

Review of allergen analytical testing methodologies: Evidence gaps in allergen management and testing

Aside from the evidence gaps in methodological testing capability of the various commercial test kits available (detailed in Table 1, Appendix 1), other gaps in allergen management are discussed below.

4.1. Current evidence gaps in testing

4.1.1. Determination of reference doses

While allergen consumption thresholds are available based on clinical studies, more work is required to determine the threshold of foods. Most recently, the ad hoc Joint FAO/WHO Expert Consultation on Risk Assessment of Food Allergens recommended reference doses for the global priority allergens, but other important allergens that are listed in UK and European regulations have not yet been assigned reference doses. Here, VITAL® 3.0 levels can serve as a guide.

4.1.2. Implementation of testing within the supply chain

(i) There is a gap here in that the level of control in the supply chain needs to be more tightly controlled to mitigate issues concerning undeclared allergens. While larger manufacturers and larger suppliers tend to invest in allergen risk assessment and confirmatory allergen testing, there are risks associated with many small-to-medium sized businesses which often perform no risk assessment and little or no testing.

(ii) Allergen testing costs approximately £150-250 per allergen by ELISA, which screens for a single allergen in each test. Multi-allergen tests are required to reduce costs and test times. Infrared spectroscopy (IR) methods have been developed for on-site detection or screening to detect multiple allergens. However, the sensitivity of IR methods is not sufficient (being in the percentage range rather than the required parts per million or parts per billion range) to support the required levels, given the threshold levels/reference doses suggested by the Joint Food and Agriculture Organisation and World Health Organisation (FAO/WHO) Food Standards Programme (FAO/WHO) Expert Group (WHO, 2021).

(iii) Methods are under development by loop-mediated isothermal amplification (LAMP) assays which could provide confirmative on-site testing of ingredients. The false positive rate of such a test (much like lateral flow tests) is low.

(iv) Methods of point-of-use testing of ingredients by technologies including LAMP and Near-Infrared Spectroscopy (NIR) are under development for areas such as quality control and authenticity of products, for example protein, sugar and fat composition. It is anticipated that new, validated low-cost methods will be available for point-of-use ingredients testing for allergens

within 5-10 years. This may permit manufacturers to gain fast knowledge regarding their raw material safety and quality at the time of use in the factory.

(v) Increased energy costs and shortage of supplies (for example linked to issues in transportation, and/or to the war in Ukraine) are causing changes in supply chains. Manufacturers are increasingly resorting to spot buying of ingredients when regular (and trusted) suppliers cannot meet demand or are using alternative (undeclared) ingredients. The practice of spot buying can inherently result in a reduced level of audit data and can result in increased risks of food fraud and safety and quality concerns.

4.1.3. Analytical gaps and testing service provision

(i) Those in the supply chain must become better-versed regarding the testing types which they request from the laboratories and which are fit-for-purpose for their sample types. For raw materials this is not a severe issue as many testing methods are fit-for-purpose for raw ingredients. However, when foods are processed, the proteins can be altered and, in general, it is more difficult to detect the allergenic protein or peptides, and detection can be reduced or the method may no longer be fit for purpose. For those needing accurate testing results of processed products, it must be understood which types of testing methods are fit for purpose. For example, there are laboratories that offer testing services by PCR for egg to detect egg-specific DNA. Here the challenge is that an entire egg contains only a single copy of DNA in the egg yolk, which is very little for detection by most DNA-based detection methods currently used. In addition, the food industry used fractionated products, e.g. egg white powder, which contains no DNA but large quantities of (allergenic) proteins that can trigger severe allergic reactions in susceptible individuals. Therefore, DNA-based detection methods such as PCR may not be fit-for-purpose for detecting the presence of egg and its derivatives like egg white powder. Similarly, PCR methods for milk tend to lack sensitivity. Alternative methods to PCR, e.g. ELISA, should be offered and non-fit-for-purpose tests should not be offered.

(ii) Full validation of testing methods is required (involving a series of studies to determine accuracy, precision, sensitivity [meaning the slope of the calibration curve], specificity, robustness, applicability, repeatability, reproducibility, LOD, LOQ and range of LOQ studies) for a wide range of matrices. The risk of false negative results would need to be investigated, especially for matrices with a high risk of false negatives. An example of such matrices includes tomato-based matrices due to the low pH of tomato, and tomato ketchup which also includes vinegar. At low pH, the DNA auto-catalyses and DNA may not be detectable by PCR, a false negative result may be yielded by PCR. In such cases, analysis by Mass Spectrometry or ELISA would be preferable. PCR testing is also less useful when a matrix has a high protein load and a low DNA load, for example egg.

(iii) As discussed in more detail under Harmonisation Activities (Section 3), there is a need for RMs or, as a minimum, quality control material against which all testing laboratories should calibrate their methods. Such RM is needed for each allergen, and ideally prepared in a range of applicable food matrices for each allergen to account for matrix interferences of certain ingredients and/or processing conditions. As discussed elsewhere in this report, RMs are currently completely lacking for most allergens. There are difficulties in preparing RMs to cover all possible food matrix types. Producing a RM for matrices such as mayonnaise for example (egg and mustard allergen concern) which contains significant levels of oil and has low pH is challenging due to challenges in extracting protein from such oil-based samples.

(iv) There are also requirements to consider the fraction of a protein which is being tested. For example, in milk, there are commercial kits containing antibodies against the casein protein. However, if the milk is fractionated during processing, and the whey proteins fraction is being used for food production, the casein allergen may no longer be present and thus cannot be detected, which does not mean that no allergens are present. Also, incorrect levels may be

determined if a particular protein fraction that the antibody recognises is enriched or depleted during fractionation.

(v) Further method development is required. A reliable method of evaluating the performance of a particular method is for the method to be the subject of an inter-lab trial. Examples of inter-lab trial data are provided in the literature review section of this report but, as an example, twelve laboratories participated in a trial to determine sesame in a mayonnaise containing 47 mg/kg sesame, which is a high level of sesame given that the reference dose is 2 mg. Only two of the twelve labs detected sesame, and both under-estimated the level by approximately 50%. (Besler-Scharf, 2021) Mayonnaise is a challenging matrix due to its acidic pH and oil content, but this demonstrates that further method development is required to protect consumers, to detect even high levels of allergens in some matrices.

(vi) It is important that more method validation data and quality data are included in the manufacturers' kit instructions so that it is clear exactly which matrices have been used in their validation studies to inform users. However, several of the allergenic commodities are so versatile that they are an ingredient (in one form or another) in many foods. For allergens such as soya, it is estimated that soya and its derivatives can be found in over 30,000 products worldwide. (personal comms) As an example, soya can be used as flour, lecithin, oil, phospholipid fraction, phytoestrogen. It is likely that milk and its derivatives are contained in a similar number of products. While method validation requires investment, the more products for which validation data is available, the more is known about the applicability of each method/test kit. In an ideal situation, such validation data could be held in a central database, allowing other laboratories to access it, thereby significantly reducing the workload and sharing the efforts across laboratories. However, this will only work if validation has been performed to national or international standards.

(vii) Similarly, a gap exists in that manufacturers are currently not obliged to publicise their day-to-day allergen issues so many issues are resolved before recalls are required. Therefore, the information the public and governments can access may not reflect the real situation. Changes are required so that all allergen-related issues are logged centrally so that we are aware of the scale of allergen issues in the UK.

(viii) ELISA is currently the most popular method for allergen detection, due mainly to the simplicity and wide availability of commercial kits. In addition, ELISA tend to be appropriately sensitive and specific for allergen proteins, particularly in raw ingredients. The acceptable cost and required instrumentation make this technique attractive for laboratories, in addition to the comparatively low level of expertise that is required by laboratory staff to conduct the method and evaluate the results. Given that there are gaps in the testing capability, some ELISA kit manufacturers however include a caveat in the user guide to their methods of using PCR as a confirmatory method. PCR is a lower cost method compared to ELISA and is often available and well-established in laboratories which also use ELISA, which may be the reason that kit manufacturers suggest this confirmatory methodology to users. As described above, PCR is not always an appropriate confirmatory method. Furthermore, it is wise to apply a confirmatory method which can detect the allergenic substance i.e. the peptide or protein, rather than aiming to detect the DNA of the host ingredient. However, a more appropriate confirmatory method would be liquid chromatography mass spectrometry (LC-MS) as this method detects the allergenic peptides and proteins themselves. Arguably, fewer laboratories which use ELISA would also have LC-MS facilities which tend to be high cost in terms of purchase, running and maintaining, and require specialist training outside of the molecular biology skills used for PCR and ELISA. It seems that there is a funding gap for developing the use of LC-MS/MS for allergen detection and quantitation compared to the development of PCR and that there should be more focus on future work to prepare LC-MS methods, which also have the benefit that they can be multiplex methods to detect multiple allergens in a single analysis. EFSA has recently funded a project (please refer to section 5 on EU Project ThRAII) with the goal to develop reference (harmonised)

methodologies for the detection and quantification of allergens in foods using mass spectrometric approaches.

(ix) Point-of-use testing could be improved for use in factories. The Titan Project (providing digital technologies that increase transparency throughout the food value chain to save money, resources, people, and the planet) includes the development of emerging nanotechnology devices for use by manufacturers in order to reduce the burden of allergen testing on the manufacturer.

Sampling regimes in factories require careful planning. Single-point sampling is a risk. A two-prong method of testing may be preferable:

- (1) Perform tests on incoming raw materials to check for cross-contamination or mislabelling
- (2) Environmental mapping on site, performing point-of-use testing of areas identified as high-risk areas on site and also on the production line to check for contamination.

The Titan project will test these scenarios. Artificial Intelligence methods may also prove beneficial to access historical data to determine particular areas of a site, or specific suppliers, where the risk has been shown to be elevated, to manage and prioritise testing efforts.

4.1.4. Method Performance Criteria

(i) Lessons can be learned from other countries to improve method performance criteria for allergen management. In Germany, a working group exists to support allergen management in the food chain, comprising approximately 75% members from government and 25% from industry. The aim of the working group is to develop official government control methods (analytical methods) for the detection of food allergens. While, as previously mentioned, ELISA is by far the most commonly deployed detection method for food allergens, very few commercial ELISA methods have been validated by this group, which is in part because the government aims to avoid commercial imbalance by validating only one or two commercial kits for an allergen while there are more ELISA kit producers offering detection kits for the allergen of interest. Also, since many of the validated methods are submitted for international standardisation, preferring certain methods over others would make it difficult for newer, potentially even better, methods to be accepted. As this is a general problem for methods which are standard methods, an approach at Codex Alimentarius was launched to establish Method Performance Criteria (MPC), also referred to as Method Performance Requirements (MPRs), rather than standardising individual methods. Setting such MPCs/MPRs would allow new methods to be accepted as long as they fulfil the criteria. AOAC International has adopted this route and developed an SMPR® (Standard Method Performance Requirements) program for different types of methods and commodities, including food allergens. Here, AOAC SMPR® 2017.020 and AOAC SMPR® 2018.003 lay down the requirements under which such methods could be accepted by AOAC, either as a Performance Tested Method (PTM) or Official Method of Analysis (OMA). Irrespective of the approach, single method validation or setting method performance criteria, the availability of appropriate RM may facilitate these approaches.

However, according to work by Rzychon et al 2017, RMs will not necessarily improve measurements where this lack of correlation is observed, although their use will highlight the variation which will be of interest to risk assessors. (Rzychon et al., 2017) With correlation between test kits, RMs improved comparability of results.

However, beneficial effects were not observed equally by all kits or even for all matrices on the same kit. While this work was based on gluten ELISA tests, the conclusions may well apply to any protein ELISA.

(ii) Auditing within the food chain in the UK and EU is deemed to be of a high quality. It is estimated that contractual agreements between buyers and sellers on safety and hygiene standards and of food certificate checking further afield would yield improvements in allergen management and safety, with independent auditing of overseas suppliers. It is known for example that, as part of their risk assessment, some companies in non-European countries do not test for all allergens held in their facilities or handled in their factories, due to the financial burden of testing. An example is a company outside of Europe known to handle much dried fruit but also handles tree nuts in the same facilities, although this is not declared on the dried fruit labels (personal comms). Without audit and inspection, we cannot be sure of the quality standards within the supply chain with its ever-growing complexity. There is also a trade-off here as more affluent countries such as the UK can afford to invest in high-end detection equipment such as LC-MS/MS but poorer exporting countries will rely on lower cost technologies, which may or may not be appropriate for testing the particular ingredient or compound. Audits evaluate the status of a commodity at a point in time only and are used to confirm that measures have been implemented.

For this reason, the key here again is education about the risks of food allergens and their adverse health impact, especially at the beginning of the food supply chain, so that the correct checks and tests can be implemented. With this knowledge, then the appropriate analytical testing can be applied and high-quality audits could be implemented to verify conformation to high standards. Without audits, the UK is unaware of practices in the global supply chain, since financial issues, education and perception of food-associated risk differ across the globe.

4.2. Emerging risks

The main emerging risk at present in terms of allergen management concerns alternative proteins. In order to feed a growing global population, much innovation is currently underway worldwide to prepare proteins from alternative sources compared to those used today, or to use the same protein sources but in new ways, such as from livestock meat, dairy foods, fresh vegetables, grains, nuts, beans, pulses and seeds. Insect proteins are being widely considered and developed to appeal to Western palates. Much in the same way as a much higher proportion of Asian consumers are sensitive to milk compared to Western consumers, there are considerations here that when new protein food sources such as insects or more highly processed vegetable proteins are introduced to a new population of consumers, data may emerge of increased levels of inherent allergen incidence due to the biology of this particular population. Also, insects such as cockroach contain tropomyosin which is a protein structurally largely identical to the tropomyosin of crustacean, a known allergen. Therefore, allergen labelling of insect foods or insect matrices used in food production is recommendable to protect crustacean-allergic consumers.

Aside from insect protein, novel ways to use plant-based proteins are being developed. Plants including soya and pea are being processed in novel ways to produce vegetable ingredients which simulate the texture of meat to offer a meat-free alternative to products such as beef steak. During this processing, the proteins are often enriched. It is important that we understand how processing is affecting the protein, and thereby the allergen, even for plant proteins we are familiar with but in other, differently processed, forms. As an example, a protein containing a sequence or structure known to trigger an allergy, may be 'hidden' and non-reactive inside the folded protein when in its native form. However, processing may release the allergenic sequence/structure or simply increase the level of allergen within the ingredient which could lead to an increased risk of allergic reactions.

It is already known that some consumers are sensitive to pea proteins, however, insufficient cases have been reported to initiate a response in declaring pea protein as a food allergen of concern. Pea protein is of major interest to current novel protein innovations. There is a requirement to be proactive to develop testing methods for pea before the alternative proteins market grows to the stage where the prevalence increases to a level which would justify declaring pea as a regulated food allergen.

Also, since there are risks that processing methods may change the allergenicity of a commodity, there is a need to educate those who are developing these innovations, often small enterprises, so that risk assessment and testing strategies can be implemented to avoid innovation of products which unintentionally increase the risk of eliciting an allergic reaction. An example of such failed innovation in the GMO area is the transgenic soybean which carried a Brazil-nut allergen (albumin). (Nordlee, Taylor, et al., 1996) This product was never marketed.

4.3. Conclusions

Evidence gaps in allergen testing have been discussed above. Necessary improvements to testing services have been highlighted. Improvements and gaps in the methodologies used for allergen testing have been highlighted here and are also discussed in detail in the literature review section (Section 2). Reliable, independent auditing to the same standard is required for all global suppliers in the UK supply chain. Finally, we must act now develop tests and risk assessment approaches to inform risk management regarding issues that may arise from emerging risks such as novel foods.

As highlighted by many stakeholders and our expert consultants, there is a need for the development and commercialisation of a fast multi-allergen test, which can be used at point-of-use in factories to mitigate cross-contamination risks. Funding to develop such a test is required. In addition, more funding to improve current multiplex mass spectrometry methods would support rapid, single test confirmatory testing of allergens in foods.