingredients. This production has to be standardized to guarantee both the homogeneous distribution of the allergen in the product and consistency between lots.

What Is the Importance of Using Either Spiked or Incurred Material for Testing and How Does It Relate to Thresholds?

Although spiked matrixes are more representative of food products contaminated without or after food processing, incurred materials are more representative of processed products contaminated before or at some stage during processing. The allergen in the incurred material is therefore exposed to further physical and chemical conditions that can lead to modifications of its structure, stability, and solubility, such as Maillard reaction products.

Several processing activities are used for food production. Thermal processing is one of the most commonly used by the food industry. Two main categories of thermal processing are distinguished by the use of water as a heat transfer media: dry (oven roasting, oil roasting, infrared heating, and ohmic heating) and wet (pasteurization, microwave cooking, pressure cooking, extrusion, blanching, boiling, and steaming). Nonthermal processing procedures include irradiation, soaking, germination, milling, fermentation, mixing, homogenization, high-pressure processing, dehulling and dehiscing, and grinding.

Resulting from food processing, allergens can be denatured, hydrolyzed, precipitated, aggregated, and bound to other food components. In processed foods, allergens may be present in several of these forms. For example, a cookie may have a mixture of native, aggregated, denatured, and glycosylated form of a particular allergen, and the most drastically modified proteins are those present in the surface of the cookie where the exposure to heat is higher. It is necessary to emphasize that reference materials such as dry milk powder and roasted peanut flours used for spiking and to produce incurred materials have already undergone some type of food processing. The consumption of real raw materials is mostly limited to fresh

fruits and vegetables, which are not subjected to specific labeling requirements. Consequently, the use of incurred materials is more desirable for validation and testing of foods contaminated before processing. Although the use of incurred materials is currently used as a substitute for spiked materials, which is traditionally used in performance/validation studies, spiking should still be a valid approach since contamination also occurs after food processing.

Protein modifications due to processing may affect protein solubility, which is critical in preparing the extracts mentioned above and used for some clinical and in vitro testing. Also, modifications concerning the structure of the protein may affect its recognition by the human immune system, specific IgE in allergic individuals, and by antibody-based analytical techniques. The diagnosis by provocation studies such as DBPCFC has the advantage of using the full material (no extraction needed). However, for other types of tests only the soluble protein fraction can be used. Because provocation studies use the entire material, individuals are also exposed to the insoluble protein fraction. However, the allergenic potential and the bioavailability of this fraction as well as other protein complexes (e.g., protein aggregates, protein-polyphenol complexes) is unknown. Studies on the modification of allergenicity due to processing have been carried out mostly using purified soluble proteins in in vitro systems. Although these studies indicate potential allergenicity, it is difficult to extrapolate their conclusions to actual individual responses of patients to those specific allergens, which also involve the effects of other food components and food processing on the overall response.

Some current expert discussions are addressing the question of whether a reference or incurred material should be validated first in human subjects or by detection methods. It is clear that both need to be done and their advantages and limitations need to be acknowledged. Detection methods are usually reproducible. However, although comparison of different commercial brands of analytical techniques (e.g., ELISA) shows variability, they offer the advantage potential for improvement and standardization, which would help to decrease assay variability. However, this is not an option for allergic individuals since human responses in clinical studies are not always predictable; for example, the same individual can respond differently to the food he is allergic to in different episodes. Therefore, clinical variability within individuals and within the allergic population makes clinical validation of testing materials difficult to interpret and normalize.

Critical characterizations for using either spiked or incurred reference materials are the characterization of the allergenic food ingredient (e.g., milk powder, egg powder, peanut flour, etc.), the standardization of protocols for producing these materials, and its suitability for use in clinical and analytical tests. Currently there are numerous efforts to produce and validate incurred materials. However, they are not standardized in the sense that different projects use different allergenic food ingredients and different ways to produce incurred materials. The lack of standardization in the use of reference materials for use in clinical studies and

validation of ELISA kits has been historically criticized as a source of variability and as an impediment for assay comparison. As long as there is no consensus in the use of reference materials, it will be very difficult to correlate clinical and analytical results, which becomes more complex with the use of incurred materials. Once reference materials and incurred materials are appropriately characterized and validated, they could be used to provide data needed to correlate clinical data and analytical values.

Quantitative Detection Methods

It would be ideal if allergenicity of food samples could be measured. In vivo testing in animal models is a theoretical option. However, there are no animal models available with the purpose of evaluating the potential allergenicity of allergens in food samples. Even if they were available, it would be very expensive and impractical. In vitro testing using human IgE would be another option, but the lack of available human sera and individual variation make quantitative IgE-based testing practically impossible. With the current technology available, it is only possible to detect the presence of either the offending allergens, regardless of their allergenicity status, or their markers. Therefore, correlation of allergic reactions and analytical results can only be based on the detection of targets (extracted from foods) of undefined allergenicity potential.

Quantitative detection methods are critical to provide useful information for risk and safety assessment activities and to enforce and comply with regulatory labeling requirements once thresholds are established. The quantitative feature of detection methods is useful if they have been validated using a validated reference material and if the deficiencies of each method for particular matrixes are known. Validation guarantees that the assays perform properly in trained hands within established criteria.

The success of detection methods for food allergens depends on several elements. Understanding the role of these factors in the overall assay facilitates the development and improvement of analytical techniques. Moreover, it provides the basis to allow a robust interpretation of the potential clinical and regulatory implications of the outcome values.

Sample Type

The degree of recovery of the target analyte from the sample is influenced by the food composition and the type and degree of processing conditions.

(a) *The characteristics of sample (e.g., pH) and its composition (e.g., fat, sugars, polyphenolic compounds).*—Food components like fat, sugar, and polyphenolic compounds are known to interact with proteins and DNA (typical target analytes in allergen analysis). Food processing conditions may also have a negative effect on the extractability and availability of the target for testing. Maillard reactions take place when sugars interact with proteins in food undergoing thermal treatment, which can make allergens unavailable for testing.

(b) *Food processing (e.g., heating, hydrolysis).*—All these factors affect the recovery of the sample and therefore the analytical value obtained. For each analytical method, the same sample composition may have different impacts; for example, DNA (PCR analysis or equivalent) is strongly degraded in a low pH environment while the target proteins may still be stable. On the other hand, some proteins are known to be heat labile while the DNA is to a lesser extent.

Sample Preparation

In this step, the sample is homogenized and the target is extracted from the sample by using the appropriate sample extraction solution. This is a critical step that has gained attention during the last few years, as it severely affects the recovery of the allergen from the sample. The features of sample extraction solution, such as chemical composition, ionic strength, and pH, determine the ability of the buffer to extract the allergen from the manufactured product or food ingredient and minimize or reduce the effect of other potential interfering food components and the matrix. Also, the extraction solution has to be compatible with the analytical technique. Commercial detection methods for food allergens do not include homogenization protocols, and using homogenizers in sample preparation and extraction is encouraged. Difficult samples, such as chewy granola bars, present a challenge for the analyst to obtain representative samples. Currently, several research projects are aiming to improve food allergen sample preparation by focusing on extraction since this is a very critical step for further analysis, detection, and quantification.

Analytical techniques can only detect the target if it is in the sample extract. Some samples lead to poor allergen recoveries. In some situations, targets are not recovered because the sample extraction procedure is not optimized. However, there are occasions where analytes cannot be extracted because of the conditions of either the target or the sample or both, regardless of the extraction procedure used. For example, heating has been shown to have a negative impact on the recovery of milk, egg, and peanuts, regardless of the extraction procedure used (7–10), and low pH of salad dressing leads to low recoveries of mustard, egg, milk, and gluten in ELISA testing (11). Therefore, an analytical technique cannot always be blamed for not detecting an allergen that is not present in the sample extract.

Analytical Techniques

Two major groups of techniques are used for the detection of food allergens: those that detect the allergenic/antigenic protein itself (ELISA and MS) and those that detect the species-specific DNA (e.g., PCR), which is considered a marker for the presence of the allergen in the sample. These techniques have to be specific, sensitive, accurate, and reproducible. Assay sensitivity has to be as low as regulatory threshold levels, which should be below the minimum level triggering allergic reactions (clinical thresholds). However, since regulatory thresholds do not exist, with the exception of

the Japanese regulation, LOQs may serve as practical values for management purposes.

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA has been the traditional assay used for the detection of food allergens in foods. It is a very versatile analytical technique that allows allergen quantification at concentrations suggested by experts as safe below clinical thresholds. The advantages and limitations of ELISA are determined by its biological nature. ELISA uses biological molecules, antibodies produced in animals or in cell cultures, as target-capturing and detecting components. As a biological assay, ELISA is sensitive to variations in assay conditions, which include the target as well, and is more susceptible to modifications of allergen structure. Because of their multiple specificities, polyclonal antibodies allow a certain degree of allergen modification compared to monoclonal antibodies, although sensitivity may be affected. The versatility of immunoassays allows the establishment of different approaches for assay improvement. Generally, the assay design combines the optimization of antibody type and specificity, assay type (competitive, sandwich), and assay buffers, including sample extraction buffer.

Antibodies, which are also proteins, are susceptible to environmental assay conditions, and therefore the buffers and other solutions used in the assay need to preserve the antibody tertiary structure and favor their binding to the target molecules. Traditionally, extraction buffers used in immunoassays are characterized by having neutral pH or slightly alkaline and moderate ionic strength. New extraction solutions have been developed to improve the extraction of food allergens from processed food samples (12). These solutions contain denaturing and reducing components that are aimed to solubilize aggregated allergens. In principle, these buffers are incompatible with the assay, with the antibodies in particular, but they are usually diluted so that their components are present in concentrations that do not pose any problem in assay performance. When such solutions are used, antibody specificity needs to be redefined to target the denatured and reduced form of the protein. Reducing and denaturing solutions have led to the improvement of ovalbumin and γ -lactoglobulin recovery, which tend to aggregate during heating. However, they do not show any improvement in the extraction of casein from baked foods when compared to traditional buffers (9). Although this new extraction approach seems to work well for some allergens, collateral effects of the diluted solution need to be explored in depth, such as the effect in potential refolding of proteins and stability of the antibody-allergen binding. Although antibody-based assays are very versatile, the nature of the assay may lead to unwanted false positives due to antibody cross-reactivity to nontarget proteins or nonspecific binding to other food components.

ELISA can be used quantitatively, although results are known to vary among commercial assays. This variability is mostly due to the lack of standardization, including antibody specificity, calibration material, sample extraction solutions,

and assay reporting units. Japan is the only jurisdiction where authorities have established a standardized protocol for the development of ELISA kits for the detection of food allergens. It includes the use of common reference materials aimed for antibody production as calibrating material and in kits as standards for quantification purposes. The standardized protocol involves the sample extraction protocol and reporting units. Japanese officials have also established the criteria for validating these assays.

In addition to the advantages such as versatility and quantitative capabilities, ELISA can be used as a high throughput technique. Another antibody-based assay is the lateral flow device, which is a qualitative single-use test usually used for screening purposes. ELISA is an available tool with some restrictions and can be used to enforce regulatory thresholds in most situations since assay sensitivity allows allergen detection at levels well below those considered safe for allergic sufferers. Although accurate quantification of allergens in highly processed foods is still an issue, current ELISA kits are able to indicate the presence of allergens semiquantitatively at low, medium, or high concentrations in this type of sample. However, it is evident that these assays do not determine the allergenicity status and bioavailability of allergens in these samples, which have unknown clinical implications.

Mass Spectrometry (MS)

MS, usually combined with LC, is an area of increasing interest with great potential for the detection of food allergens. Mass spectrometers have been used for the detection, identification, and characterization of small molecules like mycotoxins, veterinary drug residues, and forbidden dyes, but the latest improvements in the technology allow for the application of this powerful equipment to detect larger molecules, such as proteins or peptides.

There are several major advantages of MS over ELISA. MS has the ability to directly detect the allergens since it does not rely on any intermediary detecting component such as antibodies in ELISA. It also provides an unambiguous detection of the allergen, eliminating false positives due to cross-reactivity, a common issue in ELISA testing. In the most common MS testing approach (MS/MS), the allergen is enzymatically digested into several peptides. A careful selection of the peptides of interest, used as allergen fingerprint, determines the unequivocal presence of the allergen in the sample. The selection of peptides is based on their abundance and stability in processed foods. Another advantage of MS over ELISA is that the detection of allergens is not affected by structure modifications. Moreover, with MS it is possible to elucidate some structural changes, such as the addition of other molecules like glycosylation and phosphorylation.

The extraction of food allergens from food samples, especially from those that have been highly processed, is a major challenge for the detection of allergens regardless of the analytical technique used. In principle, the use of MS allows for a wider range of extraction possibilities than ELISA,

including those that would affect allergen and antibody configuration and binding.

Some of the issues that MS yet has to overcome are that sensitivity may not reach low ppm levels, which have been suggested as potential thresholds, and quantification is not accurately possible yet. However, new approaches have shown promising results in terms of sensitivity and quantification potential. In addition, MS has a great potential for multiallergen screening. Looking at the European Regulation, the 14 groups of food ingredients regulated for labeling under Commission Directive 68/2007/EC include 26 allergenic food targets for which the identification of appropriate precursors for MS analysis can presumably be analyzed in a single analysis run. Because MS is a confirmatory analysis with the robustness to stand up in court, it has a very high potential as a detection method in the near future.

DNA-Based Technologies

The detection of DNA has been used as a marker for the presence of allergens in food products. PCR, real-time PCR (RT-PCR), and ligation-dependent probe amplification are the techniques used to detect residues of allergenic commodities in food. Although some of these techniques (RT-PCR) can be used for quantification purposes, they are mainly used as qualitative assays for allergen detection. Several publications have shown consistency between PCR and ELISA for some allergens in some food matrixes. Currently, DNA-based assays are the only option for the analysis of some food allergens for which ELISA is not available. These assays are easier to develop than ELISA because all necessary components are commercially available and do not require animal facilities or maintenance of cell lines. However, PCR assays require laboratory infrastructure, such as dedicated rooms, that is not practical for many food facilities or standard food laboratories.

DNA-based assays are an option for enforcement purposes as a qualitative tool, but some aspects need to be considered before contemplating them as a testing tool. The application of these assays to the detection of allergens is controversial because they do not detect the food allergen itself and the concentration of DNA may not correlate with the presence of the allergenic protein or its concentration. Several factors contribute to this possible inconsistency. The expression of allergens may vary between plant varieties or under different growing conditions. Due to food manufacturing, the DNA may be present while the protein is not or vice versa. DNA and protein may have different stability properties in different food processing procedures. Moreover, there are products formulated with protein fractions that may contain a reduced concentration of DNA. The detection of egg and milk residue by PCR is not considered an option for two reasons. First, the abundance of DNA in these two products is very low, which jeopardize assay sensitivity. Second, DNA is not tissue specific, and the use of PCR for the detection of egg and milk could provide false negatives. For example PCR can be positive for milk due to the presence of beef meat in a product. The quality and integrity of the DNA template and the

presence of assay inhibitors are key elements for a successful assay. Like protein-based assays, food processing and food matrix may negatively impact the assay, and a proper sample preparation is a requirement to ensure the target availability for further analysis. It is very likely that after improvement of the MS technology and its quantification capabilities, MS will substitute the use of DNA-based techniques as an alternative to ELISA.

Interpretation of Results

The interpretation of results seems an easy task a priori. However, the complexity of allergen detection and the lack of clear regulatory definitions for the mandatory labeling of the presence of food allergens and regulatory testing requirements lead to the use of different reporting options and difficulties in comparing data from different sources. Standardization at this stage of the detection methodology is needed to ensure assay accuracy and consistency among results from different assays.

To fully understand the variability, accuracy, and meaning of testing results, two aspects need to be considered in addition to assay specificity and protein recovery. These factors, which are commonly overlooked or not fully understood, are the use of conversion factors, and the calibration material and how it is used to calibrate the assays. Assays detect either protein or DNA. However, because regulatory requirements from some jurisdictions define food allergens as a whole food commodity instead of the allergenic protein or DNA, tests report the results either as whole commodity or as protein with an associated conversion factor. Conversion factors are constants and are based on the concentration of total protein in the commodity (w/w). Protein determination methods are not standardized either. However, not all the entire protein fraction is soluble and its solubility depends on the type of extraction solution used and the degree of processing of the sample. Therefore, conversion factors should only consider ratios based on soluble protein. For example, raw peanuts contain about 25% protein (ratio 1:4). However, raw peanuts are rarely used in the food industry. Peanut flour with different defatting and roasting degrees is more common. Peanut flour contains between 33% (partially defatted flour) and 50% (defatted flour) protein (ratios 1:3 to 1:2), respectively. However, this is total protein, and ELISA tests only determine soluble protein. Because soluble protein varies with the extraction solution used and it is reduced through processing, the number in the ratio denominator varies as well (1:*n*). Conversion factors can introduce additional uncertainties to analytical results. Another example is the use of a default factor of 2 to convert gliadin into gluten, which is based on the gliadin/gluten ratio in some wheat varieties. However, this ratio varies depending on the cereal species and their varieties (13). Therefore, conversion factors are not really constants and their use should be handled with caution.

The fact that assays may be calibrated against different calibration material, such as whole commodity (e.g., peanut flour, egg powder, milk powder) versus proteins (e.g., casein,

-lactoglobulin, ovalbumin) and the use of conversion factors to transform units may impact the final values and contribute to assay variability. Moreover, the units used by the manufacturer to report results make comparison of results from different kits difficult. For example, commercial ELISA kits for egg allergens differ in the reporting units they use, e.g., ppm whole egg, ppm egg white protein, and ppm egg white. Assays with different units cannot really be compared, making validation studies more difficult to design. Powdered commodities are usually used to spiked food matrixes for validation purposes. However, if an assay has been calibrated against purified protein and it reports the result as ppm protein, recovery cannot be calculated unless the content of the target protein in the reference powder material is known or, alternatively, a conversion factor for that material is used. To guarantee the accuracy of validation results, it is key to know what material was used to calibrate the assay, how the assay was calibrated, what the reporting units are, and how close the calibration material used by the manufacturer is to the material used for validation. The availability of reference materials and assay standardization, including calibration protocols and reporting units, is needed in order to make kits comparable and to ensure the performance of the assay in validation studies.

An additional aspect is the distribution of the allergen in the product in sporadic contamination, e.g., by random presence of particulate allergen in the final product, such as pieces of peanuts in a chocolate bar, where peanut is not an ingredient. In cases where the allergen is heterogeneously distributed in the product, it is likely that analytical results do not correlate with the clinical reactions. For example, a consumer may have a reaction after eating the part of the product containing the allergen in high concentrations, while the remaining portion used for testing (if available) may contain undetectable or low concentrations of the offending allergen.

Approach to Establish Threshold Levels Based on Analytical Testing

Whichever threshold or action level legislators may set, the actual wording needs to be carefully selected because it is currently not possible to determine the true content of allergens with any of the technologies for the majority of samples. One option could be to evaluate several key matrixes (e.g., chocolate with high/low cacao, biscuits, and ready-to-eat meals) with all available technologies to determine the variability in results, determine the overall CV, and then assume the worst case scenario, i.e., the highest deviation, as still acceptable for any value determined for this kind of food matrix.

Another alternative could be to allow any result that is reproducible with the same validated method (in-house or multilaboratory validated). This means that if, for a target analyte, three validated assays are available, and the result has been produced with one of the three, the same assay needs to be used with the aim to reproduce the result. If the result obtained can be reproduced by another laboratory using the

same kit, this result is considered confirmed. This kind of approach would ideally be built into the legislative wording to allow open platform technologies as long as trueness determination for allergen content is not possible.

Conclusions

If regulatory threshold or action levels are introduced, the three techniques mentioned (PCR, ELISA, and MS) may be still the best options for compliance and enforcement. Commercial analytical techniques currently used for the detection of food allergens, especially ELISA, provide quantification capabilities in ranges of allergen concentrations thought to be safe at least for the majority of the population. Although accuracy may be compromised in highly processed foods, it is unknown whether the availability and potential allergenicity of allergens in the product have the same effect as in a less-processed food. So far, most clinical studies have involved lightly processed products, and it is not entirely clear how processed (e.g., glycosylated, aggregated, or insoluble) proteins contribute to triggering an allergic response. The results obtained and any threshold set based on these studies may only be accurate for similarly lightly processed products, while most baked, pH, or pressure-treated material may have a completely different effect. ELISA has been traditionally used to detect food allergens because of its simplicity of use and ability to detect the allergenic protein, and because of the lack of alternative detection methods. ELISA is sensitive to modifications of the protein structure jeopardizing the antibody-allergen binding. However, it is more limited than other technologies in the number of sample extraction buffers that can be used, which is mainly due to incompatibility issues. A promising alternative to ELISA, MS is emerging as an application for food allergen analysis with great potential for screening, confirmation, and quantification purposes, and this proteomic approach should be encouraged. Although all techniques will suffer from recovery issues, more effort should be focused on sampling plans and extraction protocols. This would help recover the protein from difficult samples (e.g., low pH, presence of polyphenolic compounds) or from those that have been highly processed, and would ultimately provide more accurate analytical results for these samples. Because these approaches are not possible at this time, only matrix-matched standards can provide accurate information on how much allergen is actually present in the matrix that may affect the allergic consumer.

It is important to acknowledge not only the limitations of analytical techniques but also their advantages. Analytical techniques may not be the only options to ensure safe foods, considering the allergen measures taken by the food industry, but they are the only option to actually demonstrate the presence of allergens. In addition, most unexpected allergic reactions occur in public places such as restaurants where labeling and identification of the allergen is currently not regulated. However, analytical techniques can always be optimized and improved, and advances in technology may

allow fine-tuning of some of the current and emerging technologies.

Levels to be set as regulatory thresholds need to be enforceable. Therefore, even if threshold or action levels are set in legislation, any technology will inevitably only estimate the true content of the allergen, which may ultimately lead to controversies when results from different methods are being used unless legislators or regulators define the conditions for testing and operational limits of the different analytical platforms.

Although not all of the methods are perfect and some have more drawbacks than others, the introduction of a threshold or action levels would be beneficial to all. Thresholds are currently unofficially used by most enforcement agencies and food-producing companies. The lack of thresholds is leading to more conservative approaches pursuing zero tolerance. Like those used for food contaminants, the definition and establishment of thresholds should be based on the reasonable certainty of no-harm principle and the assessment of risk versus benefits. The lack of commitment of regulatory agencies to define and implement thresholds is partly a result of focusing primarily on the risk with very little consideration to the benefit they would bring. Some of the benefits include the establishment of fit-for-purpose allergen control strategies in the food industry (e.g., as part of Hazard Analysis Critical Control Points and Good Manufacturing Practices), minimization of unnecessary recalls, facilitation of trade and harmonization of testing options, as well as improvement of the options to communicate the risk to the consumer through more accurate labels that would ultimately have a positive impact in consumers choices, confidence, and quality of life. Regulatory agencies and food producers should also pay attention to the effectiveness of regulatory and industry initiatives (i.e., the Japanese government and Australian VITAL system) that are already implementing threshold levels based on risk assessment and are setting a precedent.

We recommend the introduction of threshold or action levels; however, the regulatory framework surrounding these levels needs to be carefully defined to accommodate for the uncertainties still existing due to the present limitations of analytical methodology used to determine those values.

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