

## **Food Allergen Detection: A Literature Review 2004 – 2007**

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### **Summary**

This review of the recent literature indicates that ELISA and DNA techniques dominate laboratory testing for allergens. ELISA kits are available for most but not all major allergens but quantification can be problematic. DNA based methods have been criticised because they do not target allergenic proteins and data handling practices remain to be standardised. Published peer reviewed independent validation studies for either technique are lacking for all but a few allergens. Nevertheless, each of these approaches compliments the other in terms of target analyte and both applications have generated substantially more publications than any other techniques. This review has also elicited areas of good practice and common problems that must be addressed when evaluating and validating kits and methods. Liquid chromatography coupled with mass spectrometry is a powerful confirmatory technique successfully and routinely applied to contaminant and residue analysis. With growing databases of allergenic protein amino acid sequences it is possible to envisage bringing this powerful technique to bear on allergen identity confirmation.

### **Introduction**

The prevalence of food allergy appears to be increasing with about 10 deaths (and potentially many more near misses) every year in the UK in food allergy related incidents<sup>1</sup>. The detriment to the quality of life of food allergic consumers and their families is well documented<sup>2</sup> while food allergy hospital admissions are rising steeply<sup>3</sup> with concomitant costs to the NHS. Existing<sup>4, 5</sup> and more recent<sup>6</sup> legislation is being brought to bear on the problem. Cross-contamination may trigger general principles of European and UK food law which make it an offence to sell food that is unsafe for, or not of the nature, substance or quality demanded by allergic consumers, if intended for their consumption. Extensive guidance has been published by the Food Standards Agency on cross-contamination, 'may contain' and other labelling<sup>7</sup>. For prepacked foods, if any of 14 specified allergens are incorporated in their formulation, UK law, implementing European Directives, requires, with certain limited exemptions, a labelling declaration. The House of Lords Science & Technology Committee has published<sup>8</sup> a major report highlighting the many unresolved problems surrounding allergy in general including food allergy.

Food alerts triggered by allergens are increasing with some 150 tracked by the Government Chemist in world wide monitoring in 2007<sup>9</sup> causing increasing costs to food businesses. There is informal surveillance activity by enforcement authorities, and the food industry is putting significant effort into dealing with and avoiding damaging incidents. There is thus considerable analytical activity; however there are

relatively few published validated methods and international consensus on validation protocols for ELISA and DNA allergen methods remain in development. Potential confusion can also arise as different methods or kits report their results in different ways – for example different combinations of milligrams (mg) of allergen, mg of total protein or copy number (DNA methods) per total solids or weight of the food itself. The potential for error in an area where the science and data handling have yet fully to mature is clear bringing with it the potential for divergent results and conflicting opinion. The mis-identification of an allergen in food could be fatal for an allergic consumer and have devastating consequences for food businesses.

The Government Chemist must be in a position to give authoritative advice to the analytical community in resolving disputes on the assessment of compliance, for example in cross-contamination, labelling or ‘allergen-free’ claims. In addition should informal enforcement surveillance escalate to formal sampling and disagreements occur between enforcement and food industry findings the Government Chemist’s statutory responsibilities as referee analyst may be called upon\*. Thus a programme of work commenced in 2007 to build capability in the Government Chemist Programme to address these issues. This review of the recent literature on the topic is an initial output of the work.

## **Literature Review**

### **Literature Pre 2004**

The literature on allergen detection was reviewed (to the end of 2003) in a key paper by Roland Poms, C. L. Klein and Elke Anklam of the European Commission Joint Research Centre (JRC) Geel<sup>10</sup>. This paper contains much that remains of interest and repays full reading. For brevity only a short summary is given in Table I.

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\* The responsibilities of the Government Chemist are set down in UK law and consist essentially of an independent, expert service to resolve disputes between food control authorities and food traders on analytical results and their interpretation, the ‘referee function’. The service also provides a supplementary expert opinion if required as set out in EU law and is often called upon by importers. The Government Chemist directs research and development work aimed at preventing disputes arising. If a dispute does arise he oversees referee analysis of the retained portion of a formal food or feed control sample to resolve the issue.

**TABLE I Techniques for Allergen Detection (based on Poms *et al.* <sup>10</sup>)**

Technique	Comments
ELISA	Routinely used, capable of standardisation, quantified results, good precision but results can be kit dependent. Targets proteins (in some, but not all, cases allergens) therefore of direct relevance to allergic consumers. Relatively inexpensive if operated batch wise.
PCR	Detects DNA rather than protein therefore not probative of the presence of an allergen but sensitive. DNA more stable than proteins under harsher extraction conditions. Not quantitative.
PCR-ELISA	PCR but with ELISA endpoint. As for PCR but can be laborious owing to the extra steps introduced by the ELISA.
Real time PCR	Advantages and disadvantages of PCR. Moreover it is quantitative but more expensive.
Biosensors	Trade examples are Biacore, (surface plasmon resonance) Bioveris, Luminex, and can be used to detect protein or DNA. Some applications – hazelnut, egg and milk noted. Expensive set-up costs but rapid real time throughput achievable.
RAST (Radioallergosorbent) EAST (Enzyme allergosorbent)	<i>In-vitro</i> diagnostic tests but have been applied to food analysis.
SDS-PAGE Immunoblotting	Used to characterise new allergens. Elaborate and time consuming, use human sera but antibodies can be raised in animals after allergen characterised.
Rocket Immuno-electrophoresis (RIE)	Has been used but not widely owing to elaborate procedures necessary.
Dot immunoblotting	Simple and inexpensive screening of food samples, semi quantitative.

#### Literature 2004 - 2007

The literature was substantially enhanced by Kristina Williams, (US FDA) in a Guest Editor Section of JAOAC International published in late 2004. There is an introduction by Williams<sup>11</sup> and 16 peer reviewed papers covering a range of topics:

- detection methods then available or under development;

- properties of food allergens than can impact on detection methods;
- effects of manufacturing processes on allergenic proteins;
- methods for the identification of potentially allergenic proteins, and
- regulatory issues.

ELISA and PCR represent the main detection methods described; one paper deals with Biosensor (Microarray) Methods and SDS-PAGE and Western blotting are also referred to in several papers evaluating protein and antibody quality.

## ELISA

An excellent overview of Immunossay (ELISA) is given by Phillip Goodwin of Tepnel®<sup>12</sup>. He describes ease of homogenisation and protein solubilisation as important for sample preparation. Breaking up and releasing allergenic protein for analysis presents increasing difficulty as follows: [milk, eggs, gluten] < [fish and crustacean tissue] << [treenuts, peanuts, sesame and soya]. There are also matrix problems, e.g. allergenic proteins may bind to other proteins and to carbohydrates. Complex matrices with relatively low protein content such as plain chocolate are difficult to extract allergens from as are adsorbent materials such as powders and flours. Sample extracts may yield a complex solution often with a high concentration of non reactive protein. Goodwin also draws attention to the avoidance of laboratory cross contamination and the difficulty of obtaining blank food matrices that are truly free of a particular allergen, and keeping them that way. Protein is notoriously difficult to clean from surfaces, blenders and laboratory overcoats. Moreover the analyst's own diet should be taken into account with access to the food allergen laboratory avoided if the target analyte has been consumed recently, as is the practice with regard to certain antibiotics and veterinary residues analysis. Careful segregation is therefore required with, for example, separate (colour coded) laboratory overcoats, air handling considerations and maximum deployment of one-use disposable equipment.

Turning to antibody quality, Goodwin notes monoclonal (reacting to a single antigenic epitope) and polyclonal (reacting to multiple epitopes) are available and that antibodies raised to an allergenic protein of high purity that is robust to food processing will yield more easily interpretable data. He goes on to describe the development of a simple double antibody sandwich ELISA for peanut protein and its validation. Schmitt *et al.*<sup>13</sup> (USDA) similarly describes the development of a competitive inhibition ELISA for peanut Ara h 1 and Ara h 2.

Goodwin<sup>12</sup> also gives a readable description of the functioning of lateral flow devices (LFD, immunoassays similar to pregnancy test kits); their advantages being rapidity and ease of use. They are mainly qualitative, although semi-quantitative results can be obtained within broad ranges.

Claims for ELISA kits are generally confined to statements that protein, rather than a specific allergen is detected, however work in the USA by Nogueira *et al.*<sup>14</sup> confirmed that four kits (Veratox/Neogen, Ridascreen/R-Biopharm, ELISA-Tec/Biokits/Tepnel and Prolisa/Prolab diagnostics, Canada) do in fact detect peanut allergens Ara h 1 and Ara h 2. Interestingly, Nogueira notes that kits use animal IgG antibodies that may not follow the same affinity pattern as human IgE antibodies.

Williams *et al.* (FDA)<sup>15</sup> describe the use of an egg standard reference material (SRM) (NIST<sup>†</sup> SRM 8415) to evaluate an ELISA while Shriver-Lake *et al.*<sup>16</sup> discuss an evanescent wave fluoroimmunosensor method. The methods detect egg white rather than yolk and the recovery of the SRM from a range of foods was low (ca. 29%) owing to lower than expected solubility of the proteins.

Westphal *et al.*<sup>17</sup> deal in more detail with extraction techniques evaluated using a NIST peanut butter SRM. No single extraction buffer is universal and careful evaluation is required in kit development and use. The solubility classification of allergens is broadly:-

- Albumens – soluble in aqueous buffers,
- Globulins – better extracted into saline solutions,
- Prolamines – mixture of water and alcohol is best.

The influence of food processing, pH and ionic strength combined with the structural diversity of allergen proteins play a part. Kit manufacturers should be asked if they have optimised the extraction buffer, which must also be compatible with the immunoassay (retaining intact antibodies) although some incompatibilities can be neutralised or diluted out.

Standardisation should include the characterisation of processed and non processed allergens. Two papers deal with this topic in depth. Poms and Anklan<sup>18</sup> review the effects of chemical, physical and technological processing, and Maleki and Hurlburt<sup>19</sup> (USDA) deal with thermal processing.

Poms and Anklan<sup>18</sup> note that although food allergens can range in size from 5 – 70 kDa, many form oligomers with molecular masses >200kDa. Resistance to degradation in the acidic and proteolytic conditions of the digestive tract is one reason why some proteins are allergenic and others not, although little is known in depth about why some proteins are resistant. Epitopes can be linear (short 12 – 18 amino acid peptide fragments) or conformational (dependent on the 3D structure). One important feature of the latter is the presence of disulphide bonds which several enzymes can reduce to yield new epitopes. Structural changes can also reveal new epitopes previously buried within the molecule. ELISA detection sensitivity could be increased, decreased or abolished by changes in the target protein structure. Allergenicity can vary similarly (with implications for the production of hypoallergenic foods). Loss of structure as a function of temperature is described along with Maillard and other possible chemically induced changes. The effects of processing on various foods are also detailed, with a view to finding combinations such as high pressure processing and irradiation that could yield hypoallergenic foods.

The thermal stability of allergens can be classified as follows:

Heat stable	Milk, egg, fish, peanuts
Partially heat stable	Soya, cereals, celery, tree nuts
Heat labile	Fruits, carrots

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<sup>†</sup> National Institute of Standards and Technology, a US federal agency, <http://www.nist.gov/>

The possible reasons for such differences were explored by Maleki and Hurlburt<sup>19</sup>. It is well known that roasted peanut extract binds serum IgE from peanut allergic individuals at about 90 fold higher levels than that from raw peanuts. These authors found thermal treatment to render Ara h 1 much less soluble, although the effect on Ara h 2 was less marked. This is suggested to be due to the formation of intermolecular cross linking to form covalently bound trimers and hexamers. Additionally a protein domain homology search found Ara h 2 to have similarities with trypsin and  $\alpha$ -amylase inhibitors and roasting was found to increase the trypsin inhibitor activity of peanuts 4-fold. Both oligomer formation and trypsin inhibitor activity imply increased resistance to digestion enabling oligomers to pass intact across the small intestinal lumen to interact with the immune system and elicit an allergic response. Some Maillard reaction end products still attached to the proteins may also enhance a pre-existing tendency in a protein to allergenicity.

Finally, in this series of AOAC papers on ELISA the development of a soya protein assay is described by Koppelman *et al.*<sup>20</sup>. Previous assays developed for food authenticity purposes proved not to be sufficiently sensitive for the detection of soya as a potential allergen. The benefits of raising antibodies in animals fed a diet free from the antigen are described. A pH 12 extraction yielded good results and cross reactivity to wheat could be diluted out but the method requires further validation. L'Hocine *et al.*<sup>21</sup> also studied soy allergens, finding two commercial ELISA test kits (Tepnel Bio systems and ELISA systems) to quantify soy at lower than claimed detection limits but with narrower than claimed concentration ranges. There was positive cross reactivity with chickpea proteins and reduced immunoreactivity by papain and bromelain hydrolysis, by protein glycation and for non heat treated soy isolates compared with the treated ones.

## **FAPAS**

During 2006, the UK Food Analysis Performance Assessment Scheme, FAPAS® conducted five allergen proficiency tests. The allergens and matrices investigated were gluten in chocolate cake mix, soya in infant milk formula, hazelnut in chocolate, gluten in infant cereal and sesame in infant cereal. The participants for each of these rounds used ELISA kits from several different manufacturers, the most popular being those supplied by R-Biopharm and Tepnel. The results submitted for each round were divided into groups by FAPAS depending on the brand of kit used. According to FAPAS<sup>22</sup> “this separation was considered necessary because previous experience in FAPAS allergens tests has shown that results from ELISA kits from different manufacturers are from different populations and hence it is not wise to carry out a single statistical assessment of all of the results”. The grouping of results according to manufacturer may be due to different antibodies being employed in the kits resulting in the determination of different proteins. The results that were obtained using kits that had been used by few participants were not assigned z-scores as the number of data points was “too small to permit meaningful statistical assessment” although “this does not negate the accuracy and/or validity or otherwise of these results...” This practice continued in 2007 and for example, a round on peanut in chocolate yielded assigned values of 12.5 mg/kg for one kit and 16.2 mg/kg for another.

## DNA based methods

Goodwin<sup>12</sup> notes that over 180 food allergenic proteins have been identified and for many their DNA and amino acid sequences have been determined. It is important to ensure an amplicon size of <400bp to cope with degraded DNA. Other practical issues are described (e.g. avoiding complementarity in bp sequence near the 3' (extending) end. Tepnel are said to have developed a variety of specific primers offering high specificity and limits of detection (LODs) in the low parts per million range (mg kg<sup>-1</sup>) to bovine milk, peanut, soya, fish and crustaceans in raw or processed food. The first three had been validated by 2004. Precautions to avoid cross contamination are described, and finally problems noted are lack of certified reference materials (CRMs) and suitable proficiency testing schemes.

PCR techniques are also described in a paper by Poms, Anklam and Kuhn<sup>23</sup>. A specific DNA fragment flanked by two oligonucleotide primers is amplified through 25 – 45 cycles, by a thermostable DNA polymerase. The amplified product can be detected by agarose gel electrophoresis. The technique can be made semi quantitative using internal standards. Verification of the PCR products by restriction enzyme polymorphism with gel electrophoresis, specific hybridization with a tagged DNA probe or by sequencing is important. PCR – ELISA and real time PCR include product verification by DNA probe. Real time PCR combines the specific hybridisation of the DNA probe, amplification of the target DNA and fluorescence quantification in one step. It is more expensive but highly specific, potentially quantitative and less labour intensive. A table of PCR methods and kit suppliers available in 2004 is given (pp 1394 and 1395). Pros and cons (including the presence of PCR inhibitors in some foods) are listed. DNA methods are said to be immune from seasonal and geographic variations. The authors were unaware (in 2004) of any published PCR validation studies but were aware of an in-house validation for peanut.

The development of DNA methods is assisted by databases of allergen protein sequence resources described by Gendel<sup>24</sup> (FDA). He lists and describes the principle characteristics of the following databases:

- BIFS <http://www.iit.edu/~sgendel/fa.htm>
  - FARRP <http://www.allergenonline.com/default.asp>
  - Allergome <http://www.allergome.org/>
  - Protall <http://www.ifr.ac.uk/protall/>
  - SDAP\* <http://fermi.utmb.edu/SDAP/>
- \* Structural Database of Allergenic Proteins

In the 2007 FAPAS® peanut in chocolate round, only six participants returned results based on DNA methods against 30 returns by ELISA<sup>22</sup>. Although each participant using DNA methods correctly identified a blank material as not containing peanut the results for the spiked material appear by inspection to be quite variable.

## Other Techniques and Applications

Microarray methods are described by Shriver-Lake<sup>16</sup>. A planar waveguide (microscope slide) is patterned with capture antibodies. A fluidics unit enables sample/sample extract to be run over the surface, incubated and detected by

fluorescence labelled antibodies, by use of laser light shone down the wave guide. An optical image is captured by a charge coupled device camera. Signal intensity and spot location provide information. Egg ovalbumin was detected apparently with a LOD of  $1.3 \mu\text{g kg}^{-1}$  ovalbumin in non-egg pasta. A peanut assay by SPREETA (Texas Instruments Ltd) is referred to. Evanescent wave fluorimetry is described and its exquisite selectivity for surface bound fluorescence is claimed to require little if any sample pre-treatment.

Some interesting other papers in the JAOAC Int. guest editor section describe:

- the assessment of the allergenicity of proteins introduced into GM crops (e.g. brazil nut into soya bean for animal feed)<sup>25</sup>;
- the potential allergenicity of novel proteins as assessed by murine models<sup>26</sup>;
- animal models for intervention therapy following sensitisation<sup>27</sup>.

The application of protein quantification, ELISA and real time PCR used to evaluate industrial cleaning procedures to avoid cross contamination with peanut and celery are reported from Germany by Stephen *et al.*<sup>28</sup>.

Finally in the guest editor Section of JAOAC International the effects of regulatory initiatives in reducing the incidence of peanut protein allergen food recalls over a ten year time period are detailed by Beb-Rejeb *et al.*<sup>29</sup> of Health Canada.

Other contributions to the literature since 2003 include the following. A useful summary of current thinking was given in 2007 by Arjon van Hengel<sup>30</sup> (JRC IRMM Retieseweg) who noted in particular:

- broad specificity methods (e.g. for a range of fish species) are unlikely to exhibit equal reactivity between each species and the standard;
- genetic variability can occur between varieties of the same species (contrast this with previous thinking on DNA methods);
- recently available IRMM-481 test material CRM consists of 5 types of peanut varying in their variety, geographic origin and processing treatments;
- peanut protein kit validations are referenced;
- hydrolysates are an increasing challenge – being used extensively in nutraceutical, pharmaceutical and food products - it remains to be seen if current detection methods react to allergen hydrolysates.

Health Canada maintains a website resource - Canadian compendium of Allergen Methodologies<sup>31</sup>. Of the more recent studies cited in the brief list of references therein Ben-Rejeb *et al.*<sup>32</sup> describe a multi-screening immunoassay for the detection of protein markers of peanut and four tree nuts in chocolate.

### **Thresholds of Elicitation**

The limits of detection required of allergen detection methods need to have regard to human elicitation thresholds<sup>33</sup>. This is a highly problematic area although some work has been carried out<sup>34, 35, 36, 37</sup>. While definitive information may never be achieved and some individuals, by a combination of immunology and circumstance, will remain exquisitely sensitive to some allergens, LODs at the low  $\text{mg kg}^{-1}$  range seem

currently fit for purpose for many allergens. However, more sophisticated treatments of elicitation thresholds, including the possible application of safety factors to clinically determined thresholds, may render current limits of detection wanting. An evaluation of a method should always have regard to the best information on elicitation threshold concentrations.

### **Mass Spectrometry based techniques**

Although currently there are few routine applications there may well be scope for the development of mass spectrometry based techniques to address lower limits of detection that may be required in the future. Coupled with liquid chromatography such techniques may also provide independent verification of the presence and quantification of food allergens in a product. LCMS and LC-MS/MS are commonly applied in LGC to veterinary drug and pesticides residues analysis and in the Government Chemist's Programme LC-MS/MS is routinely used for mycotoxin detection. These and other MS methods have been described for the identification of proteins or peptides, mainly for identity confirmation purposes since quantification seems to be difficult and validation studies are lacking. A promising approach is claimed to be multi-dimensional separation/identification of proteins using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS). Matrix Assisted Laser Desorption Ionization Mass Spectrometry (MALDI) is also potentially applicable. Milk and gluten have been identified by MS methods. By way of illustration of the potential of the techniques a selection of papers is cited from a 2006 AOAC Europe presentation by Jacqueline van der Wielen<sup>38-42</sup>. Interestingly Chassign, Norgaard and van Hengel<sup>43</sup> have also described a proteomics based approach to the detection of major allergens in processed peanuts by reversed-phase capillary liquid chromatography with electro-spray ionisation quadrupole time-of-flight tandem mass spectrometry (nano-ESI Q-TOF MS/MS).

Thus mass spectrometry based approaches, with sufficient analytical development have the potential to provide confirmation of identity and routine analysis for peptides characteristic of allergen proteins, given the sequence information available in the database described by Gendel<sup>23</sup>. The Measurement for Biology programme in LGC has enabled development of mass spectrometry based methods for quantifying peptides with high accuracy, for example using a new high resolution MALDI-ToF/ToF instrument, standards to help characterise glycoproteins and a guide to mass spectrometry for proteomics<sup>44</sup>. It is envisaged that the 2008-2011 Government Chemist's programme will include a scoping exercise on the application of similar techniques to selected allergen proteins.

### **Quality Aspects of Food Allergen Detection Analytical Methods**

The period under review saw the promulgation of a number of official references to the performance required of analytical methods in general. These are equally relevant to the detection and possible quantification of food allergens. European legislation<sup>45</sup> promulgated in 2004 included reference to methods for sampling and analysis used in the context of official controls. Article 11 of 882/2004 states that methods of sampling and analysis must comply with relevant Community rules or if no such rules exist,

(and there are none as yet for allergen detection) with internationally recognised protocols, for example those of the European Committee for Standardisation (CEN) (see below) or those agreed in national legislation. Otherwise, methods must be fit for the intended purpose or developed in accordance with scientific protocols, e.g. single laboratory validation according to an internationally accepted protocol. Wherever possible, methods of analysis shall be characterised by the criteria set out in Annex III, reproduced below:

- (a) accuracy;
- (b) applicability (matrix and concentration range);
- (c) limit of detection;
- (d) limit of determination;
- (e) precision;
- (f) repeatability;
- (g) reproducibility;
- (h) recovery;
- (i) selectivity;
- (j) sensitivity;
- (k) linearity;
- (l) measurement uncertainty;
- (m) other criteria that may be selected as required.

The Annex goes on to add that the precision values shall either be obtained from a collaborative trial which has been conducted in accordance with an internationally recognised protocol on collaborative trials (e.g. ISO 5725:1994 or the IUPAC International Harmonised Protocol) or, where performance criteria for analytical methods have been established, be based on criteria compliance tests. The repeatability and reproducibility values shall be expressed in an internationally recognised form (e.g. the 95 % confidence intervals as defined by ISO 5725:1994 or IUPAC). The results from the collaborative trial shall be published or freely available. Methods of analysis which are applicable uniformly to various groups of commodities should be given preference over methods which apply only to individual commodities. In situations where methods of analysis can only be validated within a single laboratory then they should be validated in accordance with, for example, IUPAC Harmonised Guidelines, or where performance criteria for analytical methods have been established, be based on criteria compliance tests. Methods of analysis adopted under this Regulation should be edited in the standard layout for methods of analysis recommended by the ISO.

### **Validation studies**

Validation of analytical methods is now well described<sup>46, 47, 48</sup>. Manufacturers of food allergen test kits have carried out extensive in-house validation studies, but few have been published in the peer reviewed scientific literature. Goodwin's work on peanut ELISA validation is referred to above<sup>12</sup> and included such attributes as ruggedness. Park et al<sup>49</sup> describe the multiple laboratory validation of Neogen Veratox<sup>R</sup>, R-Biopharim RIDASCREEN<sup>R</sup> FAST, and Tepnel® Biokits commercially available ELISA kits for peanut. The foods examined were breakfast cereal, biscuits, ice cream and chocolate. The validation required 60 analyses of blank samples and 60 samples

spiked at 5 mg kg<sup>-1</sup>. All three kits successfully identified the spiked and peanut free samples in industry, international and U.S. Government laboratories. The authors calculate a 50% probability that a test sample contains an allergen given a prevalence rate of 5% and a positive test result using a single test kit analysis with 95% sensitivity and 95% specificity (as demonstrated for these kits). When two test kits are run simultaneously on all samples the probability becomes 95% and the authors recommend all 'field' samples are analysed with at least two of the validated kits.

In 2006 Matsuda *et al.*<sup>50</sup> published the results of an interlaboratory (10 laboratories) validation in Japan of two ELISA kits for the detection of egg, milk, wheat, buckwheat and peanut in a variety of highly processed foods. Both kits (FASPEK and FASTKIT) correctly determined egg and milk protein. Similarly both kits dealt with wheat, buckwheat and peanut but with higher interlaboratory variations. Both featured an improved extraction buffer that can extract insoluble proteins produced by processing and antibodies that bind to the denatured proteins extracted with the new extraction buffer. Neither kit could determine wheat protein in jam. A notable feature of both kits is a unified extraction solution and unified standard calibration solutions making comparisons easier.

Differences in the experimental design of various validation studies were noted by van Hengel<sup>30</sup> who went on to advocate a common protocol. It is understood this is currently being taken forward by a working group drawn from the Food and Drug Administration (FDA), Health Canada, Food Allergy Research and Resource Program University of Nebraska (FARRP), Food Products Association (FPA), and the European Commission's Institute for Reference Materials and Measurements (EC-JRC-IRMM).

Validation of ELISA, DNA and LC-MS/MS based analyses of food allergens should cover at least the performance characteristics outlined in Annex III of 882/2004. BSI and CEN are developing standards for the detection of food allergens by immunological and by molecular biological methods<sup>51, 52, 53</sup>, however as these documents are still in draft no information has been reproduced from them in this review. This review does however suggest a number of additional factors that should ideally be addressed and they are listed below. It is recognised that not all of this information will be available or released by the Intellectual Property (IP) holder.

1. The Allergen(s):
  - a. Has the allergen been identified as a protein or group of proteins?
  - b. Is the analyte detecting the allergen or a different marker?
  - c. What is the abundance of the analyte in the food?
  - d. Have the proteins been characterised in terms of structure, function, amino acids?
  - e. Has the gene expression for the protein been sequenced?
  - f. Are purified allergen proteins available?
  - g. How does food processing affect the allergenicity of the protein?
  - h. Are allergen hydrolysates used in the food industry and will the method detect such material?
  
2. ELISA Antibodies
  - a. Monoclonal or polyclonal?

- b. Raised to identified purified allergen proteins or crude allergen extracts?
  - c. Raised to processed or unprocessed antigen or both?
  - d. Raised in animals fed a diet free from the allergen?
3. Matrix Interferences and Extraction
- a. Have matrix interferences been investigated (e.g. for inhibition of extraction) in sufficient sample types and by spiking of unprocessed and processed foods?
  - b. Has the extraction been optimised for recovery of allergenic protein or DNA?
  - c. Is the optimum extraction chemistry compatible with the immuno assay or PCR and if not what steps were taken to avoid damaging the antibodies?
  - d. If a number of allergen/kit/methods are being compared the optimum approach is common extraction chemistries and calibration standards.
4. Cross reactivity
- a. Cross reactivity studied in a statistically designed experiment against taxonomically related and/or unrelated *genera*, species, varieties or cultivars?
5. Robustness
- a. Evaluation to variations in extraction, ELISA, PCR etc?
  - b. Dilution Parallelism investigated?
  - c. Storage stability of kits investigated?
6. DNA based methods
- a. Is the size of the detected genome known?
  - b. Has matrix PCR inhibition been investigated?
7. Reference materials and PT schemes.
- a. Is there a reference standard available with the kit and if so how was it developed?
  - b. Are CRMs, and/or SRMs available and from where?
  - c. Is there a PT scheme and what results have been achieved in it?
8. Elicitation threshold concentrations in allergic individuals.
- a. Literature threshold trigger levels in allergic individuals should be used to gauge required performance characteristics of the method.

A theoretical consideration of optimum sample sizes for validation of allergen – screening methods was discussed by McClure and Graves<sup>54</sup> in 2003.

### **Influence of sampling**

Having validated a method ultimately the significance of results will ultimately depend on sampling and an interesting paper by Trucksess and colleagues<sup>55</sup> examined the sampling, sub sampling and analytical variances for peanut contamination in

chocolate based foods, a known difficult sample matrix. A commercial peanut ELISA test kit was used. Sampling and sub sampling variability, as a percentage of the total testing variability, was found to be 96.6% and >60% for energy bars and powdered milk chocolate respectively. The variability could be reduced by increasing sample size, sub sample size and number of analyses.

## Conclusions

This review of the recent literature indicates that ELISA and DNA techniques dominate laboratory testing for allergens. ELISA kits are available for most but not all major allergens but quantification can be problematic. DNA based methods have been criticised because they do not target allergenic proteins and data handling practices remain to be standardised. Published peer reviewed independent validation studies for either technique are lacking for all but a few allergens. Nevertheless, each of these approaches compliments the other in terms of target analyte and both have generated substantially more publications than any other techniques. This review has also elicited areas of good practice and common problems that must be addressed when evaluating and validating kits and methods. Liquid chromatography coupled with mass spectrometry is a powerful confirmatory technique successfully and routinely applied to contaminant and residue analysis. With growing databases of amino acid sequences in allergenic proteins it is possible to envisage bringing this powerful technique to bear on allergen identity confirmation.

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## Disclaimer:

Reference to any manufacturer or trademark does not imply endorsement by the Government Chemist. No responsibility can be taken for the use made of any view, information or advice given. In particular, any view, information or advice given should not be taken as an authoritative statement or interpretation of the law, as this is a matter for the courts.

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