Review of allergen analytical testing methodologies: Allergen testing workflows to support incident management

RMs play a crucial role in allergen analysis to provide a means of:

(a) deriving conversion factors, especially in relation to mass spectrometry methods where there is a need to convert from peptide mass to mg allergenic food protein;
(b) supporting effective test method validation; and
(c) harmonisation of test method results as has been shown for determination of gluten. (Rzychon et al 2017)

They can include the allergenic ingredient itself alone and incurred in a food matrix. There are at least allergen CRMs available i.e. materials which have been certified (for example, ISO 17034) and demonstrate traceability to national or international standards and provide a statement of uncertainty. Although CRMs for allergens have been prepared in the past, we have identified issues with these materials no longer being available. Furthermore, there is a limit to CRMs which are incurred. Where such CRMs are lacking, in the interim, QC materials can be used, and are available for a wide range of allergenic food ingredients and incurred food matrices, e.g. surplus materials from proficiency testing providers. They may also be prepared in- house to provide closer matrix matching of food products. However, these may lack an assigned allergenic protein content, limiting their usefulness.

Since allergen testing is impacted by processing and its effect on the detectability of the protein allergen target, and due to the inherent lack of knowledge regarding the level of processing when presented with a sample suspected of eliciting allergenic reactivity, rather than relying on a single test to determine if an allergen is present, a workflow comprising multiple complimentary tests must be implemented.

Workflows include testing by validated fit-for-purpose methods. Since the allergenic hazard comprises proteins (apart from sulphites and sulphur dioxide), methods used should target the allergen proteins or their constituent peptides and provide test results in mg allergenic ingredient protein/kg food product, as recommended by the

FAO/WHO expert consultation. Only when no such method is available should test methods targeting non-protein measurands, such as DNA-based methods, be considered. As discussed previously, PCR methods are not suitable for egg and are less sensitive for certain allergens such as milk. As demonstrated by incidents in the supply chain linked to cross-reactivities displayed by allergen testing kits, workflows must include more than one test method in order to gain confidence in a negative result and to protect allergen-sensitive consumers (Walker et al 2018, see Section 2). Ideally testing will target different analytes and will also target the same or different analytes using more than one applicable technology. Testing only for the allergen protein/peptide is important for example when testing beef products for cow’s milk allergen or chicken products for egg.

Testing should be conducted by an ISO 17025-accredited laboratory using a validated test method and the sample should be analysed in duplicate. Incurred RMs with an established uncertainty factor must be extracted and analysed in the same batch to verify method

performance and to build up QC plots to track variations in kit performance between kit batches and over time.

The laboratory's performance in the most recent proficiency testing rounds must be transparent on the test report and must be at least 'satisfactory' for correct identification in qualitative analysis. For a quantitative method the proficiency test z- score must be ?±2. The methods used should all be validated, with validation data published, including all performance criteria, the composition and preparation conditions of the samples, the RM used.

For the purposes of this project, the Action Level, where available, corresponds to those recently published in the FAO/WHO 'Risk Assessment of Food Allergens Part 2: Review and establish threshold levels in foods for the priority allergens' (WHO, 2022). The consultation also identified associated test method performance criteria for the global priority allergenic foods identified by the expert consultation. These action levels are based on health-based guidance values for global priority allergenic food ingredients that have been identified by the expert consultation and the food consumption data. Published data are available for other allergenic food ingredients on which health based guidance values may be identified using a similar approach (Houben et al 2019) which could allow an interim action level to be derived for other priority allergenic foods such as soybean.

In terms of incident management, in all cases where the food allergen is a protein (so excluding sulphites and sulphur dioxide), all testing should use only methods which target the allergenic proteins or their constituent peptides. PCR methods would ideally only be used as a surrogate testing method where an alternative protein-based method does not exist, as is the case for celery for example. At present, these methodologies are represented by ELISA and peptide LC-MS/MS. A combination of orthogonal methods would improve the robustness of the testing. While LC-MS/MS methods currently lack sensitivity, they benefit from enhanced specificity. As highlighted in this report, ELISA methods are sensitive and are specific but for a range of epitopes, as shown in Section 5. Given the current lower sensitivity of LC-MS compared to ELISA methods, we recommend that the initial analysis should be based on ELISA, specifically a kit which can detect allergens down to the Action Levels prescribed by FAO/WHO for the priority allergens, as detailed in Table Section 9-Table 1. In the absence of action levels for the other recognised UK food allergens, there is a need for indicative Health-Based Guidance Values (HBGVs) to be set. The specific method for use would consider the sensitivity, specificity, alignment of the LOD with the ED10 (while further work on reference dose derivation for ED05 continues as recommended in FAO/WHO 'Risk Assessment of Food Allergens Part 2: Review and establish threshold levels in foods for the priority allergens) and the performance of the kit on the particular matrix.
Some kits are preferred by users due to the rapidity of the testing, however methods with more involved extractions, for example, often perform better on processed products for example kits manufactured by Morinaga Institute of Biological Science Inc., so may be more suitable to protect consumers. In order to provide meaningful data to inform suppliers, producers and enforcement, the kit would allow reporting of the data in mg of allergenic protein per kg of food. When a suspect sample is under investigation following an allergen incident, it must be determined whether the sample contains the allergen or is negative for the allergen. In most cases, primary analyses by ELISA kits will be most suitable, using a range of kits targeting different allergenic proteins when available, with further investigative analysis by LC-MS if the ELISA tests provide a negative result. In the eventuality of a negative result for LC- MS/MS, this should be confirmed by an alternative ELISA kit, selecting a kit based upon a different antibody to that used for the initial analysis. This testing regime is designed to avoid yielding false negative data which can be the case when only single tests are applied (Walker et al 2017).

Due to the reduced cost of testing, ELISA and PCR testing is more appropriate in the first instance. In the case of egg, PCR is not suitable, as discussed previously and consideration must be given to the sensitivity of the testing method. PCR methods targeting milk for example often

lack sensitivity and ELISA should be performed in the first instances.

9.1. Recommended workflow for allergen incident management

When interrogating a suspect sample (for example a product for which testing has given a non-routine unexpected result, or is linked to an allergy incident, product recall or complaint), believed to have elicited an allergic response for an allergen, a representative sample of the product should be taken (100g-1kg) and homogenised into powder (or slurry, if a liquid) prior to analysis, taking validated laboratory precautions to avoid cross-contamination. At least two sub-samples should be taken for analysis, of at least 1g in mass each and extracted alongside suitable positive and negative RMs, ideally a CRM and analysed alongside a blank (ELISA well containing only the kit dilution buffer). A third aliquot of the sample should be spiked with allergen and tested, as described above.

In the first instance, an ELISA test should be conducted for the allergen(s) under suspicion. Where feasible, multiple ELISA tests should be worked through, ideally until an ELISA has been performed for each available target protein for that allergen, although this information is not always disclosed or is not known. The information is not always known when the polyclonal antibodies underpinning the method have been raised against the allergenic food as a whole, so the precise protein/epitope is not known. Testing must also encompass kits which support the appropriate level of processing (typically 1-3 different tests). Known cross-reactivities of the kits must be considered for the matrix in question. Table 15 of the FAO/WHO Risk Assessment of Food Allergens Part 2: Review and establish threshold levels in foods for the priority allergens (WHO, 2022) has been adapted (Section 9-Table 1) to inform regarding suitable available methodologies to pursue in the workflow for the allergens for which an Action Level is available. Should the suspect sample still test negative after ELISA analysis, an LC-MS method to target the allergen should be sought. Where there are gaps in capabilities to meet action levels, temporary action levels need to be set.
For the rare instances for which only PCR tests are available (e.g. celery), a PCR should be performed. Should the data be negative, then an alternative PCR, if available, targeting an alternative DNA sequence, should be instigated. For some of the tree nuts, the ELISA kits available are limited. For example, for Brazil nut, only one ELISA test is in common use, along with lateral flow tests and PCR, so these alternatives should be applied as secondary methods following a negative result from an ELISA. Ideally LC-MS methodology would then be sought and the sample analysed alongside RMs.

9.2. An example workflow for egg

Samples, as detailed above, should be tested alongside RMs. RMs available are NIST SRM 8445 (whole egg), ThRAll RMs (hen’s egg in broth and in chocolate), FSA RMs prepared under FSA-funded projects (FS101206,egg white in chocolate). QC materials comprising egg in a range of matrices such as cake mix are available from proficiency testing providers. A third sub-sample of the matrix should be over- spiked with allergen and tested to determine matrix effects and the recovery of the allergen in that matrix.

As discussed previously, ELISA testing must be used for determination of egg and not PCR. The first test kit should target an egg white protein. Should a food sample be found negative for that allergen, a second ELISA test should target a different egg white protein. Consideration should be given to performing ELISA using kits which are more suited to hydrolysed products, for example the Morinaga test kits. Finally, a confirmatory test by LC-MS is required to provide a robust

framework targeting the detection of allergen proteins and peptides. The LC-MS method developed during the EFSA ThRAll project (Detection and Quantification of Allergens in Foods and Minimum Eliciting Doses in Food-Allergic Individuals) has the sensitivity required to quantify egg at the action levels identified for these foods by the recent FAO/WHO expert consultation (FAO/WHO, 2022).

9.3. An example workflow for milk

For the determination of milk, ELISA methods must be used in preference to PCR methods. The first test kits should target casein and ?-lactoglobulin. Should a food sample be found negative for that allergen by these methods, an alternative test provider's kit should be used to determine whole milk. Finally, a confirmatory test by LC-MS is required to provide a robust framework targeting the detection of allergen proteins and peptides. The LC-MS method developed during the EFSA ThRAll project (Detection and Quantification of Allergens in Foods and Minimum Eliciting Doses in Food-Allergic Individuals) has the sensitivity required to quantify milk at the action levels identified for these foods by the recent FAO/WHO expert consultation (FAO/WHO, 2022). Applicable RMs available include ThRAll RMs (skimmed milk in broth and in chocolate), RMs prepared during FSA-funded projects (FS101206, chocolate paste containing skimmed milk). QC materials comprising egg in a range of matrices such as cake mix are available from proficiency testing providers.

9.4. An example workflow for peanut

Reference materials are available for peanut and should be used during testing, namely NIST SRM 2387 (qualitative) incurred peanut butter and ThRAll RM (quantitative for incurred chocolate). For the determination of peanut, ELISA tests must be used, first of all targeting the highest number of known target analytes such as a kit which is sensitive to Ara h1, Ara h2 and Ara h3. Should allergen not be detected, an alternative kit should be applied to screen for alternative target proteins such as kits which detect the Ara h2 and Ara h6 combination. Should a suspect sample continue to be found as negative, a PCR test could be applied before confirmatory testing by LC-MS. The LC-MS method developed during the EFSA ThRAll project has the sensitivity required to quantify the allergens from egg, milk, peanut, almond and hazelnut at the action levels identified for these foods by the recent FAO/WHO expert consultation (FAO/WHO, 2022), while further refinement to improve the sensitivity by approximately 3-fold would be required to enable the method to be fully deployed in line with the FAO/WHO expert consultation recommendations for test method performance.

9.5. An example workflow for mustard

In the case of mustard, less information is available regarding the target proteins of the ELISA kits, and a widely accepted confirmatory LC-MS method has not been developed. CRMs are not available but reference materials from proficiency testing companies are available, and should be used in the absence of a CRM. Also, little information is available regarding the identity of the target proteins of the available ELISA kits. It is therefore recommended in the scope of this review that one of the two ELISA tests available will be applied and, if mustard is not detected in a suspect sample, one of the two PCR kits is applied. Failing detection, the remaining ELISA and PCR kits could be applied. This will be the scenario until a confirmatory test is available, although no suitable LC-MS methods have been identified by this review that have undergone an inter-lab validation.

These workflows, combining available methods and preferring ELISA over PCR unless ELISA is not available, can be applied to all food allergens other than sulphur dioxide and sulphites and available methods are detailed in Table 1 (Appendix 1).

For detection of sulphur dioxide and sulphites, users should refer to official methods (for example AOAC Official Method 990.28, OIV-MA-AS323-04A). Suitable RMs are detailed in Section 9-Table 2.

Section 9-Table 1. Action levels and desired kit LOQ for allergens depending on matrix. Taken from Table 15 of FAO/WHO Risk Assessment of Food Allergens Part 2: Review and establish threshold levels in foods for the priority

Allergenic food ingredient	Matrix	P75 intake (Portion size for the 75th percentile of consumers)(g)	Proposed action level (mg protein/kg food)	Desired method LOQ (mg protein/kg food)	Are at least two ELISA methods available at this LOQ? (target)	L a c (
Milk	Cookies/ biscuits	50	40	13.3	Yes (casein, ?- LG)	P e g L C 2
Milk	Chocolate	40	50	16	Yes (casein, ?- LG)	V l r f t C 2
Egg	Cookies/biscuits	50	40	13.3	Yes (ovalbumin, ovomucoid, lysozyme)	E r t c P e c r r a L M 2

Allergenic food ingredient	Matrix	P75 intake (Portion size for the 75th percentile of consumers)(g)	Proposed action level (mg protein/kg food)	Desired method LOQ (mg protein/kg food)	Are at least two ELISA methods available at this LOQ? (target)	L a c L
Peanut	Chocolate	40	50	16	Yes (Ara h 1, Ara h 2, Ara h 3, Ara h 6)	P e c L S C 2
Almond	Chocolate, ice cream, pasta sauce	40	25	8.3	Yes (target undisclosed)	P a L P 2
Almond	Cookies	50	20	6.6	Yes (target undisclosed)	P a L P 2
Almond	Pasta sauce	80	10	3.3	Yes (target undisclosed)	P a L P 2
Almond	Ice cream	100	10	3.3	Yes (target undisclosed)	P a L P 2
Hazelnut	Bread roll	120	25	8.3	Yes (target undisclosed)	P e h b t v 2

Allergenic food ingredient	Matrix	P75 intake (Portion size for the 75th percentile of consumers)(g)	Proposed action level (mg protein/kg food)	Desired method LOQ (mg protein/kg food)	Are at least two ELISA methods available at this LOQ? (target)	L a c L
Hazelnut	Chocolate	40	75	25	Yes (target undisclosed)	B r a L
Hazelnut	Cookies	50	60	20	Yes (target undisclosed)	P a L P 2
Hazelnut	Tomato sauce	80	35	11	Yes (target undisclosed)	P a L P 2
Hazelnut	Ice cream	100	30	10	Yes (target undisclosed)	P a L P 2
Walnut	Chocolate	40	25	8.3	Yes (target undisclosed)	P e L a P 2
Walnut	Cookies	50	20	6.6	Yes (target undisclosed)	P e f r c r

Allergenic food ingredient	Matrix	P75 intake (Portion size for the 75th percentile of consumers)(g)	Proposed action level (mg protein/kg food)	Desired method LOQ (mg protein/kg food)	Are at least two ELISA methods available at this LOQ? (target)	L a c L
Walnut	Sauce	80	10	3.3	Yes (target undisclosed)	P e f r c r
Walnut	Ice cream	100	10	3.3	Yes (target undisclosed)	P e f r c r
Walnut	Bread roll	120	8	2.6	Yes (target undisclosed)	P e f r c r
Pecan	Chocolate	40	25	8.3	Yes (target undisclosed)	P e L P 2
Pecan	Cookies	50	20	6.6	Yes (target undisclosed)	P e L P 2
Pecan	Sauce	80	10	3.3	Yes (target undisclosed)	P e is P 2

Allergenic food ingredient	Matrix	P75 intake (Portion size for the 75th percentile of consumers)(g)	Proposed action level (mg protein/kg food)	Desired method LOQ (mg protein/kg food)	Are at least two ELISA methods available at this LOQ? (target)	L a c L
Pecan	Ice cream	100	10	3.3	Yes (target undisclosed)	P e is P 2
Cashew	Chocolate	40	25	8.3	May depend on level of processing (target undisclosed)	Y n t P 2
Cashew	Cookies	50	20	6.6	May depend on level of processing (target undisclosed)	Y n t P 2
Cashew	Sauce	80	10	3.3	May depend on level of processing (target undisclosed)	Y n t P 2
Cashew	Ice cream	100	10	3.3	May depend on level of processing (target undisclosed)	Y n t P 2
Cashew	Bread roll	120	8	2.6	May depend on level of processing (target undisclosed)	Y n t P 2

Allergenic food ingredient	Matrix	P75 intake (Portion size for the 75th percentile of consumers)(g)	Proposed action level (mg protein/kg food)	Desired method LOQ (mg protein/kg food)	Are at least two ELISA methods available at this LOQ? (target)	L a c L
Pistachio	Chocolate	40	25	8.3	Yes (target undisclosed)	Y r t P 2
Pistachio	Cookies	50	20	6.6	Yes (target undisclosed)	Y r t P 2
Pistachio	Sauce	80	10	3.3	Yes (target undisclosed)	Y r t P 2
Pistachio	Ice cream	100	10	3.3	Yes (target undisclosed)	Y r t P 2
Pistachio	Bread roll	120	8	2.6	Yes (target undisclosed)	M f c f

Allergenic food ingredient	Matrix	P75 intake (Portion size for the 75th percentile of consumers)(g)	Proposed action level (mg protein/kg food)	Desired method LOQ (mg protein/kg food)	Are at least two ELISA methods available at this LOQ? (target)	L a c L
Wheat	Cookies	50	100	33	Yes, (gliadin) for hydrolysed, fermented and unhydrolysed foods. Apply kits which differ in the antibody used (R5 and G12 antibodies).	D r r
Wheat determined as gluten	Infant semolina	200	25	8.3	Yes, (gliadin) for hydrolysed, fermented and unhydrolyzed foods	D r r
Fish	Wine	283	15	5	Only when the species is cod (parvalbumin)	D r r
Fish	Soy sauce	30	150	50	Only when the species is cod (parvalbumin)	D r r
Fish	Chicken meatball	126	35	11	Only when the species is cod (parvalbumin)	D r r
Fish	Pork meatball dumpling	126	35	11	Only when the species is cod (parvalbumin)	D r r

Allergenic food ingredient	Matrix	P75 intake (Portion size for the 75th percentile of consumers)(g)	Proposed action level (mg protein/kg food)	Desired method LOQ (mg protein/kg food)	Are at least two ELISA methods available at this LOQ? (target)	L a c L
Fish	Vegetable and chicken soup	400	10	3.3	Only when the species is cod (parvalbumin)	D r r
Fish	Tofu soup Mushroom soup	400	10	3.3	Only when the species is cod (parvalbumin)	D r r
Fish	Soy sauce	30	150	50	Yes (parvalbumin)	D r r
Fish	Almond coconut muesli	60	80	26	Only when the species is cod (parvalbumin)	D r r
Fish	Chicken corn soup	400	10	3.3	Only when the species is cod (parvalbumin)	D r r
Crustacean shellfish	Fish ball sausages	150	1000	333	Yes (tropomyosin)	P u c t r c t
Crustacean shellfish	Chicken meatball	130	1500	500	Yes (tropomyosin)	P u c t r c t

Allergenic food ingredient	Matrix	P75 intake (Portion size for the 75th percentile of consumers)(g)	Proposed action level (mg protein/kg food)	Desired method LOQ (mg protein/kg food)	Are at least two ELISA methods available at this LOQ? (target)	L a c L
Crustacean shellfish	Freeze- dried egg soup	400	500	166	Yes (tropomyosin)	P u c t r c t

Section 9-Table 2. Suitable RMs and QC materials.

Food allergen type	Reference/Descriptor	Matrix	Reference material status	Incurred status	Availability
Milk	MoniQA MQA092014	Negative and positive skimmed milk powders	CRM	Incurred	No longer available
Milk	NIST SRM whole milk 1549	-	CRM	Incurred	Not currently available
Milk	ThRAll RM	Chocolate	EFSA ThRAll RM	Incurred	Project ongoing
Milk	ThRAll RM	Broth	EFSA ThRAll RM	Incurred	Project ongoing
Milk	Product code LGC7421	Skimmed milk powder	RM, FSA-funded	Powder, not applicable	Available in kit reference LGC746-KT

Food allergentype	Reference/Descriptor	Matrix	Reference material status	Incurred status	Availability
Milk	Product code LGC7462	Chocolate paste containing milk egg white hazelnut powder walnut powder	RM, FSA-funded	Spiked	Available in kit reference LGC746-KT
Egg	NIST SRM 8445	Whole egg	CRM	Incurred	Available
Egg	NIST SRM 8415	Whole egg powder	CRM	Incurred	Not currently available
Egg	ThRAll RM	Chocolate	EFSA ThRAll RM	Incurred	Project ongoing
Egg	ThRAll RM	Broth	EFSA ThRAll RM	Incurred	Project ongoing
Egg	Product code LGC7422	Egg white powder	RM, FSA-funded	Powder, not applicable	Available in kit reference LGC746-KT
Egg	Product code LGC7462	Chocolate paste containing milk egg white hazelnut powder walnut powder	RM, FSA-funded	Spiked	Available in kit reference LGC746-KT
Peanut	NIST SRM 2387 (qualitative)	Peanut butter	CRM	Incurred	Available

Food allergentype	Reference/Descriptor	Matrix	Reference material status	Incurred status	Availability
Peanut	ThRAll RM	Chocolate	EFSA ThRAll RM	Incurred	Project ongoing
Peanut	ThRAll RM	Broth	EFSA ThRAll RM	Incurred	Project ongoing
Soya	ThRAll RM	Chocolate	EFSA ThRAll RM	Incurred	Project ongoing
Soya	ThRAll RM	Broth	EFSA ThRAll RM	Incurred	Project ongoing
Hazelnut	ThRAll RM	Chocolate	EFSA ThRAll RM	Incurred	Project ongoing
Hazelnut	ThRAll RM	Broth	EFSA ThRAll RM	Incurred	Project ongoing
Hazelnut	Product code LGC7425	Hazelnut powder, partially defatted	RM, FSA-funded	Powder, not applicable	Available in kit reference LGC746-KT
Hazelnut	Product code LGC7462	Chocolate paste containing milk powder, egg white powder, hazelnut powder (partially defatted), walnut powder (partially defatted)	RM, FSA-funded	Spiked	Available in kit reference LGC746-KT

Food allergentype	Reference/Descriptor	Matrix	Reference material status	Incurred status	Availability
Almond	ThRAll RM	Chocolate	EFSA ThRAll RM		Project ongoing
Almond	ThRAll RM	Broth	EFSA ThRAll RM		Project ongoing
Almond	Product code LGC7424	Almond powder	RM, FSA-funded		Available in kit reference LGC746-KT
Almond	Product code LGC7462	Chocolate paste containing milk powder, egg white powder, hazelnut powder (partially defatted), walnut powder (partially defatted)	RM		Available in kit reference LGC746-KT
Walnut	Product code LGC7426	Walnut powder, partially defatted	RM, FSA-funded	Powder, not applicable	Available in kit reference LGC746-KT

Food allergentype	Reference/Descriptor	Matrix	Reference material status	Incurred status	Availability
Walnut	Product code LGC7426	Chocolate paste containing milk powder, egg white powder, hazelnut powder (partially defatted), walnut powder (partially defatted)	RM	Spiked	Available in kit reference LGC746-KT
No allergenic ingredients	LGC7461	Chocolate paste	RM, FSA-funded	Not applicable	Available in kit reference LGC746-KT
Celery	QC materials available from proficiency testing providers	e.g. soup powder	QC Material	Various, spiked or incurred	Available
Fish	QC materials available from proficiency testing providers	e.g. cod muscle, fish in sauce	QC Material	Various, spiked or incurred	Available
Cereals containing Gluten	QC materials available from proficiency testing providers	e.g. soya formula, cake mix, oat-based food, cumin powder	QC Material	Various, spiked or incurred	Available
Lupin	QC materials available from proficiency testing providers	e.g. wheat flour	QC Material	Various, spiked or incurred	Available

Food allergentype	Reference/Descriptor	Matrix	Reference material status	Incurred status	Availability
Molluscs	QC materials available from proficiency testing providers	e.g. soup powder, sauce	QC Material	Various, spiked or incurred	Available
Mustard	QC materials available from proficiency testing providers	e.g. soup powder	QC Material	Various, spiked or incurred	Available
Sesame	QC materials available from proficiency testing providers	e.g. cumin powder	QC Material	Various, spiked or incurred	Available
Crustaceans	QC materials available from proficiency testing providers	e.g. sauce	QC Material	Various, spiked or incurred	Available
Other tree nuts	QC materials available from proficiency testing providers	e.g. chocolate	QC Material	Various, spiked or incurred	Available
Sulphites	QC1541	Water	CRM	Incurred	Available
Sulphites	Calibrants from titration instrument manufacturers	For use with wine	Unknown	Not known	Available