

Development of reference materials: Achievement of deliverables and milestones

7.1 D1.1 Publication - allergen targets including verified allergen sequences databases

A systematic review of analytical targets used in ELISA, PCR and MS methods was carried out ensuring synergies, in particular, with iFAAM and ThrALL. A joint project meeting was held on the 22 March 2018 between ThrALL and FS 101206 project partnerships. Having well defined allergen sequence sets is crucial to support effective allergenic food biomarker identification and characterisation. The curated allergen sequences were analysed to understand how the protein and peptide markers identified in part 1 relate and whether they cover major isoforms and how factors such as post translational modifications and processing-induced modifications, such as the Maillard reaction, may impact on the generation and detection of allergen peptide biomarkers by mass spectrometry. The results of the study were published in 2020 covering four of the five allergens represented in the RM kit. Financial support from the UK Food Standard Agency (FSA), Contract FS101206 Development of Quality Control Materials for Food Allergen Analysis was acknowledged. The authors, title and abstract are given in Appendix 2, paper D1.1.

7.2 D1.2 Publication on characterization of exemplar tree nut ingredients

Although the RM kit is fully characterised from the point of view of SI traceability, homogeneity, stability and assigned values it is also important to have regard to the allergen proteins represented by the RM. Thus, of the five allergens in the RM kit, three, hazelnut, walnut and almond ingredients were studied. The results of the study are due to be published. Financial support from the UK Food Standard Agency (FSA), Contract FS101206 Development of Quality Control Materials for Food Allergen Analysis is acknowledged. The authors, title and abstract are given in Appendix 2, Paper D1.2

7.3 M1.1 Kick-off meeting and stakeholder workshop

7.3.1 Kick-off meeting

A kick-off meeting was held at LGC Teddington on 23 February 2018 with Gill Holcombe, Thierry le Goff, Gavin Nixon, Malvinder Singh, Michael Walker, Will Webster (LGC); Jesus Alvarez-Pinera† (FSA); Anuradha Balasundaram*, Victoria Lee*, Clare Mills* (UoM); Adrian Rogers* (Romer),

† via telephone; * via Skype.

The Agenda was as follows:

- Introduction to LGC – Gill Holcombe (LGC)
- the need for allergen reference materials – Michael Walker (LGC)
- Project Overview – Michael Walker

- making allergen reference materials – Gill Holcombe
- UoM deliverables – Clare Mills, Anuradha Balasundaram, Victoria Lee (UoM)
- LGC deliverables – Gill Holcombe

The stakeholder meeting and consultation were discussed, followed by sourcing of ingredients for the RM and homogeneity and stability studies. FSA outlined their reporting requirements and project management was discussed. Minutes of the meeting were circulated to all attendees.

7.3.2 Stakeholder workshop

An all-day stakeholder workshop was held on 23 March 2018 at the University of Manchester Institute of Biotechnology in the form of a Food Allergy Network (MFAN) Stakeholder Meeting on the topic 'Reference materials and reference methods for food allergen analysis'. The meeting was held in conjunction with the EFSA funded ThRAII project (GP/EFSA/AFSC, 2017/03) with a substantial portion of the discussions devoted to project FS101206).

Minutes of the meeting were forwarded to FSA. The discussion sessions were used to gather stakeholder views on allergen RMs which included:

- synergy rather than duplication with ThrALL and MoniQA
- the utility of RMs except when ELISA platforms measure entirely different epitopes
- priority allergens were milk, gluten, egg, soy, peanuts, nuts and fish
- the form used in the RM must be representative of an industrial-scale allergen food and would also want matrix-matched calibrator
- there was a discussion regarding the use of whole egg or egg white powder
- the matrix should be simple or of medium analytical difficulty, problematic matrices included takeaway food, baked goods, other processed foods in complex recipes, cooked and raw materials and spices
- incurred concentrations should be between the LoQs of test kits, and levels of clinical effects
- conversion factors to mg kg⁻¹ of the relevant allergen protein are important
- any usable RM should be stable for around 3 to 5 years

The agenda is shown below, in two parts, morning and afternoon sessions.

| Morning | Event | Speaker |
|--------------------|--|---|
| 9 to 9:30am | Registration and coffee | - |
| 9:30am to 9:35am | Welcome | Clare Mills, The University of Manchester UK |
| 9:35am to 9:55am | Detection and quantification of allergens in foods and minimum eliciting doses in food allergic individuals (EFSA-project ThRAII) | Clare Mills, The University of Manchester UK |
| 9:55am to 10:15am | Towards reference materials for food allergen analysis (FSA project FS101206) | Michael Walker, LGC, UK |
| 10:15am to 10:35am | Reference materials - the MoniQA experience | Roland Poms, MoniQA, AT |
| 10:35am to 11am | Allergenic ingredient and incurred food matrices | Christof van Poucke, ILVO, BE |
| 11am to 11:30am | Coffee break | - |
| 11:30 am to 1pm | What makes a usable reference material? Short presentations from Gill Holcombe (LGC) Structured discussion on reference materials What is needed? In what format? How should they be used? | Facilitator Michael Walker (LGC) and representatives of Contracting analysis Laboratory Research Laboratory Public Analyst Allergen testing commissioning organisation |

- need for advice on how to use an allergenic ingredient for use in preparation of in-house QC materials
- unit size was discussed; for "raw" ingredient about 1 g would be an acceptable if spiking into a matrix. For the incurred matrix a larger number of smaller units is preferable, or

multiple units per kit would be beneficial.

7.3.3 Further stakeholder consultation

Following the stakeholder meeting a further emailed consultation exercise was carried out on the format and content of the RM. Emails were sent to 41 stakeholders (Figure 2) outlining the background to project FS101206, information on the proposed allergen RM kit, relevant reference doses and the statement that “We intend to incur each allergen at 10 mg kg⁻¹ as allergen protein. Do you agree? Or can you suggest an alternative concentration?”

The afternoon agenda session agenda was as follows:

| Afternoon | Event | Speaker |
|------------------|--|---|
| 1pm to 2pm | Lunch | - |
| 2pm to 2:20pm | Can mass spectrometry provide the reference method for allergen analysis | Gavin O'Connor, JRC, BE |
| 2:20pm to 2:40pm | Standard methods for allergen analysis - AOAC perspectives | Linda Monaci, ISPA, IT |
| 2:40pm to 3pm | The perspective of a routine analytical laboratory | Nathalie Gillard, CER, BE |
| 3pm to 3:30pm | Coffee Break | - |
| 3:30pm to 4pm | ELISA and PCR analysis - how to integrate with MS methods | Olivier Tranquet, INRA, FR Marc de Loose, ILVO, BE |
| 4pm to 4:30pm | Integrating ELISA and MS analysis of peanut | Vicki Lee, The University of Manchester, UK |
| 4:30pm to 5pm | Topics for future meetings | All |
| 5pm | Meeting close | - |

There were 13 responses (to 22.11.2018) which can be summarized (Figure 3) as follows:

- most (9/13 = 69%) broadly agreed 10 mg kg⁻¹ as allergen protein (10 mg kg⁻¹ AP)
- some noted 10 mg kg⁻¹AP was high but commented RM could be diluted
- some preferred 5 mg kg⁻¹ AP, or even lower for egg

It was pointed out that with ELISA recoveries of typically 30% – 50%, 10 mg kg⁻¹ AP incurred would probably result in 3 – 5 mg kg⁻¹ AP on typical ELISA calibration curves and it was agreed that would be suitable for analysis by most ELISA kits

Figure 2: Stakeholder groups consulted on the RM format and content

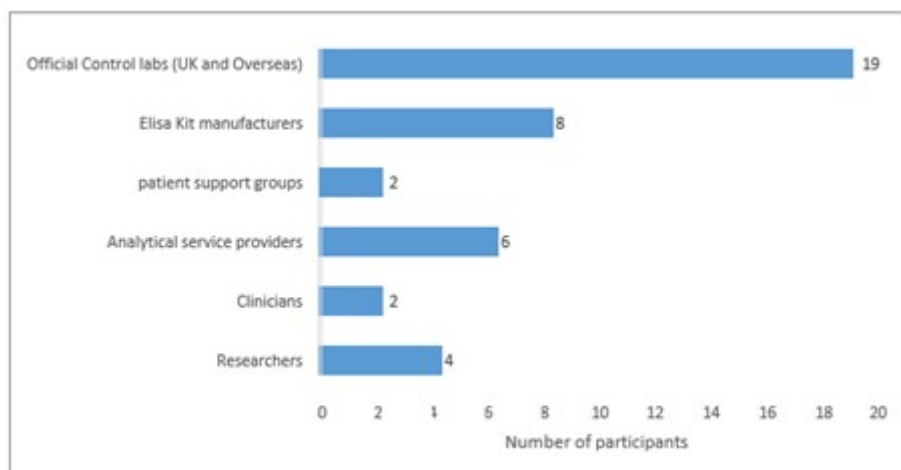
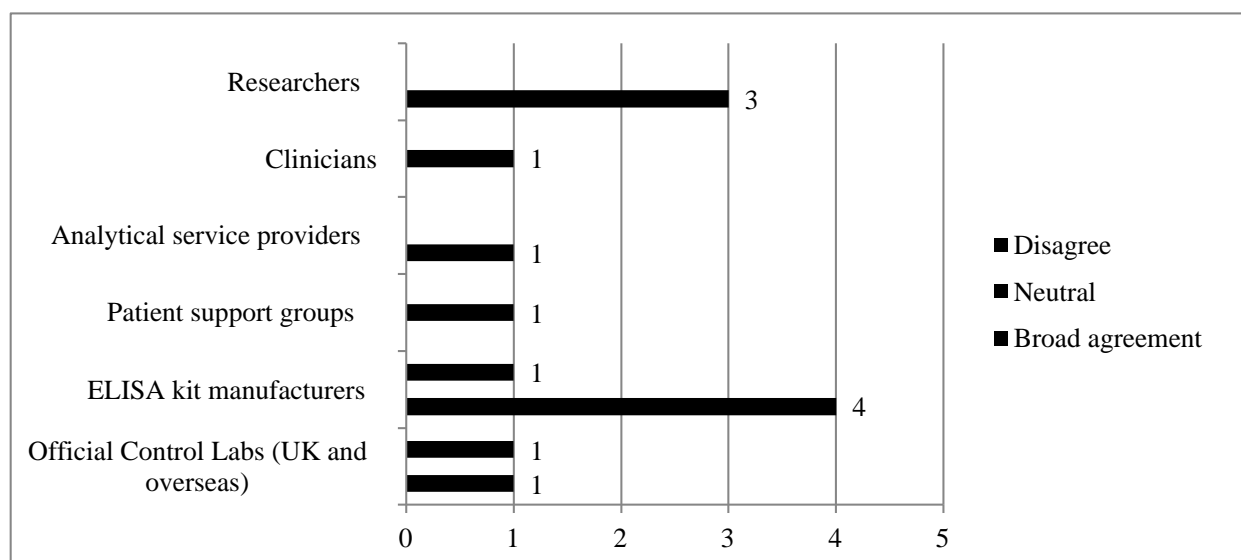


Figure 3: Stakeholder responses summary RM format and content



Based on the stakeholder feedback and initially aiming at European and North American populations it was decided that a RM kit should be prepared aimed to be of medium analytical difficulty and complementary to other RMs available (for example, from MoniQA). The RM kit should contain the following:

A food matrix based on the EuroPrevall chocolate dessert mix paste gravimetrically incurred with 5 allergens at clinically and industrially relevant concentrations, (10 mg kg⁻¹ expressed as protein, for example, each of five allergenic foods was added such that the incurred material contains 10 mg kg⁻¹ expressed as protein of each)

The food matrix devoid of the target allergens, and the allergen raw materials which should be

- hens' egg white powder,
- skimmed cows' milk powder,
- almond powder
- hazelnut powder, and
- walnut powder.

7.4 M1.2 Ingredients sourced

The ingredients for the RM kit were sourced as described in a published paper (see 8.20). Data collected have been archived for retention in accordance with LGC document control policies.

7.5 M1.3 Water and total protein determination

The water and total protein contents of the allergenic ingredients for the RM kit were determined as described in a published paper (see 7.17). The datasets from the determinations have been archived for retention in accordance with LGC document control policies.

7.6 M2.1 Food matrix produced

The food matrix for the RM kit was determined as described in a published paper (see 7.17). The written procedures, witnessed key steps and data collected have been archived for retention in accordance with LGC document control policies.

7.7 M2.2 Incurred material produced

The incurred material for the RM kit was determined as described in a published paper (see 8.20). The written procedures, witnessed key steps and data collected have been archived for retention in accordance with LGC document control policies.

7.8 M1.4 2D-page characterization completed.

See 8.2 D1.2, publication, *Adaba et al.*, 20XX, Characterisation of hazelnut, walnut and almond ingredient for use as reference materials for allergen analysis.

7.9 M3.1 Homogeneity assessment for incurred material

The homogeneity of the elements of the RM kit was determined as described in published paper (see 7.17). The datasets from the determinations have been archived for retention in accordance with LGC document control policies.

7.10 M1.5 Immunoblotting characterization

See 7.2 D1.2, publication, *Adaba et al.*, 20xx, Characterisation of hazelnut, walnut and almond ingredient for use as reference materials for allergen analysis.

7.11 M1.6 Mass spectrometry, MS, profiling

See 7.2 D1.2, publication, *Adaba et al.*, 20xx, Characterisation of hazelnut, walnut and almond ingredient for use as reference materials for allergen analysis.

7.12 M3.2 Stability assessment for incurred material

The stability of the elements of the RM kit was determined as described in published paper (see 7.17). The datasets from the determinations have been archived for retention in accordance with LGC document control policies.

7.13 M3.3 Material characterisation completed

The stability of the elements of the RM kit was determined as described in published paper (see 7.17) and *Adaba et al.*, 20xx, Characterisation of hazelnut, walnut and almond ingredient for use as reference materials for allergen analysis, read 7.2.

7.14 M3.4 Certification meeting

A certification meeting was held at LGC on 30 July 2019.

Present: Stephen Ellison (PS/Reference materials, Chair), Gill Holcombe (Hd/RM production, RM project leader), Natasha Heath (Quality manager), Kirstin Gray (Food analyst), Malvinder Singh (Food analyst), Simon Cowen (Statistics), Laura Carrick-White (RM Production team), Nagina Junaid (RM Production team), Michael Walker (Project management committee chair; Hd/Office of the Government Chemist).

Present for the first half hour: Cailean Clarkson (Team leader, purity analysis team), Michael Tong (analyst, purity analysis team).

Information was presented by Gill Holcombe on the

- preparation of the material,
- homogeneity and stability data and their statistical evaluation
- assigned values, uncertainty and metrological traceability
- intended use and instructions for use.

All aspects of the RM were critically reviewed and a series of actions agreed.

7.15 D3.1 Release of Reference Material

On satisfactory completion of the actions agreed at the certification meeting the RM was confirmed as suitable for release for sale and statements of measurement were drawn up and agreed. The RM kit and associated RMs (raw materials) were made available in February 2020 with [a press release](#), pre-agreed with FSA on 10 February 2020. The descriptions of the RM are as follows and the statements of measurement are available open access on the LGC website (links below).

7.15.1 Allergen kit – Milk, Egg, Almond, Hazelnut and Walnut, Reference Material LGC746-KT

Kit contents, each kit contains:

One vial of each of the following raw materials, each containing ~1 g under Argon, each characterised for total nitrogen (Dumas) and moisture (Karl-Fisher):

- LGC7421 Skimmed milk powder
- GC7422 Egg white powder
- LGC7424 Almond powder
- LGC7425 Hazelnut powder – partially defatted
- LGC7426 Walnut powder – partially defatted

Five bottles, each containing ~5 g, of:

- LGC7461 Chocolate paste – no added allergenic ingredients
- LGC7462 Chocolate paste with each of the above added allergenic ingredients gravimetrically incurred by serial dilution of a high-level paste at 10 mg kg⁻¹ (expressed as protein calculated on total nitrogen in the original raw material).

The [Statement of Measurement](#) contains:

- for LGC7421, LGC7422, LGC7424, LGC7425 and LGC7426 a table of assessed values for total nitrogen (Dumas) and moisture (Karl-Fisher) with associated uncertainty, the half-width of the expanded uncertainty interval calculated using a coverage factor (k) of 2 which gives a level of confidence of approximately 95 %.
- for LGC7421, LGC7422, LGC7424, LGC7425 and LGC7426 a table of calculated values for protein content with associated uncertainty and the relevant nitrogen factor. The calculated values table has been included for information. Whilst it is known that the nitrogen factor will vary according to the source of the ingredient, no allowance associated with the factor uncertainty (which is known to be non-zero) has been included in the calculation of the protein content and its uncertainty, nor has this source of uncertainty been included in the uncertainty for the assessed and indicative values for allergen

proteins. Users may wish to include their own estimates of this source of uncertainty.

- for LGC7461 a table of assessed values for milk protein (<0.05 mg kg⁻¹), egg white protein (<0.05 mg kg⁻¹) and hazelnut protein for LGC7462 a table of assessed values for milk protein, egg white protein, and indicative values for hazelnut protein, almond protein and walnut protein based on the gravimetric preparation data with their associated uncertainty, the half-width of the expanded uncertainty interval calculated using a coverage factor (k) of 2 which gives a level of confidence of approximately 95 %, except for the hazelnut protein data where a tolerance interval calculated from log-transformed data is reported owing to an observed larger between-unit standard deviation.
- a table of results for LGC7462 obtained by analysis using two different commercial ELISA kits. In most cases the mean result measured is lower than the prepared concentration, which is not unexpected. For example, for ELISAs calibrated using 'whole' hazelnuts (not defatted), low recovery from partially defatted nut powders is well known, and may be related to the impact of lipid removal on proteins. The table shows the study design and range of individual results obtained; however these data should be used with caution, as the range is likely to be less where a small number of units are examined, and a user may obtain results outside this range in their laboratory.
- a table showing material sourcing and preparation.
- a table of conversion factors from total nitrogen to casein, hazelnut, almond and walnut.
- statements of homogeneity and stability assessments with brief comments on the study designs and outcomes.
- brief descriptions of analytical methods (nitrogen, water, DNA sequencing), and proteomics.
- a description of the intended use and instructions for use.
- a statement of the metrological traceability.

7.15.2 Allergen ingredient reference materials -

Statements of measurement are also freely available for:

- Skimmed milk powder LGC7421
- Egg white powder LGC7422
- Almond powder LGC7424
- Hazelnut powder – partially defatted LGC7425
- Walnut powder – partially defatted LGC7426

Each gives details of the nitrogen and water content of the materials with associated uncertainty and methods of analysis, the material sourcing and preparation, the homogeneity and stability assessments, the intended use, metrological traceability, accreditation, instructions for use, storage conditions and shelf life. DNA sequencing information is given for the tree nuts.

7.15.3 ISO 17034 accreditation

ISO 17034:2016, 'General requirements for the competence of reference material producers', covers the production of all reference materials, including certified reference materials. It is intended as part of the general quality assurance procedures of the reference material producer. This International Standard supersedes ISO Guide 34:2009 and is aligned with the relevant requirements of ISO/IEC 17025. Reference material producers that comply with this International Standard will also operate generally in accordance with the principles of ISO 9001:2015 'Quality management systems, Requirements'. Accreditation to ISO 17034 may only be awarded after the RM is produced, so that its production, evaluation and certification may be audited. Following such an audit in 2020, the allergen RMs were confirmed within the scope of LGC's ISO 17034 accreditation.

7.16 D4.1 Promotion of Reference Material kit

Press releases for the release of the RM kit and award of ISO 17034 were issued on 10th February 2020 and 16th October 2020 respectively. Each was advised to FSA prior to issue. The LGC website is being used to promote the RM kit which has been flagged on social media. A story on the RM was carried by 'Food Manufacture' on 10 February 2020, and on the University of Manchester and Romer Labs websites.

The release of the RM kit was publicised at the following conferences:

- FOOD ALLERGY - Human, Analytical & Regulatory Implications hosted by Institute for Global Food Security, Queen's University Belfast, Monday 21st October 2019, Riddel Hall, Belfast, Michael Walker
- NIST Food Safety Workshop, Gaithersburg, Maryland, USA, October 28 – 31 2019, Gill Holcombe and Michael Walker.
- MoniQA, Food Fraud Prevention and Effective Food Allergen Management, Rockville, Maryland, USA, 30 October – 1 November 2019, Gill Holcombe and Michael Walker.
- FAAM virtual conference on 16 and 17 October 2020. This is a major clinical and scientific food allergy biennial conference in the European Academy of Allergy & Clinical Immunology (EAACI) events calendar. It was due to be held in Manchester with Professor Clare Mills as chair of the multinational organising committee however COVID-19 forced a virtual conference which attracted over 800 delegates. Michael Walker was an invited speaker, giving a talk entitled "The pitfalls of food testing and electronic allergen information provision" which included reference to the RM kit. This talk was also selected to be included in an EAACI medical training module.

7.17 D4.2 Publication, production of Reference Materials and FSA final report

A paper on the production and characterisation of the RM kit has been prepared for publication. Financial support from the UK Food Standard Agency (FSA), Contract FS101206 Development of Quality Control Materials for Food Allergen Analysis is acknowledged. Authors, title and abstract are given in Annex 2. paper D4.2.

This report constitutes the Final report to FSA.

An internal 229-page LGC report [1] contains the complete dataset on the allergen reference materials and is available on request for RM users and at the discretion of the Head of RM Production.