Spot On

Diagnostic Solutions

Issue 2

A magazine of Romer Labs®

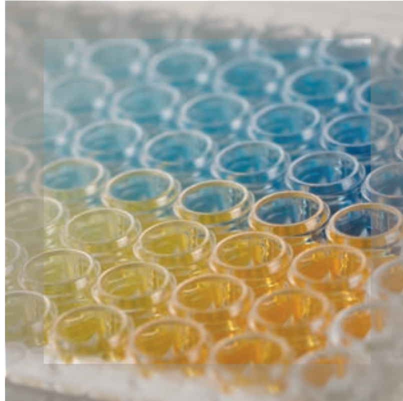
Challenges in Allergen Testing

Spiking and Recoveries

Beyond Immuno-Based Allergen Testing

Allergen Thresholds are VITAL

Content



4-7

Challenges in Allergen Testing Spiking and Recoveries

This first part of a series focuses on problems related to spiking and recovery in food allergen testing.

By **Adrian Rogers**, Senior Research Scientist, Romer Labs®

7

The Hook Effect

When a negative is not a negative:
The mystery about false test strip results.

By **Adrian Rogers,** Senior Research Scientist, Romer Labs®

8-11

Beyond Immuno-Based Testing

A discussion about different approaches to food allergen testing.

By **Kurt Brunner**, DI Dr., Division Research Officer, Romer Labs®

11

Allergen Thresholds are VITAL!

Turning away from traditional allergen concentration labeling towards relevant action levels.

By **Jasmin Kraus**, MSc, Product Manager, Romer Labs®

Spot On is a quarterly publication of Romer Labs Division Holding GmbH, distributed free-of-charge.

ISSN: 2414-2042

For a digital copy and details, visit:
http://magazine.romerlabs.com

Editors:
Cristian Ilea, Simone Schreiter

Contributors:
Kurt Brunner, Adrian Rogers,
Jasmin Kraus

Graphic:
Reinhold Gallbrunner

Research:
Kurt Brunner

Publisher:
Romer Labs Division Holding GmbH
Erber Campus 1
3131 Getzersdorf, Austria
Tel: +43 2782 803 0
www.romerlabs.com

©Copyright 2016, Romer Labs®
All rights reserved. No part of this publication may be reproduced in any material form for commercial purposes without the written permission of the copyright holder except in accordance with the provisions of the Copyright, Designs and Patents Act 1998.

All photos herein are the property of Romer Labs or used with license.

Editorial

The Caveats of Allergen Testing

Eating is necessary for survival, and also a source of pleasure. However, everyday foods like nuts, soybean, milk or sea food may cause allergic reactions.

Recent studies have revealed that more than 1 in 15 children under the age of 5 years and around 1 in 25 adults are allergic to at least one type of food. For this reason, people are becoming more concerned about food allergies. Many countries have introduced labeling regulations for such ingredients that frequently trigger allergic reactions.

Food manufacturers need to know what goes into their products and communicate any potential allergens to consumers even if only trace amounts are present. Careful testing of raw materials and final products is the only way food companies can comply with these stringent regulations.

This issue of **Spot On** *focuses on the particular challenges of applying immuno-based testing methods like ELISA or simple strip tests. Although commercial kits are highly reliable, certain procedures must be followed strictly in order that accurate results can be realised for particular food samples.*

Our lead article elucidates on finding the right test kit and explains some key steps for precise validation. The next article will discuss two alternative methods for allergen analysis, mass spectrometry and polymerase chain reaction. We also highlight some major pitfalls that are easily overlooked in allergen tests.

We hope to offer a fascinating insight into the world of food allergen testing!

Kurt Brunner

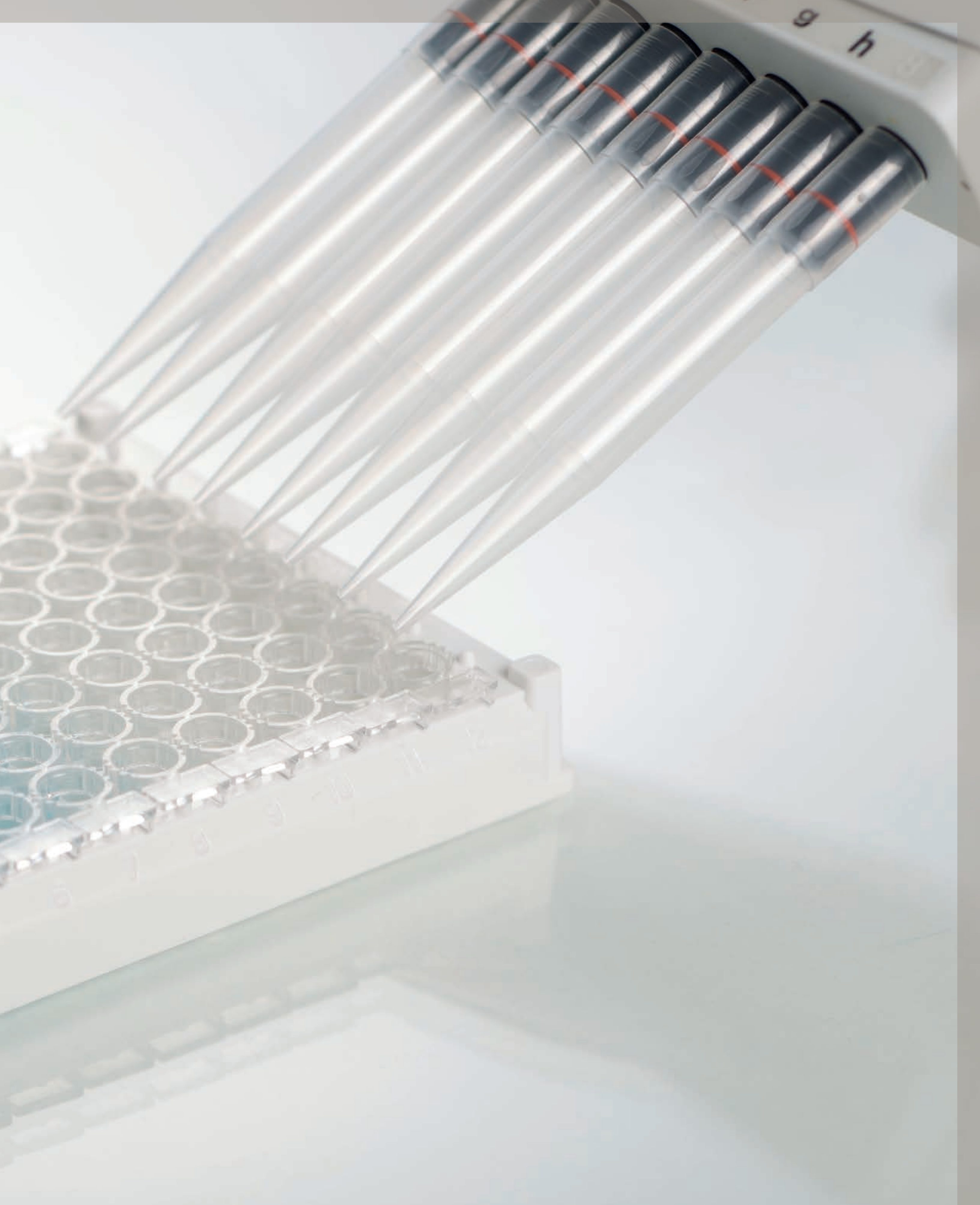
DI Dr., Division Research Officer, Romer Labs®

Challenges in Allergen Testing

Part One – Spiking and Recoveries

In this first part of a series, Adrian Rogers discusses the basics on detecting allergens in food – from finding the right test kit to methods for precise validation.

By **Adrian Rogers,** Senior Research Scientist, Romer Labs®



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 (*see box text 'Reference guideline' for guidelines on 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."

Reference guideline

In this and subsequent articles, reference will be made to this accepted published guidance with regard to assay validation:

Validation Procedures for Quantitative Allergen ELISA Methods: Community Guidance and Best Practices Abbot et al Journal of AOAC International Vol 93, No 2, 2010

About the author

Adrian Rogers has been with Romer Labs for 6 years in his role as a Senior Research Scientist. He is responsible for research and development within Romer's allergen competence centre based in the UK.

Before joining Romer Labs, Adrian was an R&D Scientist involved in the development of ELISA and Lateral Flow immunoassays for the detection of food allergens. Adrian is a microbiologist by training and has 15 years experience in the development of immunoassays, 13 years of which have been spent developing test kits for the detection of food allergens.

Over the years Adrian has been involved in a number of food allergy projects including EuroPrevall, an EU funded multidisciplinary integrated project which investigated the prevalence of food allergy across Europe. He is currently a member of the University of Manchester's Food and Health Network allergy cluster and co-ordinates Romer Labs' contribution to the "Innovate UK Knowledge Transfer Project", with the University of Manchester looking at improving soya allergen analysis.

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 6ppm. 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.

(*See box for more information on this topic*)

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.

Adding extra protein to the extraction solution may aid the recovery from difficult matrices.

The Hook Effect

Overloading the device can lead to a false negative result. This process is referred to as the “hook effect”.

The hook effect does not pose a problem in day-to-day testing using the strips.

In fact from my experience, it is only usually encountered when you are trying to verify if the LFDs are working correctly by testing 100% of the allergenic food. By doing so, the amount of allergen present exceeds the finite amount of the colored labelling material, often colloidal gold or colored latex coupled to the detection antibody.

The excess unlabelled allergen migrates along the membrane quicker than the heavier color-labelled allergen, saturating all the binding sites on the capture antibodies immobilised on the membrane surface. When the color-labelled allergen arrives, no binding sites remain, so it simply continues on to the wicking pad at the end of the test device. Since no binding sites were available, the color-labelled allergen cannot create the colored test line that would normally represent a positive result.

Beyond Immuno-Based Allergen Testing

ELISA, lateral flow, PCR or mass-spectrometry – is there a perfect test for allergens? Kurt Brunner discusses the pros and cons of each method.

By **Kurt Brunner,** DI Dr., Division Research Officer, Romer Labs®

Most commercially available kits for food allergen testing rely on the application of immuno-based methods such as ELISA or lateral flow devices (strip tests). To carry out ELISA, trained personnel are required but numerous samples can be analyzed in parallel by using 48-well or 96-well microtiter plates. In general, the analysis can take between 30 minutes and a few hours.

At present, ELISA is the most widely applied method

for the detection and quantification of food allergens. However, although many samples can be analyzed at the same time, these samples can only be tested for one analyte.

Limitations to consider

Due to the high specificity of antibodies towards only one particular allergenic protein and technology related limitations, a separate kit has to be used for each allergen. Furthermore, the high degree of specificity to one allergen might lead to false negative results.

Food processing steps like heat treatment, the addition of acidic compounds or fermentation can modify the target protein structure. These modified allergens can lose their immunological properties and the antibody – target protein complex cannot be formed anymore. This leads to false negative results or reduced quantifications.

Strip tests are inexpensive, very easy to use, do not require laboratory equipment, and give results usually in a few minutes. However, most strip tests are only qualitative and rely on antibodies as recognition elements. Therefore, they suffer from the same problems as ELISA tests with highly processed food.

In recent years, alternative analysis methods have been established to overcome at least some of the restrictions of immuno-based tests systems.

Detecting allergens with DNA

PCR (polymerase chain reaction) is a relatively fast and inexpensive method for identifying DNA. This technology, developed in the 1980s, has improved continuously since then. PCR has been used for many years in the fields of medical diagnostics, forensics, environmental monitoring, and the quantification of genetically modified organisms in food and feed.

PCR is the method of choice when cross-reactivity between similar proteins can't be overcome.

In the early 2000s PCR was applied for the first time to identify the DNA of common food allergens like hazelnut and peanut. Until now, PCR assays for most of the US "big eight" and the 14 EU food allergens have been published.

PCR amplifies small fragments of a target DNA until a sufficient number of copies are obtained for visualization or quantification. By multiplying the analytical target by a factor of 10^7 to 10^9, the few molecules of allergen DNA obtained might just be sufficient for the successful detection of allergenic ingredients.

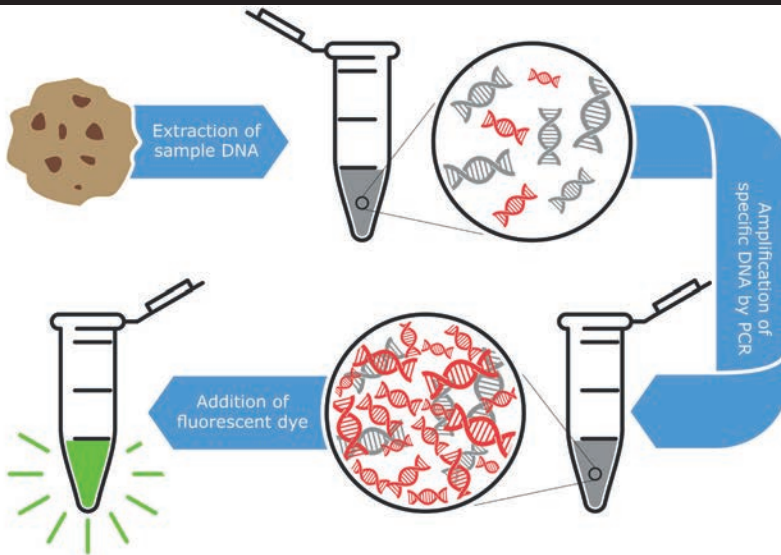
Initially developed as a qualitative method, PCR was later modified to become a tool for quantitative analysis by the application of different fluorescent generating dyes or probes.

The fact that PCR detects the extremely stable DNA molecule might be an advantage when analyzing highly processed food. DNA tends to be unaffected even by extreme conditions and can therefore still be detected even when most of the proteins have already been degraded or modified in some way. Furthermore, PCR can be used for allergens like celery which cannot be detected by antibodies. Celery has to be labeled in the EU but until now, all attempts to produce reliable antibodies have failed due to the close relationship between celery and other plants like parsley, carrot, coriander or fennel.

Over the last decade, newer DNA detection techniques have been developed. All these so-called isothermal amplification methods are in some way related to the conventional PCR but can be performed almost without any instrumentation.

A simple heating block is used to amplify the target DNA and the subsequent visual detection is realized via fluorescent dyes. Isothermal amplification is usually faster than PCR and less prone to any co-isolated impurities and in many cases even more sensitive.

Figure 1. A simplified scheme of PCR analysis



Why DNA techniques might fail

Although the detection of the DNA of allergenic food compounds might have some advantages over immuno-based methods, this approach suffers from some severe drawbacks. As DNA is the analyte of choice for PCR, it is difficult/ impossible to discriminate between egg or milk and the corresponding tissue DNA of chicken or cow as share identical DNA.

Some samples like egg white or milk only contain minor amounts of DNA but a lot of allergenic proteins and therefore, this method is not suitable for analyzing such types of samples.

Mass spectrometry: a high-end technology

An even newer technology for detecting and quantifying allergens is mass spectrometry, a high-tech method that identifies proteins and peptides with a very high level of accuracy. The first attempts at applying this technology to allergen detection began in the late 1990s but most of the results were only published in the last few years.

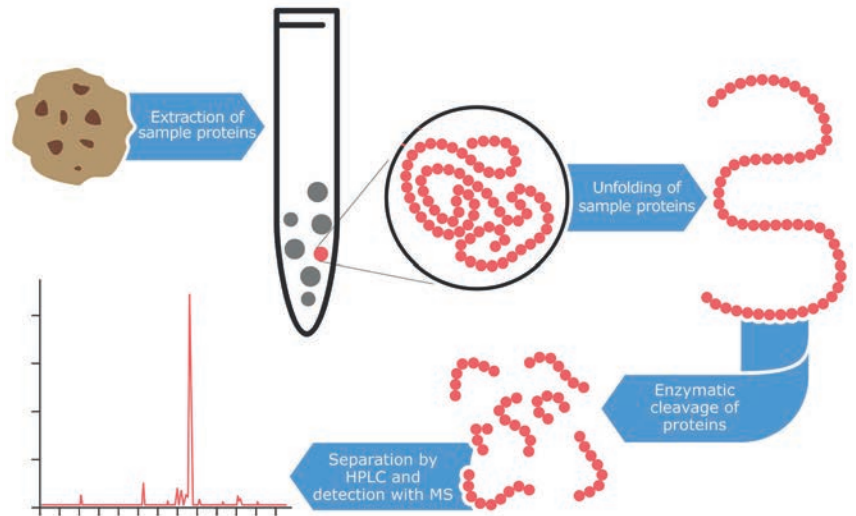
The main benefit of using this technology for allergen testing is the high level of confidence and reliability. The instruments have the capability of detecting multiple peptides per protein. Ideally, two to three fragment peptides are analyzed per allergen.

The advantage of this approach is that even if proteins are partially degraded or modified due to harsh food processing conditions, the probability of finding at least one intact fragment is quite high. These marker peptides are selected from databases or from literature and must be highly specific for the allergens to be quantified. Furthermore, they are chosen to be resistant to food processing alterations.

This multi-peptide recognition strategy of the allergen is not possible with immuno-based assays. Antibodies usually bind to only one particular (immunogenic) fragment of the allergen. If this small fragment is modified, the recognition of the target might be hampered.

Additionally, mass spectrometry is able to measure several allergens in parallel. These multi-analyte methods

Figure 2. Workflow in mass spectrometry



The best method?

Mass spectrometry can probably be considered the allergen testing method having the most potential for future improvements due to its outstanding reliability, sensitivity and the potential to perform multi-allergen analysis.

However, there is no approach without drawbacks. Mass spectrometry needs highly skilled personnel and the initial investment costs are high due to the expensive instrumentation. Furthermore, the time to result will always be much longer than for immuno methods.

have become particularly popular in recent years. This innovative strategy allows a single extraction of a sample to be screened for numerous allergens in a single analysis run.

The extraction procedure for mass spectrometry analysis is more laborious than for other approaches. First, the sample is mixed with an extraction buffer often containing dithiothreitol or urea to create a reducing environment that breaks up disulfide bonds of proteins. The remaining sample residue is then removed by centrifugation and the linearized proteins are cleaved with digestion enzymes. Some hours or an overnight duration is required to cut the allergenic proteins with these enzymes into small peptide fragments. Although the preparative steps are time consuming, the resulting peptide solution can then be analyzed for several allergens in parallel using the mass spectrometer.

Current multi-methods can quantify up to seven allergens in parallel, but it can be expected that this number might increase dramatically over the next few years. Mycotoxin multi-analyte methods started with a few analytes only some 10 years ago and today, the most advanced assay designs are capable of analyzing more than 400 toxins in parallel.

No one-size-fits-all

The perfect method, a gold standard for allergen quantification, does not exist. ELISA and LFDs are the method of choice for the majority of industrial applications. Results can be obtained relatively quickly, costs are moderate to low and personnel can be easily trained to use these tests. For some problems like highly processed testing material or specific analytes, PCR might lead to better results. Mass spectrometry is situated at the upper end of available technologies but is still in its infancy for allergen testing. However, it has, in recent years, become the method of choice for many other analytical challenges. It can be expected that this technology might experience a boost in the field of allergen analysis in near future.

Allergen Thresholds are VITAL!

By **Jasmin Kraus,** MSc, Product Manager, Romer Labs®

Allergen concentrations alone are misleading.

Legislation demands the labelling of allergens, but without reference materials, there is the omnipresent problem of lacking threshold limits.

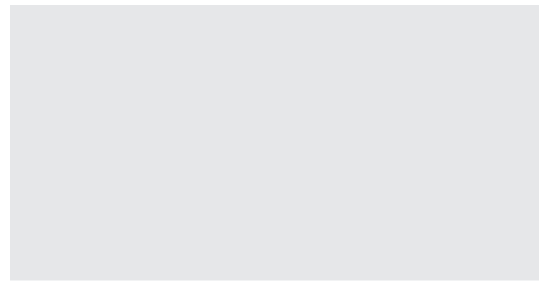
Every producer faces a dilemma in allergen labelling. Does the concentration of the allergenic ingredient found in the food product fall at, above or below the level at which the food product can be labelled allergen free? So how sensitive does the test system have to be?

More and more food producers turn away from traditional allergen concentrations towards the concept of action levels. These action levels, as originally suggested with VITAL (Voluntary Incidental Trace Allergen Labelling) by the Australian Allergen Bureau, focus on the final intake by the allergic individual.

This concept takes into account the average serving size. Hence, the same allergen concentration has a different effect on the consumer in a pinch of spices than in a portion of pasta.

Manufacturers are encouraged to assess the impact of allergen cross contamination in their products when considering detection limits to provide relevant precautionary labelling of allergens.

Your copy of Spot On



Innovative Testing Solutions.

Food Allergens?

Contact us for more information:
www.romerlabs.com | solutions@romerlabs.com

Making the World's Food Safer®