Anal Bioanal Chem (2009) 395:83–92
DOI 10.1007/s00216-009-2944-0

REVIEW

Allergen immunoassays—considerations for use of naturally incurred standards

Steve L. Taylor · Julie A. Nordlee · Lynn M. Niemann · Debra M. Lambrecht

Received: 7 January 2009 / Revised: 26 June 2009 / Accepted: 29 June 2009 / Published online: 30 July 2009

Abstract The enzyme-linked immunosorbent assay (ELISA) offers many advantages for the detection of potentially hazardous allergenic food residues that might become adventitious components of other foods during the course of food production and processing. ELISAs detect proteins, and food allergens are proteins. ELISAs are sufficiently sensitive and specific for detection of food allergen residues. ELISAs can also be produced in formats that are compatible with the industrial food processing environment. However, ELISAs also have disadvantages that should be carefully evaluated and widely recognized. Various food-processing operations can have profound effects on the detectability of allergenic food residues. ELISAs detect intact proteins but protein hydrolysates evade detection in some ELISA formats. The residual proteins present in some ingredients derived from commonly allergenic sources may also not be easily detected with ELISAs because of the nature of the protein residues remaining, e.g. lipophilic. Processing operations can dramatically lower the solubility of proteins. In some food formulations, heat processing, in particular, induces chemical modifications that can affect antibody binding to epitopes in the ELISA. The use of naturally incurred standards where allergenic food residues are incorporated into various representative food matrices and then processed in a manner similar to "real-world" food processing can reveal some of the limitations of allergen ELISAs. Methods for the preparation of naturally incurred standards in chocolate, cookie, muffin, ice cream, pasta, frankfurter, and cream of potato soup are provided as examples.

Keywords Allergen · Food · Immunoassay · ELISA · Validation · Incurred

S. L. Taylor (✉) · J. A. Nordlee · L. M. Niemann · D. M. Lambrecht
Food Allergy Research & Resource Program, Department of Food Science & Technology, University of Nebraska,
255 Food Industry Bldg.,
Lincoln, NE 68583-0919, USA
e-mail: staylor2@unl.edu

Introduction

Over the past several decades, food allergies have become recognized as a significant food-safety issue. During this period, development of analytical methods for the detection of residues of allergenic foods has been important to the protection of food-allergic consumers. These methods allow food companies to determine that incoming raw materials do not contain detectable residues of undeclared allergens and that sanitation programs are effective in removing residues of allergenic food from shared equipment and facilities, and to verify the effectiveness of overall allergen-control programs. These methods also allow public health authorities to investigate consumer complaints and monitor the presence of undeclared allergens in the food supply.

Of course, analytical methods for the detection of residues of allergenic foods must be highly specific, sufficiently sensitive, rugged enough for use in food matrices, and reliable. Because of the nature of food allergies and allergens, the choice of detection methods for allergenic food residues can be challenging and the reliability of the results obtained from the selected methods can be an important consideration.

Food allergies involve abnormal responses to specific foods and particular proteins contained therein; they occur in specific individuals and are mediated by the immune system [1]. Both IgE antibody-mediated (immediate hyper-

sensitivity) and cell-mediated (delayed hypersensitivity) mechanisms are known to occur. IgE-mediated food allergies can be provoked in susceptible individuals by one or more of a wide variety of foods. Celiac disease appears to occur via a cell-mediated mechanism and is specifically associated with gliadin (gluten) and related proteins from wheat, rye, barley, and related grains.

The worldwide prevalence of food allergies is not specifically known. However, evidence suggests that IgE-mediated food allergies occur in 3.5–4.0% of the US population [1, 2]. The prevalence of IgE-mediated food allergies is higher in infants and young children than in adults [3]. The prevalence of IgE-mediated food allergies also seems to be growing although the reasons for this increased prevalence are unknown [4]. Celiac disease occurs with variable prevalence in different countries although the differences may be partially due to the choice of diagnostic methods. In the US, celiac disease has a reported prevalence of about 1 in every 133 persons, although only about 1 in every 2000 individuals have symptomatic celiac disease on ingestion of gluten [5].

The symptoms associated with food allergies are frequently mild and easily treated, but can sometimes be severe and even life-threatening. IgE-mediated food allergies, in particular, are occasionally associated with severe reactions. Fatalities have occurred from the inadvertent ingestion of an allergenic food [6, 7]. Celiac disease is not especially life-threatening although severe sequelae such as lymphoma are associated with it [8]. Thus the undeclared presence of allergenic food or gluten residues has serious public health consequences to a reasonably large fraction of the sensitive population.

The threshold dose needed to provoke symptoms in individuals with food allergies or celiac disease is also quite low [9]. Individual threshold doses for IgE-mediated food allergies can be quite variable ranging from low milligram amounts to as much as eight grams or more [10]. For celiac disease, most evidence suggests that concentrations below 100 parts per million (ppm) in the diet are safe [11]. Analytical methods should be appropriately sensitive to protect the most sensitive individuals in the population, but greater sensitivity is not necessary and can be counter-productive if use of such assays leads to over-labeling and the exclusion of otherwise safe products from the diets of allergic consumers [12].

Immunoassays have become the most widely used approach for the detection of residues of allergenic foods that might contaminate other foods [13]. This review is oriented toward the advantages and disadvantages of immunoassays in the detection of residues of allergenic foods. However, other analytical approaches do exist, including polymerase chain reaction (PCR) and quantitative mass spectrometry [14, 15]. Of course, these methods have their own advantages and disadvantages but can occasionally be used to advantage for comparative purposes against immunoassays [15].

Immunoassays

Many different types of immunoassays exist. All immunossays use some form of antibodies to detect proteins. In the analysis of allergenic foods, the sources of the antibodies are usually either polyclonal antisera raised against specific proteins or mixtures of proteins or monoclonal antibodies usually made specific to a particular protein or peptide. These antibodies are principally IgG although IgY antibodies can also be produced in egg yolks. These antisera/antibodies are used to develop enzyme-linked immunosorbent assays (ELISAs). Alternatively, human blood serum from individuals allergic to the specific food can be used. These antibodies are IgE and are highly specific to the protein components that are allergens affecting the individuals providing the blood serum. These antisera are used to develop radioallergosorbent (RAST) assays.

ELISAs and their advantages

ELISAs are now in widespread use for the detection of allergenic food and gluten residues. ELISAs have numerous advantages for the detection of such residues. IgE-mediated food allergies and celiac disease are caused by specific proteins [16]. However, allergenic foods contain hundreds of proteins and only a few of these proteins are known to be allergens [17]. Some foods, such as peanuts and milk, have multiple allergenic proteins, while other foods, such as shrimp and fish, appear to have only one major allergen [16]. ELISAs specifically detect proteins so these assays are targeted toward the most appropriate analytes. Some ELISAs specifically detect certain proteins or allergens. Examples include a peanut ELISA that specifically detects a major peanut allergen (Ara h 1), ELISAs for casein and β-lactoglobulin, and an ELISA for shrimp tropomyosin. Other ELISAs are targeted against mixtures of proteins from the allergenic source. Examples include ELISAs for total milk, egg (often egg white), peanut, almond, and many others. The capture antibodies in sandwich ELISAs can be either monoclonal or polyclonal. Monoclonal antibodies are clearly more specific and oriented toward a particular epitope on a certain protein. One of the more prominent examples is the R5 antibody against a particular epitope from gliadin/gluten [18]. Polyclonal antisera can be directed against either a single protein or a mixture of proteins from the allergenic source.

ELISAs have considerable advantages over the RAST. RASTs are used diagnostically on the serum of human subjects to determine if these individuals have IgE antibodies against one or more foods or food proteins, a clinical indication of food allergy. Of course, sera from human subjects can be an alternate source of antibodies to use in testing foods for allergen residues. Such testing is usually done in a competitive immunoassay format known as RAST inhibition. In fact, RAST inhibition testing preceded ELISAs as a method for detection of allergen residues [19]. Certainly, human sera have relevant antibodies directed specifically against the allergenic proteins of interest. However, human serum sources can be quite variable with IgE against multiple allergenic foods leading to false positive results on occasion. For example, we experienced a false positive response during the analysis of an ice cream sample for possible peanut contamination some years ago that was attributed to the use of a serum source that had IgE against macadamia nuts which were one of the acknowledged ingredients of the ice cream (unpublished observation). Also, human sera are not readily available to most analytical laboratories and the use of radioisotopes is another disadvantage in many settings.

ELISAs can be quite sensitive. Most commercial allergen ELISAs have lower limits of quantification in the range of 1 to 2.5 ppm with gliadin ELISAs having a limit of quantification of 5 ppm gliadin or 10 ppm gluten. From a public health perspective, the sensitivity of ELISAs should be matched against the threshold dose for the allergenic food being detected. While scientific consensus does not exist regarding threshold doses for allergenic foods, the available clinical data indicate that the most sensitive individuals for peanut, milk, and egg react to levels of 0.5–1 mg of the whole allergenic food (as opposed to protein) on the basis of provocation of mild objective symptoms [9]. The threshold doses would be lower if considered on the basis of provocation of even milder subjective symptoms such as mouth itching, etc. But, a recent study indicated no difference in the prevalence of occurrence of subjective symptoms between placebo and peanut in a clinical challenge trial of peanut-allergic individuals [20]. If a typical serving size is 50 g, then 1 mg of the allergenic food corresponds to a concentration of 20 ppm. Thus, the limit of quantification of existing commercial allergen ELISAs is adequate to protect food-allergic consumers.

ELISAs have other important advantages. ELISAs are comparatively easy to use and the technology can be transferred into food industry-friendly formats such as swabs and lateral flow strips that can be used in food manufacturing facilities. In most cases, ELISA results are reproducible within the same sample/extract/ELISA kit. However, as will be seen later, different ELISAs of the same type made by different manufacturers can provide quite different results on the same sample due to differences in the ELISA format. ELISAs, especially when converted into commercial kit form, allow relatively short analysis times. Swab and lateral flow test results can be available within minutes and quantitative ELISAs can be done in less than 60 min.

Disadvantages of ELISAs

ELISAs should not be used without clear recognition of their inherent disadvantages.

For example, ELISAs can be made more sensitive than is necessary to protect public health. As noted above, detection down to a level of 10 ppm is probably adequate to protect food-allergic consumers. Detection down to 20 ppm is likely to be adequate to protect the health of celiac sufferers. When commercial ELISA kits detect in the range of 2.5–25 ppm, that is probably quite sufficient. Some commercial ELISA kits have a lower range of detection down to 1 ppm. Certainly, there is no public health justification for the development of even more sensitive ELISAs in our opinion. With the advent of “free” foods, e.g. gluten-free, peanut-free, milk-free, dairy-free, the temptation exists to develop kits with a higher degree of sensitivity. Certainly, in the absence of regulatory definitions of labeling terms, such as peanut-free, the food industry is more or less obligated to use the most sensitive ELISA, i.e. to document that the food is “free” by the most sensitive method available. However, extremely low levels detectable with highly sensitive methods may have little relationship to public health concerns as such products would be rather unlikely to provoke allergic reactions. Presently, the level of sensitivity of commercial ELISA kits is not a disadvantage but has the potential to become more of a concern if efforts are expended to make more sensitive ELISAs. If the sensitivity of test methods were beyond the limits needed to assure the health of allergic consumers, then residues of allergenic foods at very low levels would be increasingly found in other foods. The presence of these residues would be declared on the labels and food-allergic consumers would be advised to avoid these foods. The ultimate consequence would be that allergic consumers would need to avoid foods that are likely to be quite safe, thereby reducing their quality-of-life with no benefit to the allergic consumer’s health status.

ELISAs are usually highly specific for a particular epitope, protein, or source of proteins, depending upon how the antibodies are generated. False positive results rarely occur. When analyzing food products for residues, it is often desirable to have negative control samples of known uncontaminated product. We have had experiences

in which the negative control tested positive. These situations can usually be traced to some unexpected source of residues of the allergenic food in the negative control sample. In some cases, ELISAs cannot easily distinguish between closely related foods, probably because of the presence of cross-reactive epitopes or proteins in the two sources. For example, the antisera for the walnut ELISA used by our group react significantly with pecan [21] and the mustard ELISA recognizes rapeseed [22].

ELISAs are much more likely to yield false negative results. ELISAs are usually intended to detect one or more intact proteins. Thus, if the protein(s) is hydrolyzed even to a limited extent, immunoreactivity in the ELISA may be lost. Such results cannot be interpreted to mean that the food is no longer potentially allergenic because partially hydrolyzed proteins may retain their allergenicity [23]. Detection of allergen residues in fermented products can also be problematic if proteolysis occurs as part of the fermentation. Some fermentations are much more proteolytic than others so the seriousness of this concern can be variable. Competitive immunoassays can be developed to detect peptides if the antibody-binding epitopes remain intact but such assays can be difficult to quantify. A commercial competitive ELISA kit exists to detect gliadin peptides. Of course, the peptides detected in a competitive ELISA may differ in structure from the epitopes involved in IgE binding in allergic reactions. With the competitive ELISA kit for detection of gliadin, that concern does not exist because the monoclonal R5 antibody is directed against a peptide that is considered to be the primary toxic peptide in celiac disease [24]. This concern also does not exist with the RAST inhibition test because relevant human sera are used that provide immunoreactivity with the appropriate allergenic epitopes. Use of a pool of appropriate sera from several human subjects in the RAST inhibition test is preferable to assure that most of the relevant allergenic epitopes are detected.

False negative results can also be obtained if the target proteins are not extracted from the food matrix effectively [25]. The solubility of food proteins can be affected by pH, heat and other physical processing treatments, and aggregation and/or complexation. If the target proteins are not soluble, they will not be extracted effectively from the food matrix. However, insoluble proteins could potentially remain allergenic when ingested, especially if the acidic environment of the stomach or the proteolytic capacity of the gastrointestinal tract releases peptides or proteins with intact IgE-binding epitopes. The target proteins and their epitopes for ELISAs could also be affected by chemical modifications that might occur during food processing. For example, milk proteins can be chemically modified during heat processing in a manner that can affect immunoreactivity [26]. Unless the immunoassay is detecting the specific IgE-binding epitope, chemical modification could reduce immunoreactivity in the ELISA but still leave an active IgE-binding allergen in the food matrix.

ELISAs for the detection of the residues of allergenic foods have several limitations. As noted above, such ELISAs cannot be used to reliably determine the presence of potentially allergenic protein hydrolysates. Foods and ingredients produced via fermentation may also contain potentially allergenic protein hydrolysates that will not be detected by such ELISAs. Because ELISAs are aqueous systems, they do not work well with edible oils derived from allergenic sources. With edible oils such as peanut oil, the amount of residual protein is well below the lower limit of sensitivity of the allergen ELISAs (probably <0.1 ppm). Furthermore, the residual proteins present in edible oils and related ingredients such as lecithin may represent only the more lipophilic proteins from the source. The ELISAs may not detect these particular proteins very well even though some of these proteins such as oleosins may be allergenic [27, 28]. Soy lecithin contains residual protein at variable levels above 50 ppm but the proteins are not detected in existing commercial soy ELISAs.

Sometimes other analytical approaches can be used that overcome the disadvantages of ELISAs for specific applications. For example, an LC-MS method was used effectively to quantify whey proteins and peptides in both the native and denatured state [26, 29].

Calibrator effects

The results of ELISAs are affected by the nature of the standard curve including the calibrator solution that is used to calibrate the assay. Results can be based upon the whole food, e.g. ppm peanut, upon total or soluble protein, e.g. ppm peanut protein or ppm soluble peanut protein, or upon some specific protein, e.g. ppm Ara h 1, one of the major peanut allergens. Obviously a particular sample result expressed on these three different bases would be very different. From a clinical or public health perspective, the expression of results on the basis of ppm of the whole food is probably most appropriate because threshold levels are most commonly determined in tolerable amounts of the whole food. Sometimes, conversions between units are reasonably simple. For example, peanuts are approximately 25% protein so conversion from units of peanut protein to units of whole peanut is easily done. However, for some foods, the percentage content of protein is less well defined or even somewhat variable across varieties. Thus, determining the proper conversion factor to use can be more difficult. An example would be mustard where results can be expressed in ppm mustard flour or ppm soluble mustard protein [22]. The decision on the proper conversion factor

to use between these two units is complicated because protein composition results are better known for mustard seed than for mustard flour, the comparative percentages of soluble vs. insoluble protein are unknown, and published values for the percentage of protein in mustard vary by several percent [30]. When expressing results as ppm of soluble protein, the percentage of insoluble protein must also be known to compare results on a whole food basis; this can be difficult to determine and variable depending upon conditions. In some cases, results can even be expressed in terms of a specific protein from the source. But if, for example, expressing the results in ppm of a specific protein such as Ara h 1, knowledge of the percentage of Ara h 1 in whole peanuts is not precisely available. Thus, even if using antibodies directed against a specific protein, the creation of a standard curve based upon the whole food is important. A practical example of the importance of these considerations involves milk ELISAs. The antisera used in milk ELISAs can be specific for total milk, casein or β-lactoglobulin. The results of milk ELISAs can then be expressed as ppm of milk or one of the specific milk proteins. If the results are expressed as ppm of milk, the usual practice is to express them in terms of ppm of non-fat dry milk rather than liquid milk. Of course, these various units of measurement would be quite different. While there is no ideal choice for milk ELISAs or for expression of the results, it is quite important to understand the specificity of the antisera, the units of measurement, and the composition of the standard curve.

Another factor affecting the comparison of various ELISA methods is the general lack of official reference materials [31]. Such reference materials could be used advantageously to prepare or compare calibrators for different kits. The National Institute of Standards and Technology (NIST) in the USA now has reference materials for peanut, egg, and milk. Official reference materials should be evaluated for their suitability for use in spike-and-recovery experiments and for assessing recovery from naturally incurred samples [32].

The food matrix

The food matrix can have a considerable effect on allergen ELISAs. When allergenic food residues are added to or incorporated into a food matrix, numerous factors can affect ELISA results. Certainly, the quantitative extraction and recovery of the allergenic food residues from the matrix is perhaps the most important concern as will be discussed more thoroughly below. Proteins can lose solubility in food matrices for many reasons including pH, chemical modifications, and aggregation phenomena. Additionally, the food matrix may contain components that interfere with the ELISA by inhibiting antigen-antibody binding, reacting with epitopes, or having interfering enzymatic activity.

The physical form of the food matrix is also an important consideration. Some foods such as beverages, flours, and frozen desserts are reasonably uniform and any allergen residues are likely to be uniformly distributed unless the form of the allergen is particulate (see below). But other foods have a more particulate nature and allergenic residues may not be uniformly distributed. The form of the allergenic residue that is to be detected also has a major influence. Residue forms can be solids—either flours or particulates, pastes, or liquids. These various forms may not be distributed uniformly. When the allergenic residue is in particulate form, representative sampling becomes a serious concern. A good example would be whole sesame seeds which can be elusive in shared food manufacturing operations but are not likely to be found in every random sample by any means of analysis. It is unlikely that every sample of product manufactured on shared equipment will contain a sesame seed but finding no detectable sesame seed in a particular sample does not directly lead to a confident conclusion that none is present.

Spike-and-recovery methods

In the development of ELISAs to detect allergenic foods, the sensitivity and specificity of the assay is first evaluated in simple buffer systems. But food matrices are far more complex than buffer systems. Thus, spike-and-recovery experiments are often conducted next to determine if the ELISA works effectively in one or more food matrices. Ideally, several different relevant food matrices are evaluated and their selection is based upon the known uses of the allergenic food. For example, with peanuts, spike-and-recovery experiments were done with ice cream, cookies, breakfast cereal, and chocolate [33, 34]. The spiking material should also, ideally, be representative of the allergenic foods; NIST official reference standards are sometimes available for such uses. In spike-and-recovery experiments, the spiking material is made thoroughly homogeneous and put into a form that can be accurately added to the food samples. The spiking material must also be processed in a form that is typical for the material as a food ingredient. For the spike-and-recovery experiment with peanuts, the spiking material is peanut butter, a common heat-processed form of peanuts. Using a processed form of the allergenic food as the spiking material assures that the testing will determine if the antisera detect the processed form of the protein residue [25]. The selected foods are typically blended into a fine powder. The spiking material at appropriate levels is added to the food and thoroughly blended. In typical

spike-and-recovery experiments, the allergenic food residue is then immediately extracted from the food matrix and analyzed by ELISA. A good example of this approach is provided by the validation of several peanut ELISAs [33, 34]. In these studies with various foods (breakfast cereal, milk chocolate, ice cream, and cookies), either peanut butter [32, 33] or defatted peanut flour [34] were used for spiking. Despite these differences, several commercial ELISA kits yielded similar results [33, 34].

The extraction of the allergenic food residue from the matrix can be a key issue in spike-and-recovery experiments. As noted earlier, the food matrix can exert effects on the allergenic food residue that negatively influence the efficiency of extraction. Chocolate is probably the most challenging food matrix. Polyphenolic compounds in chocolate can react with food proteins in a rather non-specific way and thus make efficient extraction more difficult [25]. Commercial ELISA kits often contain extraction additives that attempt to overcome such concerns. These extraction additives often contain other proteins such as fish gelatin that react with the chocolate polyphenolics allowing release and extraction of the target proteins of interest [25]. Specialized extraction procedures can be needed for certain types of allergenic food residues or for specific food matrices. The need for such specialized procedures can be discovered through spike-and-recovery experiments. For example, specialized extraction procedures are recommended for heat-processed foods containing gliadin residues [25]. One ELISA kit manufacturer recommends an extraction buffer containing a surfactant (sodium dodecyl sulfate, SDS) and a reducing agent (2-mercaptoethanol) for the detection of egg residues, especially in processed foods [35]. However, the possible advantages of such sample treatments have not been well documented in comparative studies.

Naturally incurred standards

Spike-and-recovery experiments will demonstrate whether the ELISA, including the extraction procedure, works effectively in the food matrices tested. However, any effect of processing on the analyte is not evaluated in spike-and-recovery experiments. Thus, the ultimate evaluation of an ELISA involves the use of so-called naturally incurred standards in which the allergenic food residues are incorporated into the food formulation which is then processed in a manner that mimics industrial food processing. Naturally incurred standards evaluate the effects of processing on the allergenic food residues in the milieu of a food matrix. These effects include, but are not limited to, heat-accelerated chemical reactions including Maillard reactions and other protein-carbohydrate interactions, protein aggregation with loss of solubility, shear effects on protein structure, emulsion formation, pH effects, water activity considerations, and others. Of course, each food matrix and processing condition is different and it is impractical to evaluate all possibilities with naturally-incurred standards. However, evaluation of an ELISA with one or more typical combinations of food matrix and processing condition is an ideal way to determine if the ELISA will provide reliable results when applied to processed foods. When selecting a matrix for such an evaluation, it should be one in which the allergenic food residues might be found during typical processing. To answer this question one need simply stroll the aisles of the local grocery and look for similar foods with and without the specific allergenic food. Naturally incurred standards are usually made in a food processing pilot plant or test kitchen facility. For some food matrices, mimicking industrial food processing is quite challenging and proper facilities may be difficult to locate. Several considerations must be kept in mind when making naturally incurred standards. Homogeneity of food allergen residue incorporation into the food matrix is critical and each step of the process must be considered with regard to homogeneity. The ability to clean the processing equipment thoroughly after each batch of product is another important consideration to assure that one batch is not cross-contaminated by another.

When making naturally incurred standards, quality control becomes a paramount consideration. First, the nature of the food allergen residue that is to be incorporated into the food must be very well defined. In some instances, official reference standards may exist but these are not usually available nor affordable in the quantities needed to make naturally incurred standards. However, the official reference materials may be used for comparative purposes to define the nature of the material that is used. The issue of homogeneity of incorporation of the food allergen residue often requires multiple analyses of sub-samples of the food product at various stages of processing to assure that uniform distribution is achieved [36]. Obviously, the processing equipment must be scrupulously cleaned before use for making naturally incurred standards to avoid contamination. Contamination between batches with increasingly levels of allergenic residue can usually be easily controlled by starting with the lowest concentration and steadily increasing the levels.

The stability of naturally incurred standards is another important consideration. Certainly, the effects of processing on analyte detection by the method can be rather quickly determined. But, if the naturally incurred standards are going to be stored for later use to generate “real-world” standard curves for the method, then the shelf-life of the naturally incurred standards must be ascertained. Clearly,

the possibility exists that further reactions between the proteins of the allergenic food and components of the food into which it is incorporated could occur during storage. Because the creation of well-defined naturally incurred standards is tedious, storage of these standards would allow their future use for evaluation of new and/or modified methods. The naturally incurred standards might also be used advantageously to evaluate batch-to-batch variability for commercial ELISA kits. Few studies have been reported on the shelf-life of naturally incurred standards. Recently, in an attempt to prepare naturally incurred standards in a salad dressing matrix, the observation was made that recovery of mustard and egg residues was very poor and that shelf-life was quite short (<1–2 h) probably owing to acid precipitation of the target proteins [37].

Chocolate

For ELISAs and, probably, other types of allergen residue analyses, chocolate is perhaps the most challenging food matrix. Because of the nature of the chocolate-making process, spike-and-recovery experiments would not necessarily suffice as a substitute for naturally incurred standards. The Food Allergy Research and Resource Program at the University of Nebraska has developed naturally incurred standards for a number of allergenic food residues in chocolate including peanut, almond, casein, hazelnut, walnut, and cashew. The naturally incurred chocolate standards were prepared in the pilot plant facility of an international chocolate manufacturer. All were prepared in a similar fashion over the course of a few years. Equal-sized batches (70 lb) of first a popular milk chocolate formulation (dark chocolate for casein standards) followed by the same formulation with added allergenic food (usually at 100 or 1000 ppm) were prepared. It is important to prepare equal-sized batches so that all ingredients in each batch will be exposed to the same time, temperature, and shear effects. Ingredients (sugar, cocoa butter, milk powder, chocolate liquor) were combined in a ribbon mixer at 50–54°C until a fairly homogeneous mass was formed. The mass was refined yielding a consistent particle size (batch-dependent). Temperature in the refiner was about 48ºC. The refined material was conched at 80°C for at least 4 h with addition of vanillin and soy lecithin near the end of the conch time. Mixing time and temperature, particle size and conch time and temperature were the same for the batches with and without the allergenic food residue.

The allergenic food material could be added at different steps in the process. Optimally it would be added to the mixer, to be exposed to all of the same heat treatments as the other ingredients. Addition at the refiner stage insures that the particle size of the added allergenic food material is similar to that of the remainder of the batch. If the allergenic food is prepared to have a similar particle size, it may be added at the start of conching. Chemical alterations may occur during conching although this has not been well documented for proteins present in the chocolate during conching. Extensive mixing also occurs during the conching step. To insure homogeneity and the greatest chance of any modification, the allergenic food material must be added so that it is subjected to the entire conching step. Adding the allergenic food residue at the refining or conching steps were undertaken in most cases to avoid the inevitable extensive clean up of one or two fewer pieces of equipment in a busy industrial pilot plant.

After conching, specific part per million sub-batches were prepared by thoroughly mixing appropriate amounts of the “allergen-free” milk chocolate with aliquots of the batch containing a known amount of the allergenic food residue (either 100 or 1000 ppm). The resulting sub-batches were prepared by proceeding from low ppm levels to high ppm levels with each sub-batch being tempered, molded, labeled, and quarantined before the next higher ppm level sub-batch was mixed. During this time, the mother batches of chocolate with and without the allergenic food residues were held in a hot room at 45–50°C.

This procedure has produced very homogeneous naturally incurred standards of a number of allergenic foods in chocolate.

Other investigators have also prepared incurred standards for peanut in chocolate [34, 36, 38]. However, in those studies, the chocolate was simply melted before adding the peanut material, either peanut flour or peanut butter. This approach approximates spike-and-recovery because the peanut material was not added at the outset of chocolate manufacturing and then carried through all of the subsequent processing steps as described above. However, no studies have been conducted to determine if this makes a difference in peanut recovery from chocolate.

Cookies and muffins

A different approach must be taken to make homogenous standards with cookies and muffins (cakes) because these products do not undergo the type of intensive mixing that is typical for chocolate. In a test kitchen, naturally incurred standards in cookies and/or muffins were prepared for peanut, lupine, buckwheat, and cashew. Whether cookies or muffins were prepared from individual ingredients or from commercially available dry mixes, all of these items were pre-screened for the possible presence of undeclared residues of the relevant allergenic food using a buffer-

based ELISA. Enough individual ingredients or mixes of the same lot number(s) were obtained so that all levels of naturally incurred standards were prepared from the same raw ingredients. Sifting (multiple times) dry allergenic food materials (e.g. lupine flour) together with the other dry ingredients (e.g. wheat flour) of the formulation worked well to start the process of making a homogeneous standard. Small batches (< 1 kg) of cookies and muffins can be problematic when attempting to prepare lower-level standards (1–100 ppm). A higher ppm working spike was prepared at 10,000 ppm. After multiple siftings, the homogeneity of the working spike was confirmed by taking multiple samples, extracting and analyzing with the buffer assay. Criteria for variability within these samples were determined based on the variability of the buffer ELISA. Then the proper amounts of the working spike were sifted together with the other "allergen-free" dry ingredient components to prepare different ppm batches of cookies or muffins.

If the allergenic food does not allow incorporation by sifting, because of larger particle size (often observed with high-fat tree nuts), pulverizing in a freezer mill or an inexpensive coffee mill has been effective. Coffee mills with oval grinding bowls produce better mixing action than their round counterparts and their relatively low cost allows for having a dedicated grinder for each allergenic food. Other crystalline ingredients from the formulation such as sugar or salt make excellent grinding agents and carriers when preparing a homogeneous working spike.

Wet ingredients should be added in a way that allows for quick and even incorporation with the dry ingredients. Fats should be liquid or melted. Many kitchen style food processing machines, blenders, and stand mixers can be equipped with extra work bowls, blades and mixing paddles for making multiple batches of cookies or muffins with various levels of allergenic food residues. Each batch should be mixed for the same length of time and scrapping of bowls should be frequent. Testing the homogeneity of the raw, mixed batter with the buffer-based ELISA should be done to validate the mixing procedure. The approximate moisture loss due to baking or cooking needs to be accounted for so that the amount of allergenic food residue added to the raw batter will approximate the desired ppm levels of allergenic food in the cookie or muffin after baking or to calculate the corrected final concentration after baking. When baking, place equal-weight portions of cookie dough on the baking sheet. Evenly space these so even baking will occur. The same is true for muffins. Place equal weights of muffin batter in each muffin paper or tin. Knowing the total weight of the raw dough or batter is important so that the final moisture loss can be determined. Also, use of equal-weight portions helps to assure consistent results. Once baked, allow the cookies or muffins to cool in a dry environment. Determine the total final weight and calculate the moisture loss. Moisture loss from each individual cookie or muffin will not be the same. Calculate the final ppm level for the batch based on the amount of allergenic food added and the final total weight. Grinding and mixing of the entire cookie or muffin batch and storing frozen provide a significant cache of homogeneous standard. Note that it is best when sampling this frozen material is to remove slightly more than needed while still frozen and place in a closed container. Allow the sub-sample to come to room temperature, weigh the amount needed and discard any remainder. Do not return any material to the frozen standard. Alternatively, the standard material can be divided to multiple containers and once removed from the freezer, extra material can be discarded.

The development of naturally incurred standards in baked goods has been done successfully with several allergenic foods. Peanuts were incorporated into cookies and baked as part of the evaluation of several immunoassays for peanut [38, 39]. Lupine and walnut residues, respectively, were incorporated into muffins and baked as part of the evaluation of immunoassays for lupine and walnut [21, 40].

Ice cream

Incorporation of allergenic foods into ice cream is possible using small, electric ice cream freezers readily available in the market place. Purchase of at least two machines is advised so that one will be always be free of the specific allergenic food of interest. Try to select freezers that have removable bowls and dashers to facilitate cleaning. Motorized freezers are preferable so batch-to-batch mixing time can be standardized. It is worthwhile to make preliminary batches using the allergenic food in question. Our experience with the addition of cashew to ice cream revealed that the cashew material could not be added at the beginning of the freezing process (unpublished). The mixing speed and dasher design allowed the cashew material to settle to the bottom of the freezer bowl. The cashew material was added when the ice cream mixture was slightly thickened which prevented pieces from settling to the bottom. Ice cream freezing involves minimal processing so the recoveries from naturally incurred products would be predicted to be similar to spike-and-recovery experiments. In a study of the recovery of peanut from ice cream, the ice cream was melted, peanut was added, and then extracted for analysis [34]. While this approach differs from addition of the peanut prior to freezing, the differences in recovery are likely to be minimal.

Frankfurter

Because mustard flour is a common ingredient in processed meats, frankfurters were selected as a naturally incurred standard for mustard [22]. Frankfurters were also used as a naturally incurred standard for lupine [40] because lupine flour is often used as a substitute for soybean flour so it was surmised that processed meat applications could be relevant. Mustard flour or lupine flour was ground together with salt (10,000 ppm or 1%) as described elsewhere [22, 40] to facilitate addition of small amounts of the allergenic food material to a 1.5-kg batch of frankfurter emulsion. The allergenic food-salt mixture was added in the appropriate amounts to the cure solution of water, salt, sugar, and sodium nitrite which was then evenly distributed with the meat in a circular food cutter/chopper. The food cutter was stopped and the batter brought together in a single mass, mixed, and redistributed in the chopper bowl numerous times during emulsion formation to insure equal distribution of the allergenic food within the matrix. An 80°C water bath was used to simulate the cooking process; finished internal temperature was 71°C. Frankfurters were weighed before and after cooking to determine the cooking yield.

Pasta

Naturally incurred standards in pasta have been made for egg, using spray-dried egg [41], and sesame seed, using sesame seed flour (unpublished). Pasta can be a simple formulation of semolina flour, water, and the allergenic food material. The crucial aspect is the homogeneous distribution of the allergenic food material in the pasta dough. This can be achieved as discussed above by sifting the allergenic food material together with the semolina and mixing well with the water in an electric home-style combination pasta mixer and extruder. This allows for the consistent mixing and gluten formation at the same rate if same-weight batches are prepared and mixed for the same length of time. Naturally incurred standards in pasta have also been prepared by solublizing the allergenic food residue (ovalbumin) in the water before addition to the semolina.

Retorted potato soup

Retorting (canning) is a particularly rigorous heating process. It is important to include naturally incurred standards that have been subjected to such typical processes as actually used in the food industry for certain allergenic foods. In retorting, the matrix and the allergenic food material are subjected to pressure and heat. We found in a preliminary analysis that clam mixed with potato soup, and canned and retorted under typical conditions for this can size and the viscosity of its contents resulted in poor recovery rates of the clam using a buffer-based clam ELISA (unpublished). From practical experience using positive controls, we also know that commercial egg ELISAs do not work well in the detection of egg residues in retorted pasta products. The example of retorting certainly brings to light the importance of understanding the effects of the matrix and the manufacturing processes on the detection of allergenic food residues. Analysis of retorted foods is a limitation for at least some allergen ELISAs.

Conclusion

ELISAs have numerous advantages for use in the detection of allergenic food residues but they also have several limitations. These limitations need to be more widely recognized. The use of naturally incurred standards aids in identification of the limitations of ELISAs in the detection of allergenic food residues.

References

1. Taylor SL, Hefle SL (2006) Food allergy. In: Bowman BA, Russell RM (eds) Present knowledge in nutrition, vol2, 9th edn. ILSI Press, Washington, DC, p 625
2. Sicherer SH, Munoz-Furlong A, Sampson HA (2004) J Allergy Clin Immunol 114:159
3. Sampson HA (2004) J Allergy Clin Immunol 113:805
4. Sicherer SH, Munoz-Furlong A, Sampson HA (2003) J Allergy Clin Immunol 112:1203
5. Fasano A, Berti I, Gerarduzzi T et al (2001) Arch Int Med 163:286
6. Bock SA, Munoz-Furlong A, Sampson HA (2007) J Allergy Clin Immunol 119:1016
7. Pumphrey RSH, Gowland MH (2007) J Allergy Clin Immunol 119:1018
8. Ferguson A, Kingstone K (1996) Acta Paediatr 412(Suppl):78
9. Taylor SL, Hefle SL, Bindslev-Jensen C et al (2002) J Allergy Clin Immunol 109:24
10. Bindslev-Jensen C, Briggs D, Osterballe M (2002) Allergy 57:741
11. Collin P, Thorell L, Kaukinen K et al (2004) Aliment Pharmacol Ther 19:1277
12. Hefle SL, Furlong TJ, Niemann L et al (2007) J Allergy Clin Immunol 120:171
13. Koppelman SJ, Hefle SL (2006) Detecting allergens in food. Woodhead Publishing, Cambridge, UK, 422 pp.
14. Holzhauser T, Stephan O, Vieths S (2006) Polymerase chain reaction (PCR) methods for the detection of allergenic foods. In: Koppelman SJ, Hefle SL (eds) Detecting allergens in food. Woodhead Publishing, Cambridge UK, p 125
15. Shefcheck KJ, Musser SM (2004) J Agric Food Chem 52:2785
16. Taylor SL, Hefle SL (2002) Foods as allergens. In: Brostoff J, Challacombe SJ (eds) Food allergy and intolerance. Saunders, London, p 403

17. Taylor SL (2002) Ann Rev Pharmacol Toxicol 42:99
18. Valdes I, Garcia E, Llorente M et al (2003) Eur J Gastroenterol Hepatol 15:465
19. Nordlee JA, Taylor SL, Jones RT et al (1981) J Allergy Clin Immunol 68:376
20. Nordlee JA, Hefle SL, Taylor SL et al (2007) J Allergy Clin Immunol 119:S158
21. Niemann L, Taylor SL, Hefle SL (2009) J Food Sci (in press)
22. Lee PW, Hefle SL, Taylor SL (2008) J Food Sci 73:T62
23. Businco L, Cantani A, Longhi M et al (1989) Ann Allergy 62:333
24. Osman AA, Uhlig HH, Valdes I et al (2001) Eur J Gastroenterol Hepatol 13:1189
25. Immer U (2006) Factors affecting the effectiveness of allergen detection. In: Koppelman SJ, Hefle SL (eds) Detecting allergens in foods. Woodhead Publishing, Cambridge UK, p 330
26. Monaci L, Van Hengel AJ (2007) J Agric Food Chem 55:2985
27. Leduc V, Moneret-Vautrin DA, Kanny G et al (2006) Allergy 61:349
28. Pons L, Chery C, Romano A et al (2002) Allergy 57(Suppl 72):88
29. Losito O, Carbonara T, Monaci L et al (2007) Anal Bioanal Chem 389:2065
30. Lee PW, Taylor SL, Treloar T et al (2008) J Food Sci 73(8):viii
31. Poms R, Anklam E (2006) Reference materials and method validation in allergen detection. In: Koppelman SJ, Hefle SL (eds) Detecting allergens in foods. Woodhead Publishing, Cambridge UK, p 348
32. Trucksess MW, Brewer VA, Williams KM et al (2004) J AOAC Int 87:424
33. Park DL, Coates S, Brewer VA et al (2005) J AOAC Int 88:156
34. Whitaker TB, Williams KM, Trucksess MW et al (2005) J AOAC Int 88:161
35. Watanabe Y, Aburatani K, Mizumura T et al (2005) J Immunol Methods 300:115
36. Trucksess MW, Whitaker TB, Slate AB et al (2004) J AOAC Int 87:943
37. Lee PW, Niemann LM, Lambrecht DM et al (2009) J Food Sci 74: T46
38. Poms RE, Agazzi ME, Bau A et al (2005) Food Addit Contam 22:104
39. Van Hengel AJ, Capelletti C, Brohee M et al (2006) J AOAC Int 89:462
40. Kaw CH, Hefle SL, Taylor SL (2008) J Food Sci 73:T135
41. Hefle SL, Jeanniton E, Taylor SL (2001) J Food Prot 64:1812