Food Quality & Safety

FARM TO FORK SAFETY

Special Edition

Increase your confidence in your allergen testing results with the use of certified reference materials

Sponsored by:

ROMER

Romer Labs®

When time is of the essence,

AgraStrip® Allergen Test Kits won't keep you waiting.

- Room Temperature Shipment and Storage
- Streamlined, Fast Workflow
- Simple and Ready-to-use Buffer Bottles. No Frustrating Packets.
- Validated Protocols for Swab, Rinse Water, Raw Ingredient, and Finished Product Samples

Need Results Fast? Contact Romer Labs

Romer Labs Inc | 130, Sandy Drive, Newark, DE 19713, U.S.A.
T +1 302 781 6400 · **E** office@romerlabs.com · www.romerlabs.com

Making the World's Food Safer®

ROMER
Romer Labs®

Contents

www.foodqualityandsafety.com

BILDNACHWEIS: JENA ARDELL

CREATIVE #: 157776240

COVER PHOTO CREDIT © GETTY IMAGES, JENA ARDELL

From The Editor

By Samara E. Kuehne

Undeclared food allergens are a critical public health issue. Many allergen management plans feature analysis as part of their validation and verification process, and testing ingredients and the final product are often part of ensuring that these products don't contain unexpected allergens. Food manufacturers need reliable and effective methods of analysis to detect these allergens, and reference materials are critical in reaching accurate results in any analysis.

Wiley has partnered with Romer Labs to bring together a special collection of articles detailing why allergen reference materials are necessary for today's food professionals. In this important compendium, we bring together articles from Romer Labs and Wiley publications, including *Food Quality & Safety*, that provide a comprehensive and updated overview of what constitutes allergen reference materials, why they are essential, and how you can use them to further enhance your allergen management plan.

In addition to the importance of allergen reference materials, you'll read about common myths surrounding food allergen testing. We've also included articles on understanding food allergen ELISAs and common challenges in allergen testing.

We think this series of important articles will be a useful resource in your workflow, and serve as a valuable tool in implementing your allergen control plans.

Kuehne is the professional editor of *Food Quality & Safety*. Reach her at skuehne@wiley.com.

Adrian Rogers was with Romer Labs for 11 years in his role as a senior research scientist, specialising in the development of immunoassays for food allergen detection.
Adrian is a microbiologist by training and has 20 years experience in the development of immunoassays, 18 years of which have been spent developing test kits for the detection of food.
Adrian is currently a member of the University of Manchester's Food and Health Network allergy cluster and has recently been involved in a UK FSA funded project with the University of Manchester and LGC to develop quality control materials for food allergen analysis.

Gill Holcombe is the Head of Reference Material Production at LGC, leading global life science tools company. She is currently located in UK.

6 Common Myths about Food Allergen Testing

JASMIN KRAUS, PRODUCT MANAGER, ROMER LABS® **DATE:** 2018-05-15

Ask anyone with a food allergy and they will tell you the same thing: There's not much that's simple about a quick trip to the grocery store. They have to check every label on every product that goes into the basket to make sure that their food is free from allergens. Because there is no treatment for food allergies, there's only one thing that works: completely avoiding the allergen or allergens in question.

This makes it all the more crucial for food producers to conduct routine tests for potential allergen contamination in their products.

Yet this isn't as simple as it sounds.

Food products can range widely from straight raw materials, such as cereals, to highly processed ready-to-eat products. Their composition, moreover, varies according to the amount of protein, fat, salt and other compounds present. Test methods are expected to analyze all food sample types for allergens with equally reliable results. This, however, is often far from achievable in reality.

With all the complexity surrounding food allergen testing, perhaps it's not surprising that there are a lot of half-truths and myths out there. Here, the allergen experts at Romer Labs dispel six of the most common misconceptions about food allergen testing.

Myth #1: A test kit off the shelf works with any food matrix.

The facts:

Take the test kit from the shelf and start testing… Sounds tempting, doesn't it? The results would be quick, but are they reliable? In reality, food products are highly diverse and certain test methods may work better for certain food samples. The extent of processing adds further complexity to this equation.

With new or unfamiliar matrices, we always undertake a spike recovery validation at three different levels to make sure it works with our kits and covers the detection range of the assay. Some matrices, such as chocolate, are full of tannins and other polyphenols that bind to allergenic proteins, creating insoluble complexes from which it is difficult to extract without adding extra protein to the extraction buffer.

While implementing an allergen control plan, it is highly recommended that the selected allergen test method be fully validated on the food producer's specific food matrices.

Myth #2: "May contain…" statements can solve all our problems.

The facts:

Food allergen labeling – though intended to make the lives of people with allergies easier and safer – often causes confusion as most laws fail to state the levels above which an allergen must be labeled. Advisory "May contain…" statements are voluntary and often serve primarily to prevent the producer from having to make potential allergen-related product recalls.

Studies have shown that up to 9% of products with advisory labels in fact contained detectable levels of allergens. This means that there is a real risk of allergen contamination in products that only make a precautionary statement. As there are varying reasons why manufacturers include such statements, consumers find it increasingly difficult to interpret them.

Consumers with allergies should avoid products with precautionary labels, as the risk is not assessable. In return, food producers should avoid using a "may contain…" statement without reasonable suspicion.

Myth #3: PCR is more reliable than immunological tests.

The facts:

It depends. Polymerase chain reaction (PCR) assays are extremely sensitive and make sense when specificity is called for. For example, no antibodies have been developed that can reliably detect celery without also giving a signal for related species, such as fennel, carrot or parsley. Hence, celery detection with an immunological test is currently not possible.

How can specific species be detected with PCR? It relies on DNA extraction and amplification, which is made possible by the nature of DNA: it is a stable molecule that remains unaffected by most common food processing methods. Yet PCR has significant drawbacks: it requires specially trained personnel to perform the complex sample preparation and result interpretation. Furthermore,

the DNA molecule itself is not responsible for the allergic reaction, meaning that the presence of DNA is at best an indicator of the allergenic potential of the sample.

Immunological rapid tests are still the gold standard and should be preferred in most cases as they directly detect food allergens. However, when specificity is called for, PCR may be a great alternative.

Myth #4: Mass spectrometry will soon replace allergen rapid tests.

The facts:

Mass spectrometry (MS) is a high-end technology that is already used in several fields for routine analysis and shows some potential in allergen analysis: it can measure several allergens in parallel. However, it is still in its infancy and is currently restricted to research applications. As a result, it's not clear how MS will perform in routine analysis.

Additionally, MS is not yet able to deliver the highest level of accuracy. Its basic principle is one of fragmentation: a molecule – in this case the allergenic protein – is broken down into small pieces (peptides) and their mass is subsequently determined. However, food processing can affect the fragmentation process of proteins, resulting in varying peptide patterns.

Without a doubt, MS technology will continue to develop and improve in the future. Yet since it relies on highly trained personnel and expensive equipment, there will still be demand for fast and inexpensive in-house testing, making it rather unlikely that rapid tests such as ELISAs will be replaced.

Myth #5: All test kits on the market detect the same.

The facts:

Commercially available test kits do not perform in the same manner. For each food allergen, there is a variety of different allergenic proteins, but there is no recognized standard defining which of them must be detected. Therefore, we cannot assume that all test kits detect the same and consequently give comparable results.

Kits do have one thing in common: the overall target (e.g., peanut or casein). But the similarities end there. Different kits use different buffers and procedures, which can have an impact on the extraction process and generate diverging patterns. Furthermore, kits differ in the antibodies used, which, in an added layer of complexity, need to take the various methods of food processing into account.

So what should you do? A close discussion with the kit manufacturer is highly recommended as they can provide information about the test kit's performance specifications. Also, analysts should carefully review and summarize all the processing steps that are applied

to a food product to assess which kit is most suitable for their individual application.

Myth #6: All currently available "allergen reference materials" improve testing reliability.

The facts:

It's unfortunate but true: there are very few reliable allergen reference materials (RMs) out there, despite the claims that some producers make. In other fields of food safety, producing reference materials requires high-end technology, but the procedures for doing so are well-established. In some cases, the analyte under consideration is more easily characterizable. If we take mycotoxins for example, we have one defined molecule, allowing accurate calculations of the final concentration.

In contrast, with food allergens, there is not just one specific molecule; an allergenic commodity consists of a mixture of different proteins. To date, several allergenic proteins have been identified, but many have not yet been well characterized. Furthermore, the protein pattern varies between different cultivars of the same species. And to make matters worse, proteins can change their conformations as a result of processing, which may lead to a change in their allergenic potential.

When deciding which RMs to use, it is important to keep a few things in mind. Typically, allergen RMs are mixtures of allergenic food commodities in certain matrices. Such mixtures have their uses in checking regular test performance, provided that they are used with care and in consideration of all known limitations. The most reliable allergen RMs are a matrix incurred with specific allergens and come with a certificate of uncertainty and metrological traceability. If such RMs are not available, or the matrix of the available RM is too dissimilar to the sample of interest, an acceptable alternative may be for producers to create materials in-house using well characterized allergen sources and their own matrices. These represent the two best options until standardization bodies define specifications for reference materials.

Understanding Food Allergen ELISAs

BY **MELANIE L. DOWNS, PHD, AND JOSEPH L. BAUMERT, PHD**

These allergen-specific tests can protect your business—if you choose the right one

Undeclared food allergens are a significant food safety hazard, and manufacturers need to have practices, processes, and controls in place to prevent the presence of undeclared major food allergens in their products. The detection and quantification of food allergen residues is an important capability for robust food allergen control, and methods capable of detecting and quantifying proteins from allergenic foods can be used in a number of ways. Food manufacturers can use allergen detection methods to assess various aspects of allergen control plans, including cleaning procedures, supply chain controls, and overall allergen management. In addition, manufacturers may need to rely on food allergen detection methods to confirm an alleged instance of undeclared food allergens in a product and conduct root-cause analyses. Food allergen detection methods are also used by regulatory authorities to investigate the presence of undeclared major food allergens in products on the marketplace, either as part of research studies or as enforcement actions.

Understanding how food allergen methods work, how to select the appropriate method for a particular application, how results from these methods are interpreted, and what potential issues may arise with the methods is critical for food manufacturers when implementing allergen control plans or navigating potential allergen recalls.

How Methods Work

Currently, the detection and quantification of food allergens in finished food products is primarily conducted using enzyme-linked immunosorbent assays (ELISAs). ELISAs detect proteins from allergenic foods by using antibodies that specifically recognize the food proteins of interest. Method developers produce these allergen-specific antibodies in laboratory animals by exposing them to the food or protein target of interest. After an immune response has been developed, antibodies can be collected, screened for specificity and affinity, and subsequently used in an ELISA.

Most ELISAs utilized for food allergen detection use a sandwich ELISA format. In a sandwich ELISA, one source of allergen-specific antibody is coated onto the surface of microwells, generally in a 96-well plate format. After coating with the antibodies (also referred to as capture antibodies), the wells are coated with a blocking agent to prevent any non-specific binding of components from the sample. In commercial allergen ELISA kits, pre-coated and blocked wells are provided as one of the kit reagents.

When conducting a sandwich ELISA method, an extract from the sample of interest or method controls is then added to individual microwells. During an incubation period, any proteins present in the sample from the target allergenic food will bind to the antibodies present on the surface of the microwell. The region on the protein that is recognized by the antibody is known as an epitope.

Following incubation, the wells will be washed thoroughly to remove any unbound sample components. A second allergen-specific antibody will then be added to the wells and will bind to target proteins already captured in the microwells, forming an antibody sandwich with the target protein in the middle. In commercial assays, this second antibody will have an enzyme attached (or conjugated) to it, and the second antibody is therefore commonly referred to

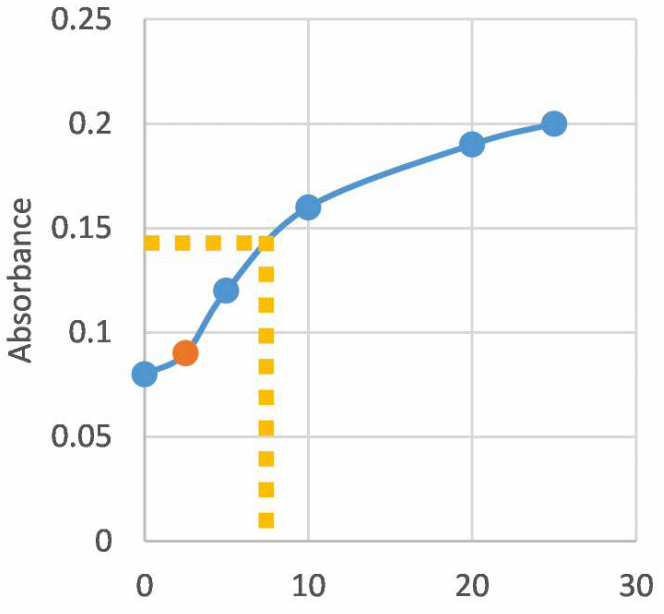


Figure 1. Quantification using a standard curve.

as the conjugate antibody. Following another washing step to remove unbound conjugate antibody, the substrate for the conjugated enzyme will be added to the wells. The enzyme present in the microwell will convert the substrate to a specific color product, indicating the presence of an intact antibody sandwich and therefore the presence of the food allergen target.

The amount of color generated in the microwell will depend on the amount of enzyme present, which in turn depends on the amount of target allergen protein present. This relationship between color intensity and amount of target allergen protein can be used to quantify the amount of target allergen in a sample.

In order to produce quantitative results, a series of standards containing known amounts of the allergenic food protein is analyzed alongside the samples. The absorbance values for both the standards and samples are measured using a plate reader. When the absorbance values from the standards are plotted against the known concentrations, a standard curve can be developed (see Figure 1). The absorbances from the unknown samples can then be used to interpolate the amount of allergen present.

Interpreting Results

Understanding how to interpret the results from a food allergen ELISA method can be challenging, as a number of different factors can impact the method's outputs.

Units and calibrators. Most commercial food allergen ELISAs report results in the concentration range of parts per million (ppm). The units of ppm indicate a concentration value for the analyte, which can also be expressed as mg analyte per kg product (mg/kg). Just using units of ppm or mg/kg does not, however, provide enough information for food allergen ELISAs. It is also important to know specifically what form of analyte the units are being expressed in. The most common analyte units for food allergen ELISAs are either whole commodity (e.g., ppm peanut, walnut, egg, etc.) or total protein (e.g., ppm peanut protein, walnut protein, egg protein, etc.). For some foods, however, there are commercial ELISA kits that express results on the basis of soluble protein from the allergenic food or a single protein analyte (e.g., ppm beta-lactoglobulin). In order to both understand the implications of a result from an ELISA and to compare results from different ELISA methods, it is crucial to have complete units expressed. The same sample analyzed by methods that use different units will have very different quantitative results, even if all other method conditions are similar.

Limit of detection, limit of quantification, and lower limit of applicability. As with many types of detection and quantification methods, food allergen ELISAs work only within a certain range of target analyte concentrations. The concentration below which a method is not able to distinguish a true positive from a true negative is known as the limit of detection (LOD). The LOD of a method, therefore, controls against false-positives arising from the food matrix and is generally estimated using a statistical evaluation of blank matrices. Because the LOD of a food allergen ELISA is highly dependent on the specific background food matrix being analyzed, it may not be as applicable across a diverse range of food products and ingredients as other method metrics.

The limit of quantification (LOQ) for a method is the lowest level at which a method can quantify an analyte with a specific level of precision (i.e., with a specific coefficient of variation, frequently 10 percent CV). The LOQ of a method as determined by statistical calculations is also dependent on the background matrix and may not represent an indication of the method performance across different food matrices. Method developers may therefore set a lower limit of applicability that better represents the performance of the method as the LOQ and establish that level by including it as the lowest positive value on the standard curve.

Selecting an Appropriate Method

One of the main considerations that needs to be accounted for when selecting a food allergen ELISA is whether the method detects the allergen-derived ingredient of concern. The ability to detect allergen-derived ingredients can depend on a number of factors. The first method characteristic that should be understood is what protein or groups of proteins the method is targeting—particularly important for allergenic foods from which the food industry produces ingredients containing different protein fractions.

The classic example of this issue is for the detection of milk residues. The food industry produces and utilizes ingredients that are composed of different milk

protein fractions, specifically whey protein and casein protein fractions, both of which pose risks to allergic consumers. Milk allergen ELISAs, however, are frequently produced to recognize specific proteins (e.g., beta-lactoglobulin from the whey fraction or α_{s1}-casein from the casein fraction) or protein fractions (e.g., caseins). If the milk allergen cross-contact of concern is due to a whey protein isolate ingredient, it would be ineffective to use a method targeting caseins for assessment or validation as the casein proteins would be present at extremely low levels, if at all, in the whey protein isolate ingredient. The opposite would be true when the source of cross-contact was a sodium caseinate ingredient, in which case it would not work to use a beta-lactoglobulin ELISA for detection. In addition to understanding the target of the ELISA, it is also important to have information about whether the allergen-derived ingredient has undergone substantial processing, which could affect detection.

The specificity of ELISA method should also be considered as a factor in some cases. Most food allergen ELISAs are incredibly specific for the target allergenic food of interest. But in some cases, very closely related foods may cross-react with the antibodies used in the ELISA method. This type of cross-reactivity issue has been observed among closely related allergenic foods such as walnut and pecan. Cross-reactivity can also be observed between foods designated as major allergens (e.g., peanuts) and related foods that are not designated as major allergens (e.g., peas). In most cases, commercial ELISA developers have screened for cross-reactivity with closely related species during development and should be able to provide users with information on the specificity of the assays. If novel food ingredients that are closely related to major allergenic foods are used in a product, it may be advisable to evaluate potential cross-reactivity to those ingredients before evaluating finished product.

Potential Food Allergen ELISA Issues

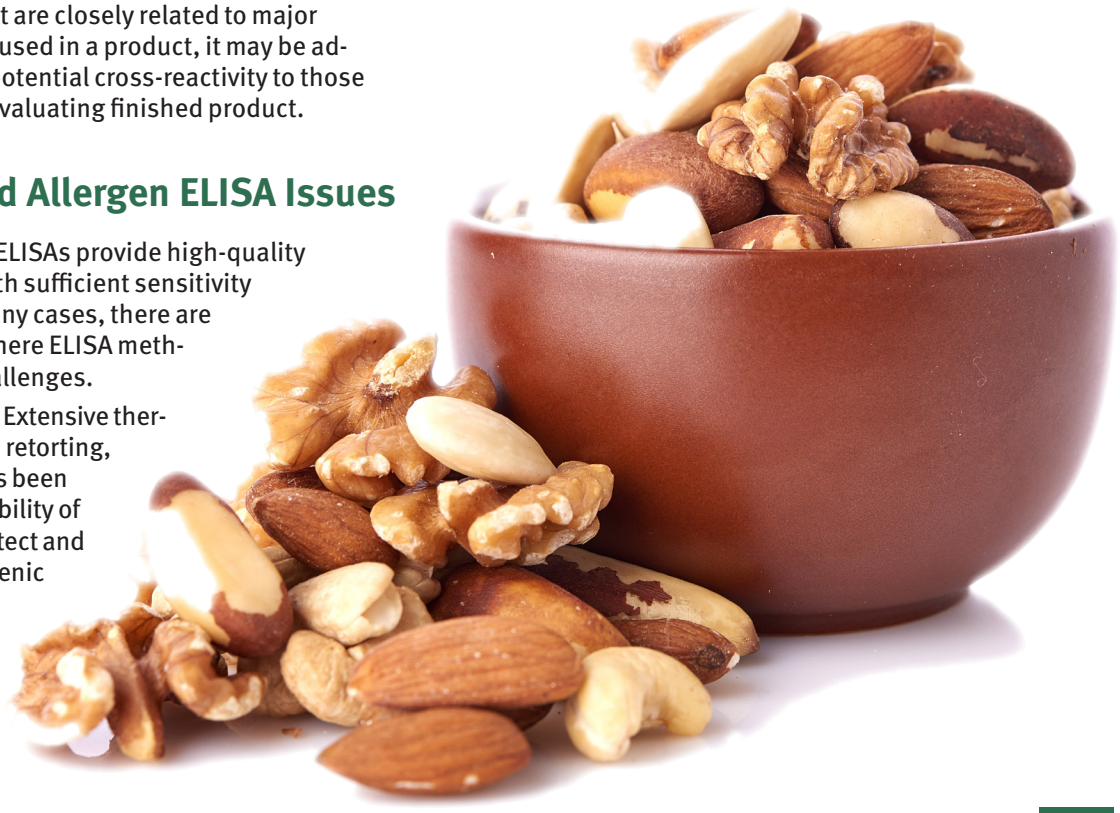
While food allergen ELISAs provide high-quality quantitative data with sufficient sensitivity and specificity in many cases, there are certain situations where ELISA methods face specific challenges.

Thermal processing. Extensive thermal processing (e.g., retorting, deep frying, UHT) has been shown to affect the ability of ELISA methods to detect and quantify some allergenic foods. The effects of thermal processing are two-fold. First, thermal processing can denature food proteins in a way that decreases the ability of the ELISA antibodies to recognize the proteins. However, these denatured food proteins are still considered to be allergenic.

The second effect of thermal processing is that it may result in target proteins that are aggregated in a way such that they are not extracted by the typical ELISA extraction procedure. If the target proteins are not extracted, they will not be included in the assay and will not be detected. Similar to denaturation, aggregated and insoluble proteins should still be considered as allergenic.

Fermentation and hydrolytic processing. Processes such as fermentation that can result in partial hydrolysis of proteins can also have a detrimental effect on quantification by ELISA. In these cases, the partial hydrolysis may result in cleavage of the part of the protein recognized by the assay antibodies. While there are circumstances where extensive hydrolysis can reduce allergenicity (e.g., extensively hydrolyzed infant formula or acid hydrolyzed vegetable proteins), it is not possible to determine allergenicity using ELISA methods. This is particularly true with sandwich ELISA methods that generally target intact proteins or large peptides, where the hydrolysis of just one part of the protein can prevent detection, as the two separate recognition areas required to form the antibody sandwich may not remain connected, even though other large pieces of the protein remain intact.

Dr. Downs is an assistant professor in the Department of Food Science and Technology at the University of Nebraska, Lincoln. Reach her at mdowns2@unl.edu. **Dr. Baumert** is the co-director at Food Allergy Research and Resource Program at the University of Nebraska, Lincoln. Reach him at jbaumert2@unl.edu.



AgraQuant® Plus Allergen Test Kits

Quantification, Simplified

- **FAST:** 1 Minute Sample Extraction
- **STREAMLINED:** Water Based Extraction; No Buffers To Prepare and Store
- **EFFICIENT:** Single Extract to Test for Multiple Allergens
- **SIMPLE TEST PROCEDURE:** Does Not Require Extensive Training or Lab Expertise

Let Romer Labs make it simple for you.

Romer Labs Inc | 130, Sandy Drive, Newark, DE 19713, U.S.A.
T +1 302 781 6400 · **E** office@romerlabs.com · www.romerlabs.com

Making the World's Food Safer®

ROMER
Romer Labs®

Challenges in Allergen Testing – Spiking and Recoveries

DATE: 2016-05-09 AUTHOR: **ADRIAN ROGERS, SENIOR RESEARCH SCIENTIST, ROMER LABS®**

Adrian Rogers discusses the basics on detecting allergens in food – from finding the right test kit to strategies used for precise validation.

When I started developing immunoassays for the detection of allergens in food, the first thing that struck me was the wide range of different food types or matrices that the assays had to work with. Coming from a medical immunoassay background, there was a limited number of different matrices to work with. In my case, this was blood serum. With food there is an almost infinite range of different sample types, each with their own specific properties.

How do I choose the right test kit?

So how do we ensure that the test kit produced is suitable for use with such a diverse and challenging range of samples? This is where sample validation comes in. The process involves adding a known amount of an allergen of interest to our matrix (**spike**) and then trying to get that allergen back out again (**recovery**).

An important thing to remember is that, as the name implies, immunoassays use biological components (antibodies) to achieve the detection of the allergenic proteins of interest. As with all biological systems, the kits are sensitive to extremes.

In the case of foods, the kits may not work as they should in the presence of strong acid or alkali, high salt, high fat, etc. Many of these extremes can be countered during the extraction process. Kits therefore use a buffered system to cope with changes in pH and the addition of the buffer to the sample helps reduce and dilute some of the other problems such as salt and fat.

Is my recovery acceptable?

When it comes to the recovery of a known amount of allergen from a sample matrix, what is deemed acceptable? Before answering this, we need to define where we are starting from. Is it an incurred sample or a spiked one?

Incurred samples are defined as samples in which a known amount of the food allergen has been incorporated during processing, mimicking as closely as possible the actual conditions under which the sample matrix would normally be manufactured.

The subject of incurred samples will be discussed in more depth in a subsequent issue of Spot On. In this article, I will concentrate on outlining a more

accessible method of spiking a known amount of allergen into a matrix as received from the supplier or manufacturer and measuring its recovery.

With regard to recovery, the guidance states that:

"Ideal percent recovery levels would range from 80 to 120%. Recovery levels are affected by both the efficiency of the extraction step and the ELISA procedure.

"With ELISA methods for food allergens, this level of recovery is not always possible, particularly when certain difficult matrixes are analysed. In addition, the recovery from incurred samples can be substantially different from those obtained using spiked samples.

"For this reason, recoveries between 50 and 150% will be considered acceptable so long as they can be shown to be consistent."

The guidelines were published in 2010 by the Association of Analytical Communities (AOAC) with particular reference to quantitative ELISA (Enzyme Linked Immunosorbent Assay) methods. Many of the key points are also applicable to qualitative or semi-quantitative LFD (Lateral Flow Device) methods.

The "science" behind spiking

When we receive or encounter a new food type that has not been tested before, we will undertake spike recovery validation to ensure it works as it should with our test kits. We will spike in at three different levels of allergen – low, medium and high – to cover the range of detection of the assay.

The low allergen spike will be close to the Lower Limit of Quantitation, LLOQ, of the ELISA (in this case the lowest value calibrator above 0 ppm) or close to the Limit of Detection, LOD, of a lateral flow device. The medium spike will be in the middle of the ELISA calibration curve, and the high spike will be at or near the Upper Limit of Quantitation, ULOQ (the highest ppm value calibrator). The sample is extracted and tested in accordance with the product insert supplied with the kit.

So for example, if we spike 5 ppm of almond into chocolate, we would expect to see a recovery of 4 ppm to 6 ppm. If the result is outside of this range, then there are steps that can be taken to help improve the recovery. From experience, chocolate is one of the most challenging food matrices to test – it is full of tannins and other polyphenols which can bind to any allergenic protein that may be present and form insoluble complexes which are difficult to extract.

Such difficulties can be overcome by adding extra protein to the extraction buffer. The excess protein binds to the polyphenols and makes the allergens available for extraction. My protein of choice is fish gelatine, although other material such as milk powder can be used to improve the extraction efficiency from high polyphenol containing foods. If using milk powder, be careful not to contaminate your laboratory space, especially if you are carrying out milk allergen testing.

Lateral Flow Devices, or strips or dipsticks as they are sometimes referred to, can be validated for spike recovery in a similar way to an allergen ELISA test kit. The thing to be aware of when choosing a high spike level is that although LFDs are capable of detecting very high ppm levels, you can actually overload the device by adding too much allergen. This can occur in amounts greater than 1% of the allergenic food.

Maintaining quality and test precision

It may be necessary for a kit manufacturer to work closely with customers who routinely test challenging food matrixes. It is important to verify that the kit is working as it should and to the customer's satisfaction. This can be achieved, as detailed above, by undertaking allergen spike recovery experiments into the problematic matrix.

In some cases it may be desirable to modify or change the standard kit method to meet the demands of the sample and/or the customer; this should always be undertaken with the guidance of the kit manufacture to ensure the quality and reproducibility of the test kit.

The Role of Reference Materials in Allergen Analysis

GILL HOLCOMBE, HEAD OF REFERENCE MATERIAL PRODUCTION, HEALTH SCIENCE AND INNOVATION DIVISION, LGC

In this article, we discuss some of the challenges in producing RMs for allergen measurement and describe some of the materials now appearing on the market.

Food allergy is a significant public health issue with mandatory labelling requirements for priority allergens in most jurisdictions. For example, in the EU, there is a list of 14 priority allergen groups in Annex II of Regulation (EU) No 1169/2011, "on the provision of food information to consumers,"[1] which applies to a much larger number of individual foods and food products. Of greatest interest are IgE-mediated allergenic symptoms which are responses to proteins or peptides present in the food; proteins are notoriously difficult to measure accurately because of their complexity and the low levels of them typically encountered in foods. Further, both their biological effects and their response to immunoassay methods depend heavily on the exact form of the protein present in the food. Food matrices are also complex in their own right and may have been heavily processed before sale. All this makes the measurement of allergens unusually challenging. A key tool in improving the reliability of these measurements is suitable reference materials (RMs) with well characterized properties so that new allergen measurement methods can be developed and tested with confidence and existing methods can be properly understood, validated and controlled.

What are reference materials and certified reference materials?

A reference material is a very useful tool in the analytical laboratory. It may consist of a single substance or a mixture of substances, and can be used to support measurements in a variety of ways.

The term "reference material" is defined in ISO Guide 30[2]. The definition is reproduced in the United Kingdom Accreditation Service (UKAS) document "TPS 57 Guidance and Policy on the Selection and Use of Reference Materials"[3]:

> Reference material (RM): material, sufficiently homogeneous and stable with reference to one or more specified properties, which has been established to be fit for its intended use in a measurement process.

Homogeneity and stability are seen as particularly important attributes of a reference material. Homogeneity refers to the effective uniformity of each unit in a batch. Products are said to exhibit stability if the properties of interest do not change over the shelf life of the product and the documentation provided with them remains valid for each unit.

A certified reference material is a 'special' type of reference material. Again, it has an ISO definition, found in ISO Guide 30[1], here reproduced from UKAS TPS 57[3]:

> Certified Reference Material (CRM): reference material characterized by a metrologically valid procedure for one or more specified properties, accompanied by an RM certificate that provides the value of the specified property, its associated uncertainty, and a statement of metrological traceability.

TPS 57 provides in its Annex 2 some further guidance to each of the above definitions. This mirrors guidance in ISO Guide 30 and includes the following:

- RM is a generic term, the properties of which can be qualitative or quantitative.
- The uses of an RM may include calibration of a measurement system, assessment of a measurement procedure, assigning values to other materials, and quality control.
- PD ISO Guide 31 gives guidance on the contents of RM certificates.
- CRM production and certification are covered in, among others, ISO 17034[4] and ISO Guide 35[5].

TPS 57 is worth consulting for further information.

Uses and applications of allergen reference materials

For laboratory analysts, the most common uses of reference materials are in method validation and quality control. Additionally, reference materials can be used for calibration, most commonly as pure substances, as well as for staff training and staff competence checks. More information on the uses of reference materials, including detailed guidance on their uses in validation, can be found in ISO Guide 33[6].

Reference materials also have applications in method development, e.g. for developing improved analytical equipment or for test kit development. This is particularly relevant for the evolving field of allergen analysis as our understanding of the topic grows. Both common reference materials and common calibrants for allergen research have a role to play in developing methods and improving the agreement between methods.

Why it's difficult to make a useful allergen reference material

Making a reference material to support food allergen measurements is not as easy as it sounds as the difficulties of reliable measurement present the producer with a host of challenges that need to be addressed. The need for increased effort in this space, and in particular the need for food allergen reference materials, has been summarized in a paper by Walker et al. [7].

One of the major problems in allergen analysis is in defining the analyte. What, exactly, are we trying to measure? Away from allergen analysis, the issue is much more straightforward as the analyte may be defined as a single element or a molecule with known composition. Those working in allergen analysis, however, may have more practical concerns: for example, they may need to know how much of a particular food is present, or the component of the food (that is, the protein which is the hazard for IgE-mediated food allergy) directly responsible for causing an adverse reaction in those affected. Another complication is where an ingredient has been denatured through food processing: does it still make sense to detect the denatured proteins if they no longer cause an adverse reaction?

Methods to detect and quantify allergens fall into three main categories: enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR) assays, and LC-MS/MS. Each approach has its benefits and limitations[7]. Analysts will need to consider how well the methods employed in their laboratory answer the analytical question of interest.

Ideally, matrix materials are incurred so that the allergenic food is present within the food structure in the same way it would be in a "real" sample, but the matrix chosen will often have an effect on the ease of preparation. It is difficult to add a small amount of one food to another evenly, more so if both are powders, so the producer generally finds other matrix types to work with. One option that has been widely employed is to prepare a biscuit (cookie) matrix: the allergen food can be baked into the matrix and then the cooked food ground to provide a homogeneous powder. Although the homogeneity of the sample portion will be better than when using a mixture of powders, the drawback of this approach is that the heat applied in baking may affect the protein structure, further complicating subsequent analyses. At the National Measurement Laboratory at LGC, we have prepared matrix materials using a low-water chocolate paste based on that used in food challenge studies by Cochrane et al[8], which is easier for mixing purposes and provides a sample with a medium level of difficulty for the user. Here, the inclusion of cocoa powder increases the complexity; cocoa contains polyphenols, which may affect analytical recoveries. It is not always desirable to make a very simple material to analyze as this may not challenge the analyst in the same way as a laboratory sample and may result in a false impression of the capabilities of a laboratory or method. Fortified allergen food materials are difficult to make with suitable between-unit homogeneity, as the level of fortification is very low in order to be at the same level as the "typical" sample, or reference dose level[9]. At a level of 10 mg/kg of the allergen protein, dispersing the minor component evenly through the matrix requires considerable effort to achieve a suitable product.

For most matrix reference materials, the reference value is determined by analysis, but in the field of allergens where the recovery of allergen food protein is very difficult, the most useful current approach is to characterize the material based on what has been added rather than what can be measured. Results gained through analysis add to our knowledge, and these results may vary considerably depending on the technique or test kit used. A gravimetrically prepared reference value with metrological traceability can be provided, but the uncertainty of this value may be higher than expected due to the need to incorporate a contribution for the uncertainty of the homogeneity, which can only be achieved through measurement.

There is a balance to be struck between the pursuit of the perfect reference material, which is time-consuming, and the need to provide something sooner that, while imperfect, advances the current state of the art. With the large number of foods of interest and the vast number of possibilities for food matrices, the reference material producer has to make a judgement on the most useful combination.

What reference materials are available?

There are three main categories of reference materials which can help in the detection and quantification of allergen protein, and hence allergen foods: peptides, the allergen foods, and matrix materials in which the allergen protein is present in a food matrix through the addition of the allergen food.

Peptides are used in mass spectrometry methods both to identify the allergen protein present and to aid quantification. There are challenges in relating the amounts of individual peptides to the amount of allergen protein present and the subsequent calculation of the amount of allergen food present. The availability of peptide standards depends heavily on the allergen of interest. Those with reliable purity values should be used, but it is also advisable to check purity by amino acid analysis.

Some allergen foods are available as reference materials, but those in common use are not necessarily intended for allergen measurement and serve rather as reference materials for other purposes. Those looking to purchase a reference material should first look at the stated intended use of a material to see if it was designed for their purpose; if not, they should consider carefully whether it is still suitable for their needs. Important considerations are the steps taken to stabilize the material, the composition of the material itself, and how representative the material is of a commercial food ingredient. Not all allergen foods listed in the applicable EU legislation[1] are currently available as reference materials.

Finally, there are very few high-quality allergen matrix reference materials currently on the market, and those with ISO 17034 accreditation are very rare. This leaves the task of assessing the competence of their potential supplier, a requirement of ISO/IEC 17025[10], with the laboratory.

A laboratory looking to use a reference material in its measurement process should buy a material with a matrix as close as possible to the samples they are analyzing and containing the allergen(s) of interest at a similar level and in a similar form. This ensures that they are assessing the performance of their method under the most appropriate conditions. This principle holds for most applications of reference materials, but is particularly important in allergen analysis where sample treatment can have a significant effect on the assay recovery.

One of the first natural matrix allergen reference materials produced was a dessert matrix material containing peanut made by the National Measurement Laboratory at LGC[11]. This was released as a quality control kit in 2014[12]. Here, the matrix is a chocolate dessert paste, and the product contains both a positive and negative control material, the positive material being fortified at a level of 10 mg/kg peanut protein in the reconstituted dessert. The documentation supplied with the kit notes the level of peanut protein obtained by analysis as well as the prepared level. A peanut flour material from the same supplier as the one used in the peanut kit is sold separately[13].

In 2017, materials produced by the MoniQA Association entered the market[14]. The MoniQA materials are designed to support measurements of milk protein and the product range comprises a skimmed milk powder, a blank milled cookie, and two milled cookie materials with the skimmed milk powder at two different levels of fortification. The items are available as separate items or together in a kit. Again, the documentation supplied with the kit notes the level of milk protein obtained by analysis as well as the prepared level. While the cookie matrix allows the allergen food to be baked into the material and therefore provides the same sort of challenge as a commercial food product, the high temperatures involved in the baking process affect the food proteins and consequently can make detection difficult.

The National Measurement Laboratory at LGC has also recently released a multi-allergen reference material kit[15] produced in collaboration with the University of Manchester and Romer Labs and funded in part by the UK Food Standards Agency and the UK Department for Business, Energy and Industrial Strategy. The allergen foods incorporated are milk, egg, almond, hazelnut and walnut, all in a chocolate dessert matrix. LGC's scope of accreditation to ISO 17034 has recently been extended to include the preparation of allergen matrix reference materials, which includes this kit. As well as the fortified material, the kit contains units of the individual foods together with units of the blank paste. The materials have metrological traceability to the SI.

How can I be sure I'm getting a reliable material?

Reference material producers make materials that can fulfill a variety of uses. UKAS TPS 57 provides a useful guide to selecting a suitable reference material and producer. It explains that rather than carrying out its own assessment, a laboratory can instead select a reference material from a reference material producer accredited to ISO 17034. In the UK, this accreditation is granted by UKAS, and internationally, agreements have been signed by UKAS under the European Co-operation for Accreditation (EA) [16] and the International Laboratory Accreditation Cooperation (ILAC) [17] for the mutual recognition of reference material producer accreditation; similarly, mutual recognition of ISO 17025[10] accreditation has been in place for many years.

Finally, there is plenty of space in the market for more matrix reference materials to support allergen method development and analysis, but it is important that all materials offered for sale are fit for purpose. A poor reference material is worse than no material. The product should be industrially relevant, i.e. it should represent samples seen in the laboratory, and preferably provide values with metrological traceability. Users should read the documentation provided by the producer to see how the material was made and how the reference values were established. Users should buy materials from a reputable producer, use them carefully, and interpret results with due caution.

References

1. Regulation (EU) No. 1169/2011 of the European Parliament & of the Council of 25 October 2011 on the provision of food information to consumers, OJ L 304, 22.11.2011, 18–63. Annex II.
2. ISO Guide 30: Reference materials - Selected terms and definitions
3. TPS 57 Guidance and Policy on the Selection and Use of Reference Materials, United Kingdom Accreditation Service, Edition 4, June 2020
4. ISO 17034: General requirements for the competence of reference material producers.
5. ISO Guide 35:2017 Reference Materials - Guidance for characterization and assessment of homogeneity and stability.
6. ISO Guide 33:2015 Reference materials - Good practice in using reference materials.
7. M.J. Walker, D.T Burns, C.T. Elliot, M.H. Gowland and E.N. Clare Mills. Is food allergen analysis flawed? Health and supply chain risks and a proposed framework to address urgent analytical needs. Royal Society of Chemistry 2015 DOI:10.1039/c5^{a}n01457c
8. S. A. Cochrane, L. J. Salt, E. Wantling, A. Rogers, J. Coutts, B. K. Ballmer-Weber, P. Fritsche, M. Fernández-Rivas, I. Reig, A. Knulst, et al., Development of a standardized low-dose double-blind placebo-controlled challenge vehicle for the EuroPrevall Project, Allergy, 2012, 67, 107–113.
9. Allergen Bureau Reference dose table VITAL 3 http://allergenbureau.net/vital/vital-science/
10. ISO/IEC 17025 General requirements for the competence of testing and calibration laboratories
11. https://www.lgcgroup.com/uk-national-measurement-laboratory/
12. LGCQC101-KT Chocolate mousse dessert – peanut protein. https://www.lgcstandards.com/GB/en
13. LGCQC1020 Peanut flour. https://www.lgcstandards.com/GB/en
14. www.moniqa.org
15. LGC746-KT Allergen kit – Milk, Egg, Almond, Hazelnut and Walnut. https://www.lgcstandards.com/GB/en
16. https://european-accreditation.org/mutual-recognition/the-ea-mla/
17. https://ilac.org/ilac-mra-and-signatories

The Need for Food Allergen Reference Materials: A Kit Manufacturer's Perspective

ADRIAN ROGERS, SENIOR RESEARCH SCIENTIST, ROMER LABS®

Food producers looking for options to test for the presence of allergens are spoiled for choice – and that's not always a good thing. One of the greatest current frustrations regarding food allergen analysis is the seeming lack of comparability between the disparate analytical methods applied by different labs and the panoply of kits brought to market by different manufacturers. Well characterised, traceable and easily adoptable allergen reference materials would help to ease the frustration of those requesting allergen analysis, the labs running the tests and the kit manufacturers who produce them.

To explain the benefits that food allergen reference materials will bring, I first need to take the unorthodox stance of highlighting the challenges that a kit manufacturer faces when developing an analytical method for food allergen analysis. Then I will explain how reference materials can help with these challenges.

Antibodies

The main workhorse of any routine food allergen testing laboratory is the ELISA (enzyme linked immunosorbent assay). With the first part of "immunosorbent," we refer to the characteristic of antibodies to selectively recognise different proteins. Yet this brings us to our first challenge: what type of antibody do we use in our test? One fundamental distinction is between monoclonal and polyclonal antibodies. While monoclonal antibodies are very specific for individual epitopes, they may miss some modified proteins. Polyclonal antibodies respond to a broader range of epitopes, but are more often subject to cross-reactivity. Related questions concern the kind of protein used to make antibodies. What are they going to target? Is it a single protein from an allergenic food, multiple proteins, a fractionated protein, or a modified synthesized protein that mimics a part of the allergenic food.

Calibrators

The next challenge we face is to decide from what material to make the kit calibrators. As with our choice with antibodies, going in one direction excludes the benefits of going in another. If we use the same material that we used to produce our antibodies, we will have high recognition; if we use something that better reflects how that allergen would be present in the food we eat, our levels of recognition with our antibodies may suffer. This is where reference materials can play a crucial role. If all analytical methods were calibrated against or at least in reference to a well characterised reference material, some of this uncertainty could be reduced. Once we have decided on a calibrator, what value do we assign to it? What are we actually measuring and how will the results be reported? Do we report in whole allergenic commodity or the amount of protein from that commodity? For example, peanut contains 25% protein, which can lead to confusion in the interpretation of test results. If, for example, an analytical test report merely states "1 ppm," is that 1 ppm whole peanut or 1 ppm peanut protein? If it is whole peanut then that would convert to 0.25 ppm peanut protein. Having a common reference material and a common way to report an analytical result would help to eliminate some of this confusion.

Extraction and recovery

Another challenge is determining the proteins in an extracted sample. Will the extraction buffer succeed in pulling out the proteins that our antibodies will recognise? How do we know that we are recovering the right amount of allergen from a sample? According to guidance from Abbot et al., a recovery of 80% to 120% is deemed acceptable. The guidance, however, concedes that this is not possible for all foods, defining recoveries of 50% to 150% to be acceptable as long as they are consistent for what they class as difficult matrices, such as those that contain high levels of polyphenols or salts, have extremes of pH or are highly processed.

LOD and LOQ

While many kit manufactures quote a limit of detection (LOD) and limit of quantitation (LOQ) for their method, what this means in practical terms is not always clear. Is a low value always preferable?

LOD is defined as the lowest concentration or mass of analyte in a test sample that can be distinguished from a true blank sample at a specified probability level. LOQ refers to the lowest level of analyte that can be reasonably quantified at a specified level of precision in a test sample. More often than not, these values reflect a best-case scenario calculated

using a blank buffer extraction. To better reflect how these assays will perform with real samples, many reference materials available on the market are supplied with a blank matrix free of any allergens. This blank matrix can be used to determine LOD and LOQ specifically for the reference material in question.

Cross-Reactivity

The final challenge I want to discuss is that of cross-reactivity. Cross-reactivity can occur when a method of analysis gives a positive response to a sample that does not contain the target allergen. Often, cross-reactivity can occur with foods that are genetically similar to the allergen you are looking for. For example, if you test a sample of wheat flour, you may get a positive response with a mustard ELISA. A bit of cursory research will show that rapeseed (canola) and wheat are often grown concurrently in adjacent fields or consecutively in the same field in adherence to a crop rotation program. Furthermore, both rapeseed and mustard belong to the Brassica family of plants. Method validation demonstrates that rapeseed will cross-react in the mustard ELISA.

Processed foods such as those that are heat-treated can evince levels of cross-reactivity different to those in the food in its native form. For example, that raw peanut does not cross-react in a soy ELISA does not mean that a roasted peanut would not. It is important that the kit manufacture's validation report include information about differing levels of cross-reactivity. If it does not, check with the manufacturer of your kit.

Conclusion

All the different methods and the challenges we face when developing a food allergen analytical method have their pros and cons. There is no one-size-fits-all answer, and each one can introduce variability into the result returned on a laboratory report. Sometimes, this can be a hidden benefit, as no single method is suitable for every allergen in every type of food matrix; having some methods that perform better in different situations opens up more options to ensure the method being used is fit for purpose.

By using traceable and well characterised reference materials, we are better able to navigate our way through the challenges I have highlighted and allow for far more accurate comparison of analytical results between different allergen testing platforms, laboratories and kit manufacturers.

Allergen reference materials in brief: questions and answers

Why there is a need for reference materials?

- The number of individuals suffering from food allergies is increasing across the world.
- With a move towards risk assessment tools and threshold levels, reliable and reproducible analytical tools are needed.
- Food allergen labelling must become more comprehensive so that producers can move away from precautionary labelling.
- Due to the nature of the analytes and their susceptibility to various processing effects, reliability and comparability of results can be a challenge.
- Reference materials are needed to assure the quality, reliability and comparability of analytical results obtained from different analytical methods.

What are the best approaches to producing and validating reference materials?

- Produced to ISO 17034 standards
- Fully traceable with in-depth characterisation of all materials used
- Using gravimetric and clinically relevant concentrations of allergenic ingredients
- Packaged to ensure the integrity and stability of the material
- Stored under controlled conditions to maintain stability
- Evaluated for homogeneity and stability by validated methods with known performance data
- Characterized and certified with documented traceability values
- Applicable to all methods of analysis (ELISA, PCR, mass spectrometry)

Where can allergen reference materials be applied?

- Method calibration
- Method verification
- Method development
- Proficiency testing
- Internal quality control within a laboratory

Certified Allergen Reference Materials

Reliable, Top Quality, ISO 17034 Accredited.

Introducing the first ever multi-allergen reference material containing five commonly tested allergens: milk, egg, almond, hazelnut, and walnut.

Developed by LGC's NML Reference Materials team and in collaboration with the University of Manchester and Romer Labs, this material supports the development and validation of allergen test methods and assists the industry in verifying the performance of technicians and test results on a day to day basis.

To learn more about this next step in your allergen management program go to: