Review of allergen analytical testing methodologies: Alignment of the project with ThRAll project outcomes

As the co-leader of the EFSA ThRAll project (detection and quantification of allergens in food and minimum eliciting doses in food allergic individuals), Clare's collaboration will align the literature review with the outcomes of the ThRAll project. Clare currently holds a joint appointment between the Universities of Manchester and Surrey. Her laboratory is based at present in the Manchester Institute of Biotechnology at the University of Manchester and is part of the Respiratory and Allergy Research team at the Wythenshawe Hospital and the Immunology Section at the University of Surrey. She led the EU integrated projects iFAAM (integrated approaches to Food Allergen and Allergy Management) and EuroPrevall (the prevalence, cost and basis of food allergy across Europe) and coordinated the European Food Safety Authority project ThRAll and currently leads the UK Food Standards Agency project PAFA (Patterns and Prevalence of Adult Food Allergy).

Clare is also a partner in a recently awarded project from EFSA led by EuroFIR on allergenicity prediction. Professor Mills is a member of the FSA Advisory Committee on Novel Foods and Processes and was involved in the recent FAO/WHO Expert Consultation on Food Allergens. Her personal research interests are focused on structure-function relationships in food proteins particularly with regards what makes some proteins, and not others, become allergens, including the effects of the food matrix and processing on resistance of food proteins to digestion and the role this plays in determining the allergenicity of foods.

5.1. Report on EFSA project GP/EFSA/AFSCO/2017/03. "Detection and Quantification of Allergens in Foods and Minimum Eliciting Doses in Food- Allergic Individuals" (ThRAll).

Project partnership: This contract/grant was awarded by EFSA to Professor Clare Mills, School of Biological Sciences, Division of Infection, Immunity and Respiratory Medicine, Manchester Academic Health Science Centre, Manchester Institute of Biotechnology, The University of Manchester (UNIMAN) UK.

Following the University of Manchester not renewing its article 36 membership the contract was transferred to Partner 1 (Dr Linda Monaci, Institute of Sciences of Food Production, National Research Council of Italy (CNR-ISPA), via Giovanni Amendola 122/O - 70123 Bari, Italy). Contractor/Beneficiary: The University of Manchester (until 18th December 2019); CNR-ISP (19th December 2019-30th September 2022).

Other Partner Organisations were as follows:
Partner 2: Flanders Research Institute for Agriculture, Fisheries and Food (ILVO), Brusselsesteenweg 370, 9090 Melle, Belgium.
Partner 3: CER Groupe, Rue du point du Jour, 8, 6900 Marloie, Belgium. Partner 4: INRAE UMR 1163 Biodiversité et Biotechnologie Fongiques (BBF), F- 13288 Marseille, France, INRAE UR1238 BIA, Rue de la Géraudière, BP 71327, 44313 Nantes, France and INRAE-CEA, Service de Pharmacologie et d'Immunoanalyse, Laboratoire d'Immuno-Allergie Alimentaire, Bât. 133-CEA de Saclay, 91191 - Gif-sur-Yvette, France.

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5.2. Summary of Results

5.2.1. ThRAll Objective 1: Develop reference (harmonised) methodologies for the detection and quantification of allergens in foods.

The focus of Objective 1 of ThRAll was the development of a prototype multi-analyte mass spectrometry-based reference method for determination of allergenic food ingredients (Mills et al., 2019). A systematic review of the literature on food allergen analysis using mass spectrometry (MS) was performed and peptide markers for the six allergenic food ingredients collated. The peptides were evaluated and filtered based on their length, their type, food matrix and level of processing investigated, and whether they were identified using discovery or targeted MS analysis. Peptides containing amino acid residues prone to modifications, such as methionine or asparagine-glycine motifs, were excluded. Peptide specificity and potential sequence similarity with homologous proteins from related species was also assessed. Based on this analysis a preliminary list of candidate marker peptides was developed (Pilolli et al., 2020).

To support further evaluation of the candidate peptides, test method comparisons and validation two difficult-to-analyse food matrices were prepared for the project based on a chocolate bar and a powdered broth. These were incurred with six different allergenic food ingredients namely cow's milk, hen's egg, peanut, soya, hazelnut and almond. These included RMs developed through the UK FSA call FS101206 Development of Quality Control Materials for Food Allergen Analysis) and RMs from NIST and MoniQA. The materials were assessed for homogeneity and the IgE-binding capacity of the allergenic ingredients assessed using in vitro test methods using serum samples from relevant food allergic subjects (Huet et al., 2022).

Analysis of these materials provided data which were then used to further filter peptide markers to give a preliminary list of around fifty candidate marker peptides

(Pilolli et al., 2021). These were then systematically evaluated with optimization using multiple reaction monitoring (MRM) experiments executed on triple quadrupole instruments. Since the broth powder proved to be very highly processed with many allergens poorly detected by either immunoassay or MS-based methods, the method optimisation was undertaken using allergens incurred into the chocolate bar matrix. The method optimisation assessed methodological parameters including extraction and purification of allergenic ingredient proteins and optimisation of digestion protocols (Henrottin et al., 2023). This analysis identified the key methodological parameters and allowed a subset of the peptide markers to be identified which were synthesised in stable isotopically labelled forms for use as external calibrants in further method validation.

The MS results will be compared with analytical results obtained on the same incurred materials using ELISA but an assessment of droplet digital PCR based methods (ddPCR) showed they were not suitable for use with such complex incurred matrices. Thus, a comparison of test method data is only possible with ELISA. Working with the community, an approach to develop harmonised conversion factors has been developed. These were then applied to analysis of an inter-laboratory assessment of the prototype test method. This demonstrated the transferability of the method, despite its complexity, across laboratories experienced in allergen analysis. The method 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). Further refinement to improve the sensitivity by approximately 3-fold will be required to enable the method to be fully deployed in line with the FAO/WHO expert consultation recommendations for test method performance. Further refinement to bring the detection of peanut and whey in line with that of egg, soyabean, hazelnut and almond is also required, perhaps developing an optimised extraction buffer. Furthermore, given that there is indication that the boiling step during the preparation of the broth powder may have impacted on the diminished detection of peanut in this matrix, work to understand the impact of extensive processing on the clinical reactivity of food among sensitive consumers would be beneficial, to determine if, for example, the highly processed peanut in this matrix would still elicit an allergic reaction.

5.2.2. ThRAll Objective 2: Generate good quality data on Minimum Eliciting Doses (MED) and Minimum Observed Eliciting Doses (MOED).

Through systematic mapping of clinical record forms, a harmonised approach for coding of food allergy data was developed which will support collation of data on minimum eliciting doses from low-dose oral food challenges undertaken in food allergic patients. This was used as the basis for developing an electronic record using the REDCap secure web application for managing online databases and surveys to which data were either uploaded directly or entered from the literature. Data gaps identified included the lack of challenge data for foods such as Brazil nut, macadamia nut, molluscan shellfish and lupin. For many other foods, fewer than 60 patient records could be identified for inclusion, as is required for best practice modelling (Klein Entink et al., 2014). Many of the foods for which data were lacking represent less prevalent food allergies which makes it more difficult for clinical studies to identify many patients to include in any threshold study.

Options for modelling dose distributions were explored using fish as a case study. Data from two published studies were harmonised, the dose distributions modelled using interval censoring survival analysis and the MEDs calculated. This analysis demonstrated the benefits of combining studies in providing dose estimates with narrower confidence intervals. The combined data set provides ED 05 values close to those published by EuroPrevall and a little lower than those published in the recent FAO/WHO expert consultation. It was not possible to apply novel model averaging approaches since the code available was designed for use with only one particular database. This approach provides a framework for the future curation of oral food challenge data.

5.3. Conclusions: Aligning the ThRAll outcomes with this review

The new LC-MS method developed in this project benefits from detection of allergens at the action levels identified for these foods by the recent FAO/WHO expert consultation (FAO/WHO, 2022). This method, along with the incurred RMs

developed, would therefore strongly support current methods (mainly ELISA), acting as a

confirmatory method during incident management, to interrogate foods for hen’s egg, milk, peanut, almond and hazelnut.