External Scientific Report

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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)

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Abstract

Mandatory labelling of allergenic food ingredients has helped allergic consumer manage their condition, but unintended allergens and precautionary allergen labels (PAL) continue to cause confusion for allergic consumers and the food industry alike. Identifying doses of food protein that are safe for the majority of allergic consumers and test methods for their analysis are essential for evidence-based application of allergen labelling. The ThRAll project addressed this by developing a multiplex prototype mass spectrometry (MS)-based reference method capable of analyzing six allergenic foods (cow's milk, hen's egg, peanut, hazelnut, almond and soybean) with complementary assessment of immunoassay and DNA-based methods. The MS method was transferable between laboratories and has the sensitivity required to quantify the allergens from egg, milk, almond and hazelnut in chocolate, meeting test method performance requirements identified for these allergenic foods by the recent FAO-WHO expert consultation. Further refinement is needed to improved sensitivity for peanut and soy. In parallel an approach for harmonizing and integrating oral food challenge data in allergic subjects was developed and data collection piloted using an on-line database. Data gaps were identified for many allergenic foods and there is an urgent need to confirm the allergenic activity of highly processed food matrices.

Key words: Food allergen, chocolate, IgE; mass spectrometry, immunoassay, PCR, minimum eliciting dose.

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Summary

Since there is currently no cure for food allergy, individuals diagnosed with the condition must practice avoidance of their problem food and those at risk of severe reactions given rescue medication such as self-injectable adrenaline, in case of accidental consumption. The difficulties of managing food avoidance and the severe, and in some case fatal, nature of IgE-mediated reactions mean the food allergies are a matter of public health concern.

The ThRAll project partnership led by the University of Manchester, UK and Institute of Sciences of Food Production, National Research Council of Italy (CNR-ISPA) in Italy worked with three other partner organisations (Flanders Research Institute for Agriculture, Fisheries and Food and CER, Belgium together with INRAe, France) to (1) develop a prototype reference (harmonised) method for the simultaneous quantification of six allergenic food ingredients (cow's milk, hen's egg, peanut, soybean, hazelnut and almond); and (2) develop an approach for integration and hamonisation of clinical data from food allergic subjects undergoing oral food challenge and pilot an on-line data base for its collection and curation.

The activities have built on the outputs of previous EU and nationally funded projects (EuroPrevall, iFAAM, MANOE, Safe&Smart, Allersens, MoniQA and the UK FSA projectl FS101206 Development of Quality Control Materials for Food Allergen Analysis). Two incurred food matrices were prepared, one based on a chocolate bar and second on a powdered soup. These were used to develop a harmonised quantitative multiplex mass spectrometry based prototype reference method for the detection of six foods allergens. Analysis of these materials, coupled with a systematic review of the literature allowed a suite of peptide markers to be identified for the six allergenic foods that met quality criteria for use in a prototype reference method. An assessment of ddPCR-based methods showed they were not suitable for use with such complex incurred matrices whilst analysis using commercial ELISA test kits showed the broth powder to be very highly processed with many allergens poorly detected. Consequently, further MS based methods development and validation was undertaken using allergens incurred into the chocolate bar matrix. Through a process of test method optimisation using multiple reaction monitoring experiments executed on a triple quadrupole MS platform a subset of candidate peptide markers was identified for method validation. Using stable isotope-labelled forms of the peptides an inter-laboratory assessment of the prototype test method was undertaken. This demonstrated the transferability of the method, and showed it was capable of providing accurate quantification of the six allergenic food ingredients the sensitivity required to quantify the allergens from egg, milk, almond and hazelnut and can perform in line with the test method performance requirements identified for these allergenic foods by the recent FAO-WHO expert consultation. Further refinement to improve the sensitivity by ~3-fold will be required to enable the method to be fully deployed for analysis of whey proteins and peanut in line with the FAO-WHO expert consultation recommendations for test method performance.

In parallel a harmonised approach for coding of data from food allergic subjects undergoing low-dose oral food challenges undertaken in food allergic patients which was implemented in a data base suitable for either automated upload or direct data entry. Data gaps identified included the lack of challenge data for foods such as Brazil nut, macadamia nut, molluscan shellfish and lupin. Many foods for which few data were identified which were below the 60 data points identified as being required for best practice modelling. Many of the foods for which data are lacking represent less prevalent food allergies which makes it more difficult for clinical studies to identify many patients to include in any threshold study. The framework developed provides a means to collate further threshold data in collaboration with the clinical community. A common need identified across the research objectives is the need to understand how food processing may impact on eliciting doses and link that with the impact of processing on modification of allergenic proteins and their determination by allergen test methods.

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1 Introduction

1.1 Background and terms of reference as provided by the requestor

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 - 31st December 2022).

Contract/Grant title: Detection and Quantification of Allergens in Foods and Minimum Eliciting Doses in Food-Allergic Individuals (ThRAll)

Contract/Grant number: GP/EFSA/AFSCO/2017/03

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.

The project was cofunded by the United Kingdom Food Standards Agency (FS101209) and Belgian Federal Agency for the Safety of the Food Chain (FASFC).

1.2 Interpretation of the Terms of Reference

OBJECTIVE 1: DEVELOP REFERENCE (HARMONISED) METHODOLOGIES FOR THE DETECTION AND QUANTIFICATION OF ALLERGENS IN FOODS

The project partnership focused on the development of MS-based multiple reaction monitoring method(s) for the simultaneous detection and quantification analysis of six foods causing IgE-mediated food allergies which are included on Annex II of the FIR, namely milk (as cow's milk), egg (as hen's egg), soybean, peanut, hazelnut and almond. Building on reference and

quality control materials together with incurred matrices, that were already available from EU and nationally funded projects (iFAAM, MANOE, Allersens, Safe&smart, MoniQA and UK Food Standards Agency Project FS101206) two incurred matrices were prepared for the project including a chocolate based product (chocolate bar), and a powdered soup. These were used to develop the methods, which were founded on published data together with advances made through the iFAAM and Allersens projects. Using this knowledge base a harmonised quantitative MS-based prototype reference method for the detection of multiple food allergens in standardised incurred food matrices was developed. The matrices included reference and/or quality control materials already available and produce additional complementary materials in a food pilot plant thus mimicking, as far as possible "real world" manufactured foods.

OBJECTIVE 2 - GENERATE GOOD QUALITY DATA ON MINIMUM ELICITING DOSES (MED) AND MINIMUM OBSERVED ELICITING DOSES (MOED)

The ThRAll project sought to develop methods and approaches, including data cleaning and curation, to harmonise oral food challenge data used for identifying minimum eliciting doses (MED's) for allergenic food ingredients. Building on clinical best-practice for undertaking double blind placebo controlled food challenges (DBPCFC) from the PRACTALL group (Sampson et al., 2012) and drawing on tools and approaches arising from the EuroPrevall and iFAAM projects (Fernandez-Rivas et al., 2015; Grabenhenrich et al., 2017) we will provide a tool box to support generation of good quality data on MED's from low-dose oral food challenges undertaken in food allergic patients. Consensus approaches will be developed for quality assessment of data, including classification of reactive, tolerant and placebo reactive patients, evaluation of symptoms (objective, subjective, persistent subjective) to support consistent definition of lowest observed adverse effect levels in individual patients, including those experiencing transient reactions during challenges. Data from the literature, EU-funded projects such as iFAAM and EuroPrevall, and nationally-funded projects in France (such as MANOE) and the UK will be collated and reviewed using these criteria to provide "cleaned" analysis-ready data sets. The focus for this work will be on foods for which data gaps were identified by the iFAAM notably tree-nuts (walnut, cashew, pistachio, almond, macadamia nut, fish, crustacean and molluscan shell fish) although other foods listed on Annex II of the FIR, such as soybean and wheat, will also be collected. Where sufficient data are collected dose distributions will be modelled using interval censoring survival analysis to calculate MED's.

The aims and objectives of the ThRAll project are described in full by Mills et al (Mills et al., 2019)

1.3 Additional information

The ThRAll project partnership brings together centres with expertise in development of allergen detection methodology using liquid chromatography-mass spectrometry (LC-MS) methodology, and the capacity to curate and analyse oral food challenge data from food

allergic patients. It is formed from five organisations in the UK, BE, IT and FR and brought together the group led by Mills at the University of Manchester in the UK which has expertise in LC-MS analysis of allergens in foods (Mills, Nitride) with knowledge of the thresholds studies undertaken in the EuroPrevall and iFAAM projects and how to curate and analyse such data (Mills). This is complemented by the analytical sciences skills, especially in the development of LC-MS, enzyme linked immunoassay (ELISA) and polymerase chain reaction (PCR) methodology in the groups led by Monaci (CNR-ISPA, IT), van Poucke (ILVO, BE), Gillard (CER, BE), Tranquet and Adel-Patient (INRA, FR).

2 Data and Methodologies

2.1 Data

Two types of data have been generated during the course of the project:

(1) MS data: Data relates to proteomics discovery data and targeted analysis. These data are available on request from the communicating authors of the associated publications.

(2) Oral food challenge data: Clinical data from oral food challenges was collated in a RedCAP challenge database. These data and the associated code book can be found at https://figshare.manchester.ac.uk/ with the DOI 10.48420/21688199.

2.2 Methodologies

2.2.1 OBJECTIVE 1 (lead Monaci, CNR-ISPA)

In order to maximise synergies with EU- and nationally funded work relevant to Objective 1, a stake holder meeting was held in association with the kick-off meeting and in collaboration with the consortium of the FSA project FS101206 "Development of Quality Control Materials for Food Allergen Analysis". Synergies were developed between the projects, in particular with tasks 1.1 and 1.2.

Task 1.1 Allergenic ingredient and incurred food matrices

In order to maximise synergies with EU- and nationally funded work relevant to Objective 1, a stake holder meeting was held in association with the kick-off meeting and in collaboration with the consortium of the FSA project FS101206 "Development of Quality Control Materials for Food Allergen Analysis". Synergies were developed between the projects, in particular with tasks 1.1 and 1.2.

The allergenic commodities (food ingredients) used for the production of the incurred food matrices need to be well defined especially concerning the total protein content and allergen composition. As far as possible appropriately qualified materials were sourced by ILVO, subject to cost and sufficient material being available and were chosen to represent the types of ingredient used widely in the food manufacturing sector.

Allergenic food ingredients were characterised in two ways. Firstly, the total protein content was verified by ILVO using Kjeldahl total nitrogen determination, taking at least three sub-samples of 5g each from the batches sourced in a manner that allowed the homogeneity of

the protein content to be assessed. Conversion factors appropriate for each different food ingredient were used from FAO/INFOODS Guidelines for Converting Units, Denominators and Expressions, version 1.0 (2012). Subsequently, to enable bench-marking of food ingredients used in the project against reference materials (where available), the protein profiles were defined by INRAe using SDS-PAGE and compared with other types of widely used allergenic ingredients. Their allergenic activity was also compared using *in vitro* methodology developed in the iFAAM project for cow's milk, hen's egg, peanut and hazelnut. Since methods were less well developed for soybean and almond, more limited data were obtained for these foods (Nitride et al., 2018; Huet et al., 2022).

In order to make a realistic assessment of the capacity of a test method to detect and quantify an allergenic food ingredient in a processed and complex matrix it is important to incur the allergenic ingredients in a food product rather than spiking protein extracts or peptides on the food matrix as is common practice. Therefore, incurred food matrices were used to take account of the impact of food processing and the food matrix on extractability and digestibility of the proteins that form the analytical targets of the test methods (Wolf and Andrews, 1995). Two different incurred food matrices were produced at ILVO's Food Pilot plant, a chocolate bar and a broth powder, both containing the six allergenic food ingredients each at a concentration of 1000 (chocolate bar) and 400 (broth powder) mg total allergenic protein ingredient/kg. Alongside the incurred food matrices, the same food matrix was produced free from the six allergenic ingredient ("blank" food matrices; 0 mg allergenic ingredient protein/kg). These were used to make serial dilutions of the incurred food matrices to provide concentrations of 2, 4, 10, and 40 mg protein/kg of each allergenic ingredient. These were chosen as being close to the candidate action levels for these food matrices identified for five of the allergenic food ingredients by the FAO-WHO expert consultation (FAO-WHO, 2022a). Two higher concentrations of 200 and 500 (chocolate bars), 200 and 400 (broth powder) mg protein/kg of each allergenic ingredient were also produced to support test method development. The ingredients were also mixed and processed in a similar manner to the broth powder to allow the development of calibrators for ELISA analysis (see section 2.2.1.2.1 below).

Incurred material were then subjected to homogeneity tests according to the criteria described by Fearn and Thompson (Fearn and Thompson, 2001). Ten aliquots of the prepared material were selected at random, ground before being extracted in duplicate and tested by ELISA by CER The test proposed by Fearn and Thompson provides estimates of the analytical (S2an) and sampling (S2 sam) variance. The allowable sampling variance entitled σ2, as well as the critical value for each test, were calculated. This value corresponds to (F1 x σ2all + F2 x S2an) using the F1 and F2 values of Fearn and Thompson (Fearn and Thompson, 2001). If S2 sam > critical value, the test indicates a lack of sufficient homogeneity; otherwise, if S2 sam < critical value, the assumption of homogeneity is accepted.

Batches fulfilling the homogeneity criteria were then packed as follows:

(1) Chocolate bars: vacuum packed, protected from light and stored at 4°C.

(2) Broth powder: portioned and vacuum packed in double aluminium laminate sacks and stored in the dark at 4°C and under controlled relative humidity.

The allergen stability of the incurred matrices was monitored at regular intervals throughout the project life at CER and samples of incurred matrices shipped to partner laboratories as required. The stability study was performed using three samples extracted separately (n=3) every 6 months. The average of all values obtained per level was calculated as well as the corresponding standard deviation (SD) and the average plus or minus two SD.

Task 1.2 Analysis of materials incurred with allergenic ingredients by ELISA and PCR (Lead Tranquet, INRAe)

Task 1.2.1 Analysis by ELISA (Lead Tranquet, INRAe)

The allergenic protein content of each of the six ingredients incurred in the chocolate bar and broth powder was determined by ELISA. Through the Manchester Food Allergy Network (MFAN) allergen test kit manufacturers from Europe, Japan and Australia collaborated to identify allergen ELISA test kits suitable for analysis of the ThRAll incurred materials. Manufacturers were sent samples of the incurred chocolate bars and broth powder for evaluation and subsequently three agreed to provide INRAe with kits for the determination of allergen content at each level in both ThRAll matrices.

Discrepancies in test results obtained using different food allergen test kits can sometimes be observed when analysing allergenic ingredients in processed matrices. One major reason for this relates to the way in which different antibody preparations show different reactivities towards the allergen targeted in the calibrant of the kit and in the processed matrices. Thus, an ad-hoc "ThRAll allergenic ingredient" was included in all assays performed on the chocolate bars. Similarly, a "processed ThRAll allergenic ingredients mix" was prepared at ILVO using a process similar to that used in the manufacture of the broth powder, which was included in all assays performed on the broth powder. The six allergens incurred in the two matrices and the two calibrators were analysed in triplicate in two independent experiments (two different extractions performed on two different days by the same experimenter). Recovery was calculated for each allergen according to their incurred content using either the calibrator of the kit or the ThRAll calibrator.

Task 1.2.2 Analysis by ddPRC (Lead: Taverniers, ILVO)

Compared to conventional PCR (1st generation PCR) and real-time or qPCR (2nd generation PCR), digital PCR has the advantages of being more sensitive, less prone to PCR inhibitors that might be present in the matrix, and independent of the use of an external standard series for quantification. Digital droplet PCR (ddPCR) is a third generation PCR application, based on

the partitioning of the PCR reaction in thousands of individual droplets in a water-in-oil emulsion. In ddPCR, the polymerase chain reaction takes place in each individual droplet, resulting in either a positive droplet – where the target is detected – or a negative droplet. Accurate, absolute quantification becomes possible using flow cytometry to count the number of positive droplets, relative to the total number of droplets.

The objective was to develop ddPCR assays for soybean, hazelnut, peanut and almond, and apply these to the analysis of ingredients as well as processed, complex matrices, including the ThRAll matrices developed within Task 1.1. Soybean-specific ddPCR assays (an in-house assay and a Generon soy ddPCR test kit) were assessed in combination with the NSF DNA extraction kit. However, the ddPCR assays currently available were only suitable for use in simple food matrices and did not work on the ThRAll incurred matrices. Consequently, activity was focussed on development of suitable conversion factors from DNA to protein.

Task 1.3 Identification of protein and peptide markers for selected allergenic foods (Lead: Monaci, CNR-ISPA).

A literature review was undertaken to identify candidate peptides for use in the project (Pilolli et al., 2020). Starting from the available information, peptide markers were experimentally verified using MS analysis in the incurred matrices (Pilolli et al., 2021). The experimental validation by untargeted MS was carried out on the two incurred matrixes analysed (40 mg allergenic ingredient protein/kg for chocolate bar and 200mg allergenic ingredient protein/kg for the broth powder) allowed confirmation of most of the markers previously validated in independent investigations, but also enlarged the list to include new options that might represent good candidates for method development.

Task 1.4 Development of extraction, purification and digestion conditions for MS methods (Lead Gillard, CER Groupe)

Task 1.4.1 Optimization of the sample preparation workflow for LC-MS/MS analysis (CER Groupe) (Lead Gillard, CER Groupe)

The development of a harmonised, reliable, straightforward, and reproducible sample preparation workflow for MS-based multi-allergen detection in processed foodstuffs was developed by taking into consideration the main and critical steps of the sample preparation protocol, namely:

- Sample preparation (*i.e.*, sample grinding, melting, or melting and defatting procedures).
- Composition of the extraction buffer (*e.g.*, concentration of urea).
- Protein purification using size exclusion chromatography (SEC) columns to remove low molecular weight contaminants on the protein extracts (and including comparison between spin and gravity elution) as described by Pilolli et al (Pilolli et al., 2017).

- Enzymatic digestion of protein was optimized in order to maximise conversion of extracted allergen proteins into peptide markers using tryptic digestion (*i.e.* digestion duration, trypsin-to-protein ratio, addition of chemical aids such as Rapigest SF).

- Peptide purification and pre-concentration using solid phase extraction (SPE) on disposable columns testing different solid phases (Huschek et al., 2016; Korte et al., 2016; Planque et al., 2016; Hoffmann et al., 2017; Monaci et al., 2020) and evaluating the use of DMSO during the evaporation step.

Minimal requirements of the instrumental set-up and parameters were also identified and optimised in terms of chromatographic separation and MS-based detection using multiple reaction monitoring (MRM) mode.

The main experiments were carried out by CER and some were replicated by CNR-ISPA to confirm the results on a different MS platform in order to provide a first assessment of the robustness of the method under development. Statistical analysis of all data collected in Task 1.4.1 was performed by CNR-ISPA.

Task 1.4.2 In-house validation of the method developed (Lead Pilolli, CNR-ISPA)

In order to develop a cost-effective method the list of markers to be monitored during the in-house method validation was refined. Two peptides per allergenic food ingredient were selected for peanut, soybean, hazelnut and almond and four peptides per allergenic food ingredient were selected for egg (either from the white or yolk fraction) and milk (from either the casein or whey fraction). The validation was carried out at CNR-ISPA on the refined list (Table 1) selected as the best performing candidates during method development (task 1.4.1) carried out jointly by CNR-ISPA and CER Groupe on two different MS platforms.

Table 1. Final list of markers monitored in chocolate bars for the method in-house validation.

Allergenic Ingredient	Protein	Allergen	Uniprot ID	Peptide Target Residue	Peptide sequence
Milk /caseinate	αS1-Casein	Bos d 9	P02662	38-49	P1 - FFVAPFPEVFGK
	αS2-Casein	Bos d 10	P02663	130-140	P2 - NAVPITPTLNR
Milk /whey	β-Lactoglobulin	Bos d 5	P02754	108-116	P3 - VLVLDTDYK
				100-107	P4 - IDALNENK
Egg /white	Ovalbumin	Gal d 2	P01012	128-143	P1 - GGLEPINFQTAADQAR
				324-340	P2 - ISQAVHAAHAEINEAGR
Egg /yolk	Vitellogenin-1	Gal d 6	P87498	1874-1884	P3 - ATAVSLLEWQR
	Vitellogenin-2	-	P02845	919-927	P4 - NIGELGVEK
Peanut	Cupin	Ara h 3	O82580	342-354	P1 - SPDIYNPQAGSLK
				355-365	P2 - TANDLNLLILR
Soybean	Glycinin	Gly m 6	P04776	411-423	P1 - VLIVPQNFVVAAR
				401-410	P2 - VFDGELQEGR
Hazelnut	11S Seed Storage Globulin	Cor a 9	A0A0A0P7E3	339-348	P1 - ADIYTEQVGR
				462-476	P2 - ALPDDVLANAFQISR
Almond	Amandin, 11S Globulin	Pru du 6	E3SH28	493-505	P1 - TEENAFINTLAGR
				388-394	P2 - ADIFSPR

Task 1.5 Inter-laboratory comparison (Lead: Nitride, UNIMAN)

The transferability of the test method was assessed in a ring trial involving four ThRAll consortium laboratories (CER, ILVO, UNIMAN and CNR-ISPA) which took place in the summer of 2022. The opportunity was explored to include additional laboratories but it was not feasible given the resources and time frame available as a result of the COVID-19 Pandemic. Based on the outputs of Task 1.4 a suite of peptide targets was identified and synthesized as 13C- and 15N- C-terminal labelled peptides and the mass of peptide determined using amino acid analysis by a commercial entity. Chocolate bars prepared in Task 1.1 and containing 0, 2, 4, 10 and 40 mg of total allergenic protein ingredient per kg of food were used in the inter-laboratory comparison. Incurred chocolate bars were sent out by ILVO and CNR-ISPA sent out labelled peptide standards for calibration curves. All laboratories performed the analysis using a triple quadruple mass spectrometry platform equipped with different chromatographic systems with their own system optimisation.

A set of standard operation procedures (SOPs) and data return sheets were developed for the prototype multiallergen MS method by CNR-ISPA and UNIMAN based on those developed for the iFAAM. Data was returned by the test laboratories and analysed by UNIMAN to provide the following assessments:

- Inter-laboratory reproducibility of the allergen determination
- Identification of the potential sources of uncertainty (fish bone diagram). To determine the total uncertainty of the methods developed.
- Comparison between the MS data and the measurements made on the same materials by ELISA from Task 1.2.1.

2.2.2 OBJECTIVE 2 (LEAD Mills, UNIMAN)

Task 2.1: Development of harmonised protocols for collection of threshold data in food allergic individuals (Lead: Mills, UNIMAN)

A mapping exercise was undertaken using the EuroPrevall/iFAAM and TRACE clinical record forms (CRFs) to identify key terminology and provide a roadmap to allow coding of such information, paving the way to its harmonisation. This was achieved through an expert clinician workshop working with EuroPrevall/iFAAM and TRACE clinical partners. Through evaluation of various clinical and food ontologies an approach for coding IgE-mediated food allergy data was developed that is based on SNOMED CT for coding of clinical data and FoodEX2 for coding of food data. An approach to coding severity of reaction was also applied. The collaborators who contributed to this exercise and the collation of data in Task 2.2 were as follows:

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The coding system can be found at https://figshare.manchester.ac.uk/ with the DOI 10.48420/21688199.

Task 2.2 Population and curation of database with historic and published data (Lead: Mills, UNIMAN)

Based on the results of mapping the CRFs in Task 2.2.2.1 an online database was built using REDCap (Research Electronic Data Capture) tools hosted at UNIMAN. REDCap is a secure web application for building and managing online surveys and databases which is free to use

for consortium members (Harris et al., 2009; Harris et al., 2019). It provides 1) an intuitive interface for validated data capture; 2) audit trails for tracking data manipulation and export procedures; 3) automated export procedures for seamless data downloads to common statistical packages; and 4) procedures for data integration and interoperability with external sources.

The database comprises four instruments as follows which are designed to capture various different types of data:

(1) ***Protocol:*** this collates data on the allergenic food used for a food challenge, the matrix it was delivered in, the ingredients used to make the matrix, type of challenges study (e.g. double blind placebo controlled food challenge, open challenge, single dose challenge or one using interspersed doses), the dose progression, dosing interval and stopping criteria.

(2) ***Demographics:*** this captures the data source, research ethics committee number and information on how the challenges are coded regarding whether objective or subjective symptoms were recorded, the country the data originated from, gender, age and BMI of study subject.

(3) ***Challenge day:*** this captures data on a challenge day and the symptoms recorded during the dose progression and their time of development. This instrument is suitable for upload of clinical data collected during a food challenge.

(4) ***Threshold dose:*** this captures data that is only available in a summarised form where the lowest observed adverse effect level (LOAEL) and no observed adverse effect level (NOAEL) is provided. This is frequently the case for published data.

The code book is available through the University of Manchester repository at https://figshare.manchester.ac.uk/ with the DOI 10.48420/21688199.

Data were entered either manually or through a bulk upload of data formatted correctly using the harmonised approach developed in Task 2.2.2.1.

3 Assessment/Results

3.1 OBJECTIVE 1

3.1.1 Allergenic ingredients and incurred food matrices

The preparation and characterisation of the incurred ingredients is described in full in (Huet et al., 2022) and is summarised below. Where reference or quality assurance material could not be obtained, food ingredients were sourced from single source suppliers or who assure the manufacturing process and potential for cross-contact with other allergenic ingredients.

Six priority allergenic foods which are responsible for the majority of food product recalls (Bucchini et al., 2016) were sourced as food ingredients as follows:

- Milk – milk was used in the form of skimmed milk powder which is the form most widely used in food manufacturing. A cow's milk reference material from the MoniQA project (SMP-MQA 092014) was used.
- Egg – egg will was used in the form of spray-dried whole egg, a form widely used in food manufacture, using a reference material certified for its total protein content (NIST – RM 8445).
- Peanut – peanut flour quality control material available from LGC Standards (LGCQC1020) which has also been used for collection of oral food challenge data in EuroPrevall, TRACE and iFAAM was used (Ballmer-Weber et al., 2015; Dua et al., 2019; Bernard et al., 2020).
- Soybean – enzyme active and non-toasted full fat soy flour was purchased from Soja Austria (a manufacturer focussed on soy) supplied through GenM-service.
- Hazelnut - powdered ground raw hazelnut flour used for oral food challenges in the EuroPrevall (Ballmer-Weber et al., 2015) projects was sourced which is available from LGC Standards (LGC7425).
- Almond – blanched almond flour ground almonds was sourced which is available from LGC Standards (LGC7425).

The protein profile of the food ingredients was fully characterised by one- and two-dimensional electrophoresis and the presence of the major allergenic proteins confirmed by immunoassay using animal antibodies and/or patient's sera, when available. This showed that ThRAll peanut, milk, egg, and hazelnut ingredients have the expected protein profiles and that the major allergens retained their IgE reactivity. The total protein content for each ingredient was also assayed by the Kjeldahl method unless specified by the producer.

The ingredients were then incorporated into two model foods selected as hard to analyse matrices. Both involve processing steps or matrix characteristics such as cooking, high fat, high polyphenols, high carbohydrate content and complex protein background, which are known to have an impact on the extractability and detectability of proteins/peptides. A

stakeholder meeting was held at UNIMAN where the choice of ingredients and incurred matrices was discussed before arriving at a final conclusion to use the chocolate bar as an example of a high fat and polyphenol rich matrix whilst the broth powder was chosen as a matrix which has undergone extensive food processing including cooking, boiling down and drying to a powder. Furthermore, the broth was made using meat and different vegetables resulting in a complex protein background from which the allergenic proteins will need to be discriminated.

The allergenic food ingredients were used to provide incurred levels at 2, 4, 10, 40, 200 and 500 mg of each allergenic ingredient/kg of chocolate bar as intermediate steps to developing the final incurred levels of 2, 4, 10, 40, 200 and 400 mg of each allergenic ingredient/kg of broth powder.

The homogeneity of the materials was then assessed by enzyme-linked immunosorbent assay (ELISA) and were as follows:

Chocolate bar: homogeneity was found to be acceptable at all dose levels for all allergenic ingredients apart from the 2 ppm peanut and soya which could not be analysed due to the lack of sensitivity of the ELISA's employed. Although the homogeneity of the peanut and soy ingredients could not be determined in this matrix at this level, it was confirmed that the egg, casein, almond, and hazelnut ingredients were homogeneously distributed at this level. Since all the ingredients were powders of similar particle size, it was highly likely that the peanut and soy ingredients were also homogeneously distributed. This conclusion was also supported by the observation that the peanut and soy ingredients were homogeneously distributed at the other, higher, levels and confirmed that the approach for the production of incurred chocolate bars was successfully executed.

Broth powder: None of the in-house ELISAs (CER) were able to detect the incurred allergenic ingredients. However, two commercial ELISA kits (one for total cow's milk and one for soybean) were able to detect the allergenic ingredients down to 4 mg allergenic protein/kg (soybean) and 10 mg allergenic protein/kg (total milk) although with poor recovery. To overcome the problem encountered by the lack of detection of other selected allergenic ingredients, two alternative in-house ELISA kits were used which has been developed by INRAE-CEA, for the detection of milk (Bernard et al., 2021) and peanut (Bernard et al., 2020). The inclusion of a chaotropic agent in the extraction step in combination with targeted antibodies allowed the detection of allergenic proteins in the broth powder. The homogeneity of milk and peanut ingredients incurred in the broth powder was proven using an ELISA for β-casein with a total milk protein calibrant and an ELISA Ara h 6 with a total peanut protein extract as a calibrant. Fearn's test for the lowest level (2 mg allergenic protein/kg) for soy was not determined because the signals were inferior with respect to the limit of quantification (LOQ); since all other levels showed acceptable homogeneity, it was postulated that the lowest level for soy was also homogenous. It was also postulated that the other three targets in the incurred test materials were sufficiently homogenous on the basis of results obtained for the three assessed contaminants and good results obtained for chocolate.

The stability of the produced materials was also assessed by ELISA:

Chocolate bar: From all obtained data, it could be concluded that incurred chocolate bars were stable for at least 37 months from the point of manufacture for all the allergenic ingredients studied since test results lay within the mean of all samples analysed, plus or minus twice the corresponding standard deviation (SD).

Broth powder: the incurred materials were stable for at least 30 months for peanut and milk, using the in-house kits developed by INRAe-CEA and for soy using the commercial kit (R-Biopharm). The lack of effective detection methodology in the highly processed matrix did not allow the stability of the other allergenic ingredients to be followed.

3.1.2 Analysis of materials incurred with allergenic ingredients by ELISA and ddPCR

3.1.2.1 Analysis by ELISA

The six allergenic ingredients incurred in the two different matrices were assayed in the INRAE laboratories using ELISA test kits from three different manufacturers (Manufacturer's A, B and C). Analysis was performed according to the manufacturer's instructions and with an additional "ThRAll" calibrator to assess whether this internal calibrator improved recovery of the allergen in the ThRAll matrices (Table 2). Although recoveries were between 50-150%, since they were consistent for a particular allergen in a given matrix, they were considered acceptable in accordance with AOAC guidelines (Abbott et al., 2010).

Chocolate bars: In almost all cases, the recovery of allergens calculated with either the kit calibrator or the ThRAll calibrator was acceptable (50-150%) at each incurred level. When using the kit calibrator the allergen content was overestimated in two cases (Manufacturer C, Soya and Almond) and underestimated in one case (Manufacturer B, Hazelnut). The use of the ThRAll Calibrator instead of the kit calibrator improve the recovery in acceptable limits in one case (Manufacturer C, Soya) and had no effect in the two other cases. It should be noted that the use of the ThRAll calibrator resulted to overestimation in one case (Manufacturer C, Almond) and underestimation in one case (Manufacturer A, Egg), which was not observed with the Kit calibrator.

Broth powder: The situation was not as favourable for allergen analysis in the broth powder. When using the kit calibrator, no acceptable recovery was ever observed for this matrix. Allergenic foods were not detected in 4/12 cases and recovery was below 50% in the other cases (8/12). Apart from the 2mg allergenic protein/kg level, the recovery improved and became acceptable in 5 of the 8 cases by using the ThRAll calibrator.

Table 2: Analysis of recovery by allergen and kit.

Allergenic food	Chocolate bars			Broth powder		
	Manufacturer	Calibrator		Manufacturer	Calibrator	
		Kit	ThRAll		Kit	ThRAll
Peanut	A	√	√	A	Recovery <50%	Recovery <50%
	B	√	√	B	Recovery <50%	OK
	C	√	√	C	NT	NT
Milk	A	√	√	A	Recovery <50%	Recovery <50%
	β-Casein	√	√	β-Casein	Recovery <50%	OK from 4ppm
	β-lactoglobulin	√	√	β-lactoglobulin	Recovery <50%	OK from 4ppm
	C	√	√	C	NT	NT
Soya	A	√	√	A	ND	ND
	B	√	√	B	Recovery <50%	Acceptable ≥4ppm
	C	Recovery >150%	√	C	NT	NT
Egg	A	√	Recovery <50%	A	ND	ND
	B	Acceptable >4ppm	√	B	Recovery <50% ≥4 ppm	Acceptable ≥4ppm
	C	√	√	C	NT	NT
Almond	A	√	Recovery >150%	A	ND	ND
	B	NA	NA	B	NA	NA
	C	Recovery >150%	Recovery >150%	C	NT	NT

Allergenic food	Chocolate bars			Broth powder		
	Manufacturer	Calibrator		Manufacturer	Calibrator	
		Kit	ThRAll		Kit	ThRAll
Hazelnut	A	√	√	A	ND	ND
	B	Recovery <50%	Recovery <50%	B	Recovery <50% ≥4 ppm	Recovery <50% ≥4 ppm
	C	√	√	C	NT	NT

√: acceptable recovery between 50 and 150%.

NA: non-applicable as some manufacturers did not produce kits for all the ThRAll allergenic ingredients.

NT: Not tested as due to strict quarantine regulation it was not possible to ship the incurred broth and hence kits were not available for this analysis.

ND – not detected.

ppm – mg allergenic ingredient protein/kg food product.

3.1.2.2 Analysis by ddPCR

A soybean-specific ddPCR assay was developed and tested on pure matrices as well as processed, complex matrices. The first part of the optimization and validation stage occurred at the level of DNA extraction. Several products such as certified reference materials (powders), maize or wheat background with soybean powder added, mixtures of species with soybean added were used. Among several protocols tested for DNA extraction, the NucleoSpin Food (NSF) kit performed best in terms of yield (ng DNA/mg product), purity (the absorbance ratio ratios at 260:280 nm indicating the presence of RNA and proteins), and amplifiability in ddPCR (a clear positive signal at low DNA amounts *i.e.*, min. 3 positive droplets). The second part of the optimization/validation process concerned deciding on the best soybean-specific ddPCR assay (in-house assay versus Generon soy ddPCR testkit), in combination with the NSF DNA extraction kit. Here, the commercially available ddPCR testkit from Generon did not perform better, in terms of sensitivity, than the in-house available *Le(1)* PCR detection system (primers plus TaqMan probe) (Platteau et al., 2011). Therefore, it was decided to apply the same combination of DNA extraction (NSF kit, Machery-Nagel) and in-house ddPCR detection methods to the analysis of the other allergens, peanut, hazelnut, and almond (Figure 1) (Bergerová et al., 2011; Platteau et al., 2011; Van Gansbeke et al., 2018) .

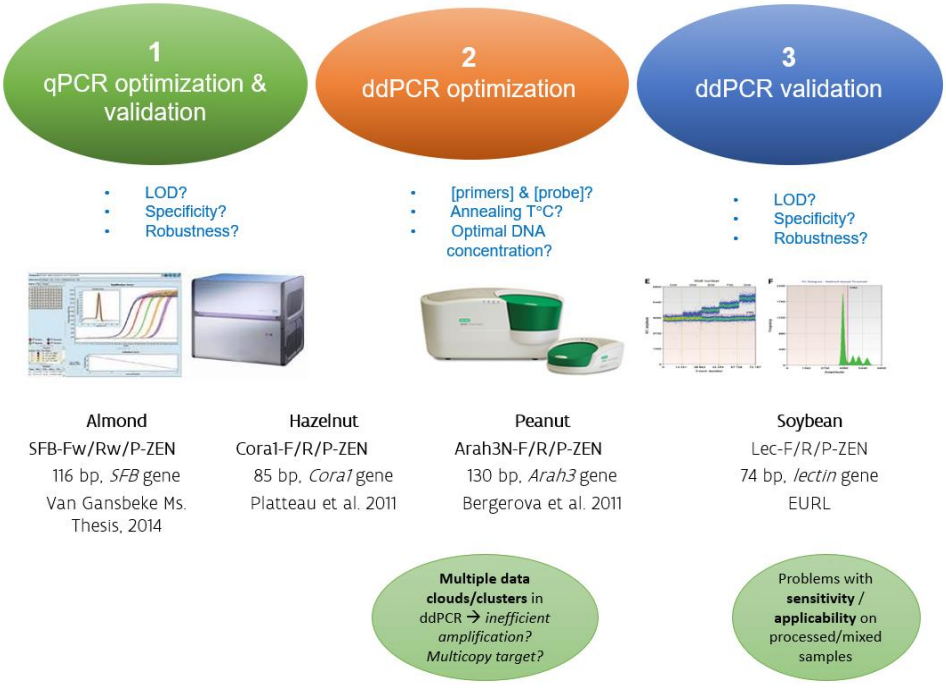

Figure 1. Flow and main criteria from (1) qPCR optimalisation and validation to (2) ddPCR optimization and (3) ddPCR validation. The aim was to adapt an existing qPCR assay towards a ddPCR assay. On the vertical axis, in the middle are the names (primers/TaqMan probe) and references of the adapted and available PCR assays for the 4 allergens. Below is

the status where the assay could be finally put, along with (in green) some issues that are typical problems when applying ddPCR.

An optimized and validated ddPCR assay is available for soybean in simple, solid products. Basic, although sub-optimal, ddPCR assays are available for peanut, hazelnut, and almond. However, there was insufficient sensitivity for complex and processed matrices, also after adapting the DNA extraction method and ddPCR assessment (e.g. trying Generon products). Due to a lack of sensitivity, the EFSA-ThRAll matrices have not been tested with ddPCR.

In the context of PCR methodologies in general, work has been done on conversion factors needed between DNA (the analytical target in PCR) and protein (the analyte in ELISA). Experimental data were generated for peanut and hazelnut commodities in two case studies. Different types of pure hazelnuts and peanuts were gathered, varying in origin (country), source, trade name, storage conditions and processing conditions. First, protein extraction and concentration measurement were performed with Bradford versus Kjeldahl assessment (mg protein / kg commodity). Second, DNA extraction was performed with Qiagen versus NSF kit, followed by DNA concentration measurement with Bio-Spec nanodrop spectrophotometetry (SFM) versus Quantus fluorimetry (FM) to obtain mg DNL per kg commodity. Conversions, expressed in [mg protein/kg commodity] / [mg DNA/kg commodity], could be graphed for eight different possible combinations (hazelnut; peanut). The combination depicting the lowest dispersion in conversions (protein/DNA) over the wide variety of tested commodities, was selected and proposed as the best conversion factor (hazelnut; peanut).

At the level of real-time PCR (qPCR), ready-to-use kits for detection and quantification of food allergens in ppm allergen (not protein) were also tested and evaluated. According to e.g. SureFood Soy Allergen detection kit (R-Biopharm), direct relative quantification in ppm allergen (not protein) would be feasible. However, this quantification is complicated by DNA extraction issues from complex, processed food matrices which may degrade DNA(Gryson, 2010) and because of the needed conversion from DNA to protein. One approach to achieving this is to directly compare the absolute Ct value for a specific allergen in a sample (based on the DNA extracted from the matrix) with the absolute Ct value obtained for the allergen in the standard used for quantification in the kit e.g. *Quantard* mixture of 7 food allergens in a maize background (based on DNA extracted from the *Quantard* material). However, by testing different DNA extracts from different matrices, comparing these extracts' Ct measurements to the *Quantard* extracts' Ct measurements, made clear that direct quantification based on this comparison is not repeatable/reproducible and thus not accurate.

Therefore, in-house validation data were gathered for qualitative detection of a food allergen by qPCR. More in particular a practical limit of detection (LOD) was determined, a specificity check was performed, and finally a robustness check. First, theoretical determination of LOD was done according to DIN 32645:2008-11, (Deutsches Institut für Normung, 2008) i.e. the LOD was calculated as the 95 % confidence level of a standard curve based on a dilution series of different known concentrations (reference material from kit). The theoretically

determined LOD was then confirmed by testing relevant concentration levels for detectability in ppm (mg target allergen/kg matrix) for limited number of certified reference materials (i.e practical LOD). Second, besides data on sequence similarity search of the DNA target against one of the major nucleic acid sequence data bases, available in the kit validation report (i.e. theoretical specificity), practical sensitivity i.e. cross-reactivity was checked for closely related and additionally relevant species, other than those tested by the kit provider. Third and last part of the in-house validation concerns double blind analysis performed as 2nd line QA controls in the lab, by testing small changes to DNA extraction or PCR protocol e.g. another qPCR instrument, other mastermix, different operators, etc.

3.1.3 Identification of protein and peptide markers for MS analysis of selected allergenic foods

A systematic review of the available literature on food allergen was performed to develop a list of candidate peptide markers for the six allergenic food ingredients. Candidate peptides were first evaluated and filtered based on four main features: (i) peptide length (7-20 amino acids in length); (ii) investigated food matrix (chocolate based and/or thermally processed incurred matrix); (iii) type of investigation reported (discovery vs targeted); and (iv) occurrence of amino acid residues prone to modifications (excluding sequences containing methionine or asparagine-glycine motifs). All available information related to the main proteins targeted by means of the proteotypic peptide markers was then collected in terms of relative abundance in the allergenic ingredient, modifications, and occurrence of isoforms and/or variants. Finally, the peptide specificity and potential sequence similarity with homologous proteins from related species was assessed. These data were then used to further filter peptide markers to give a preliminary list of candidate marker peptides (Table 3). The results of this analysis have been published (Pilolli et al., 2020).

Since the reliability of the peptide markers may be affected by several experimental factors such as the ingredient and matrix composition and processing, as well as method-dependent factors such as extraction, purification, and digestion protocols, the preliminary list of candidate markers selected by literature review was then validated by discovery high resolution MS/MS analysis on incurred food matrices from Task 3.1.1. Independent experiments on incurred chocolate bar and broth powder were carried out, comparing different sample preparation protocols to provide preliminary qualitative information on the most promising sample preparation workflow. This approach also allowed refinement of the peptide marker list and identified new peptide markers for the six allergens (see Table 4) (Pilolli et al., 2021).

Table 3: Selected signature peptides monitored in independent targeted investigations with relevant information on allergens isoforms/variants, and typical precursor ions (Pilolli et al., 2020). The presence of amino acid residues prone to modification was highlighted with a different font (bold and underlined).

Target protein	Peptide Marker		Typical Precursor	
	Sequence	**IUS allergen name**	**m/z**	**charge**
MILK				
Bos d 9 – α-S1-Casein	FFVAPFPEVFGK	Bos d 9.0101	692.9	+2
	YLGYLEQLLR	Bos d 9.0101	634.3	+2
Bos d 10 – αS2-Casein	NAVPITPTLNR	Bos d 10.0101	598.3	+2
	FALPQYLK	Bos d 10.0101	490.3	+2
Bos d 5 - β-Lactoglobulin	TPEVDDEALEK	Bos d 5.0101	623.3	+2
	VLVLDTDYK	Bos d 5.0101	533.3	+2
	VYVEELKPTPEGDLEILLQK	Bos d 5.0101	771.8	+3
	LSFNPTQLEEQ**C**HI	Bos d 5.0101	858.4	+2
EGG				
Gal d 2 – Ovalbumin	GGLEPINFQTAADQAR	Gal d 2.0101	844.4	+2
	HIATNAVLFFGR	Gal d 2.0101	673.4	+2
	ISQAVHAAHAEINEAGR	Gal d 2.0101	887.4	+2
Gal d 4 - Lysozyme C	FESNFNTQATNR	Gal d 4.0101	714.8	+2
	NTDGSTDYGILQINSR	Gal d 4.0101	877.4	+2
Vitellogenin-1	YLLDLLPAAASHR (lipovitellin-1 chain)	-	480.6	+3
	ALLLSEIR (lipovitellin-1 chain)	-	457.8	+2
Vitellogenin-2	NIPFAEYPTYK (lipovitellin-1 chain)	-	671.8	+2
	NIGELGVEK (lipovitellin-1 chain)	-	479.8	+2
	LPLSLPVGPR (lipovitellin-2 chain)	-	524.8	+2
PEANUT				
	DLAFPGSGEQVEK	Ara h 1.0101 (clone P41B) Ara h 1 - clone P17	688.8	+2

Target protein	Peptide Marker		Typical Precursor	
	Sequence	IUS allergen name	m/z	charge
Ara h 1 | Cupin (Vicillin-Type, 7S Globulin)	VLLEENAGGEQEER	Ara h 1.0101 (clone P41B) Ara h 1 - clone P17	786.9	+2
	GTGNLELVAVR	Ara h 1.0101 (clone P41B) Ara h 1 - clone P17	564.8	+2
Ara h 2 - Conglutin (2S Albumin)	**CC**NELNEFENNQR	Ara h 2.0101, Ara h 2.0201 Ara h 2.0202	863.8	+2
	NLPQQ**C**GLR	Ara h 2.0101, Ara h 2.0201 Ara h 2.0202	543.3	+2
SOYBEAN				
Gly m 5 - β-Conglycinin (Vicilin, 7S Globulin)	LITLAIPVNKPGR	Gly m 5.0101	464.6	+3
	QQQEEQPLEVR	Gly m 5.0201	692.3	+2
	DSYNLQSGDALR	Gly m 5.0201	669.8	+2
	DSYNLHPGDAQR	Gly m 5.0301, Gly m 5.0302	458.2	+3
Gly m 6 - Glycinin (Legumin, 11S Globulin)	VFDGELQEGR	Gly m 6.0101	575.2	+2
	SQSDNFEYVSFK	Gly m 6.0101, Gly m 6.0201, Gly m 6.0301	725.7	+2
	ISTLNSLTLPALR	Gly m 6.0401, Gly m 6.0501	699.9	+2
	FYLAGNQEQEFLK	Gly m 6.0101, Gly m 6.0201	793.9	+2
HAZELNUT				
Cor a 9 - 11S Seed Storage Globulin (Legumin-Like)	INTVNSNTLPVLR	Cor a 9.0101 (Q8W1C2) Cor a 9 (A0A0A0P7E3)	720.9	+2
	ALPDDVLANAFQISR	Cor a 9.0101 (Q8W1C2) Cor a 9 (A0A0A0P7E3)	815.5	+2
	ADIYTEQVGR	Cor a 9.0101 (Q8W1C2) Cor a 9 (A0A0A0P7E3)	576.3	+2
	QGQVLTIPQNFAVAK	Cor a 9.0101 (Q8W1C2) Cor a 9 (A0A0A0P7E3)	807.5	+2
Cor a 11 - 7S Seed Storage Globulin (Vicilin-Like)	LLSGIENFR	Cor a 11.0101	524.9	+2
	AFSWEVLEAALK	Cor a 11.0101	628.4	+2
ALMOND				
Pru du 6 - Amandin, 11S Globulin Legumin-Like Protein	QQGQQEQQQER	Pru du 6.0101	694.0	+2
	TDENGFTNTLAGR	Pru du 6.0201	698.3	+2

However, since the reliability of the peptide markers may be affected by several experimental factors such as the ingredient and matrix composition and processing, as well as method-dependent factors such as extraction, purification, and digestion protocols, the preliminary list of candidate markers selected by literature review was validated by discovery high resolution MS/MS analysis on incurred food matrixes. Independent experiments on incurred chocolate bar and incurred broth powder were carried out, comparing also different sample preparation protocols to convey preliminary qualitative information on the best promising sample preparation workflow. This approach allowed refinement of the peptide marker list and identified new peptide markers for the six allergens (see Table 4) (Pilolli et al., 2021).

Table 4: Candidate peptide markers experimentally validated by untargeted discovery analysis on incurred matrices chocolate bar (40 ppm concentration level) and broth powder (200 ppm concentration level) (Pilolli et al., 2021). The last column highlights the final list of candidates that were used as starting point for the method development activity (Task 1.4.1).

Protein	Peptide Sequence	Validated by discovery analysis of incurred chocolate bar	Validated by discovery analysis of incurred broth powder	Candidate marker used for method development (Task 1.4.1)
Bos d 9 αS1-casein	FFVAPFPEVFGK	X	X	X
	YLGYLEQLLR	X	X	X
	HQGLPQEVLNENLLR	X	X	X
	EGIHAQQK	X		
Bos d 10 αS2-casein	NAVPITPTLNR	X		X
	ALNEINQFYQK	X		
	FALPQYLK	X	X	X
Bos d 11 β-casein	VLPVPQK	X	X	
	AVPYPQR	X		
	GPFPIIV		X	
Bos d 12 κ-casein	YIPIQYVLSR	X	X	
	SPAQILQWQVLSNTVPAK**		X	
Bos d 5 β-lactoglobulin	VYVEELKPTPEGDLEILLQK	X	X	X
	TPEVDDEALEK*	X		X
	IDALNENK	X		X
	VLVLDTDYK*	X		X
	LSFNPTQLEEQ**C**HI		X	
Gal d 2 Ovalbumin	ELINSWVESQTDGIIR**	X		X
	ISQAVHAAHAEINEAGR	X	X	X
	GGLEPINFQTAADQAR	X	X	X
	DILDQITKPNDVYSFSLASR**		X	
	DILNQITKPNDVYSFSLASR**		X	
	YPILPEYLQ**C**VK		X	
	HIATNAVLFFGR		X	
Gal d 3 Ovotransferrin	SAGWNIPIGTLIHR		X	
Apovitellenin-1	DWLVIPDAAAAYIYEAVNK		X	
Apolipoprotein B	GFEPTLEALFGEK		X	
Vitellogenin-1	ATAVSLLEWQR	X	X	X

Protein	Peptide Sequence	Validated by discovery analysis of incurred chocolate bar	Validated by discovery analysis of incurred broth powder	Candidate marker used for method development (Task 1.4.1)
Vitellogenin 2	LPLSLPVGPR	X	X	X
	NIGELGVEK	X		X
	NIPFAEYPTYK	X		X
Ara h 1	VLLEENAGGEQEER	X		X
	DQSSYLQGFSR	X		X
	DLAFPGSGEQVEK	X		X
Ara h 2	NLPQQCGLR			X
Ara h 3	TANDLNLLILR	X	X	X
	SPDIYNPQAGSLK	X		X
	TANELNLLILR	X	X	
	SSNPDIYNPQAGSLR**	X		
	GENESDEQGAIVTVR**	X		
	QIVQNLR	X		
	SQSDNFEYVAFK	X		
	AQSENYEYLAFK	X		
	FFVPPSEQSLR	X		
	WLGLSAEYGNLYR		X	
Gly m 5 β-conglycinin	VPAGTTYYVVNPDNDENLR	X		X
	VPSGTTYYVVNPDNNENLR	X		X
	AIPSEVLSNSYNLGQSQVR		X	
	QVQELAFPGSAQDVER	X		X
	QQQEEQPLEVR	X		X
	NILEASYDTK	X		X
	LQSGDALR	X		
	SPQLQNLR**	X		
	SPQLENLR	X		
	EQQQEQQQEEQPLEVR	X		
	LFEITPEK	X		
	ESYFVDAQPK	X		
	LQESVIVEISK	X		
	ESYFVDAQPQQK	X		X
Gly m 6 Glycin	NLQGENEEEDSGAIVTVK	X		X
	SQSDNFEYVSFK	X		X
	VLIVPQNFVVAAR	X	X	X
	NNNPFSFLVPPQESQR		X	
	LSAEFGSLR	X		
	LSAQYGSLR	X		X
	VFDGELQEGR	X		X
	ISTLNSLTLPALR	X	X	X
	FYLAGNQEQEFLK	X	X	X
	FLVPPQESQK	X		
Cor a 9 11S globulin-like protein	ALPDDVLANAFQISR	X	X	X
	TNDNAQISPLAGR	X		X
	INTVNSNTLPVLR**	X		X
	QGQVLTIPQNFAVAK***	X		X
	ADIYTEQVGR	X		X
	AESEGFEWVAFK	X		
	LNALEPTNR**	X		
	WLQLSAER	X		

Protein	Peptide Sequence	Validated by discovery analysis of incurred chocolate bar	Validated by discovery analysis of incurred broth powder	Candidate marker used for method development (Task 1.4.1)
	QETTLVR	X		
Cor a 11 48kDa glycoprotein	ELAFNLPSR	X		X
	LLSGIENFR	X		X
	AFSWEVLEAALK	X		X
	VQVLENFTK	X		X
Pru du 6 Prunin	TEENAFINTLAGR	X	X	X
	TDENGFTNTLAGR			X
	ALPDEVLANAYQISR**	X	X	
	QETIALSSSQQR***	X		
	FYLAGNPENEFNQQGQSQPR	X	X	
	ISTLNSHNLPILR	X	X	
	NQIIQVR	X		
	GNLDFVQPPR	X	X	X
	ADIFSPR	X	X	X
	ENIGNPER	X		
	QQGQQEQQQER	X		
	ALPDEVLQNAFR	X		X

*Peptide identified also as core in longer sequences with missed cleavages.

**Peptide identified also with asparagine/glutamine deamidation.

***Peptide identified also with N-terminal pyroglutamate.

3.1.4 Development of extraction, purification and digestion conditions for MS methods

3.1.4.1 Optimization of the sample preparation workflow for LC-MS/MS analysis

Capitalising on approaches developed in the iFAAM (Nitride et al., 2018; Nitride et al., 2019), Allermass (Planque et al., 2016, 2017a; Planque et al., 2017b; Planque et al., 2019), Safe&Smart (Pilolli et al., 2017) and Allersens (Gavage et al., 2020; Gavage et al., 2022) projects a single procedure applicable to extraction of six defined allergens (egg, milk, soya, peanut, hazelnut and almond) was developed using the candidate peptide marker list identified using discovery MS. The method was optimised using MRM on a triple quadrupole mass spectrometer with regards optimization of

(1) Extraction and purification for the analysis of six allergenic food ingredients in the food matrices selected in the present project (CER); and

(2) Optimisation of digestion yield and single laboratory validation of the developed method to assure the adherence to the acceptance criteria the method is expected to match (CNR-ISPA).

During this sample preparation workflow optimisation, fifty target peptides were monitored in MRM mode and validated in both laboratories to trace the seven allergenic ingredients in the incurred chocolate. Based on this optimization, the following steps and parameters for the sample preparation were identified:

I. **Sample preparation and homogenization**. Several conditions were tested on chocolate bars, including grinding, melting, and melting and defatting procedures. The best results were obtained grinding using a combination of fast and short pulses together with cooling (-20°C) cycles. This procedure ensures the preparation and homogenization of the sample while being relatively simple, environmental/user friendly, and easily applied to other matrices such as broth powder.

II. **Protein extraction.** Proteins were extracted in 200 mM Tris-HCl buffer pH 9.2 containing with various concentrations of urea (2 M and 5 M), a chaotropic agent causing the denaturation and unfolding of the proteins and facilitating therefore their extraction. It was shown that 5 M urea improved allergen extraction from incurred processed food commodities (*e.g.*, processed (roasted) peanut).

III. **Protein extract purification.** The protein extract was subsequently filtered on an Acrodisc® syringe filter with a 5 µm Versapor® membrane, pre-purified on a size exclusion chromatography (SEC) disposable cartridge (5 kDa molecular weight cut-off; PD-10 desalting columns pre-packed with Sephadex G-25 M resin) and recovered in ammonium bicarbonate buffer by centrifugation. This additional step has been extensively investigated (with or without SEC pre-purification, using either spin or gravity elution) and shown to significantly affected the detection of the target peptides. Regarding the need to reduce the urea concentration ≤1M by dilution (mandatory for the tryptic digestion step), SEC pre-purification gave samples with a higher protein concentration while allowing the elimination of the urea, both resulting in an improvement of the peptide detection.

IV. **Protein extract digestion.** The extracted proteins were then denatured by heating (95°C, 15 min), reduced with dithiothreitol (DTT; 60°C, 30 min), and alkylated with iodoacetamide (IAA; 37°C, 30 min, in the dark). The resulting proteins were then digested with trypsin. Several trypsin-to-protein ratios (1/50, 1/100, and 1/200 (w/w)) and duration of digestion (1 h, 4 h, 16 h, and 24 h) were investigated in either the presence or absence of surfactant which does not modify protease activity (such as RapiGest SF). It was found that a trypsin-to-protein ratio fixed at 1/100 and digestion carried out for 16 h in the absence of surfactant, was the best set of conditions to ensure that the molar amount of all the peptide markers produced during the digestion step are representative of the moles of protein present in the original extract.

V. **Tryptic peptide extract purification and concentration.** Tryptic digestion was stopped by the addition of HCl, the digested extract centrifuged and the tryptic peptides purified on a Strata-X SPE cartridge. Sep-Pak C18 SPE was also tested and gave quite similar results. The purified peptides were eluted with acetonitrile:

methanol (1:1, v/v) containing 2% (v/v) formic acid. The solvent was then evaporated, at 40°C and under a nitrogen flow, in presence or in absence of dimethylsulphoxide (DMSO), in order to concentrate the sample and increase the sensitivity. The absence of DMSO was selected since it was shown to give slightly better results for a limited number of peptides. Finally, the sample was reconstituted in acetonitrile /0.1% (v/v) formic acid in water (5/95 (v/v); 100 µL) and centrifuged, before being analysed by ultra high performance liquid chromatography (UHPLC)-MS/MS.

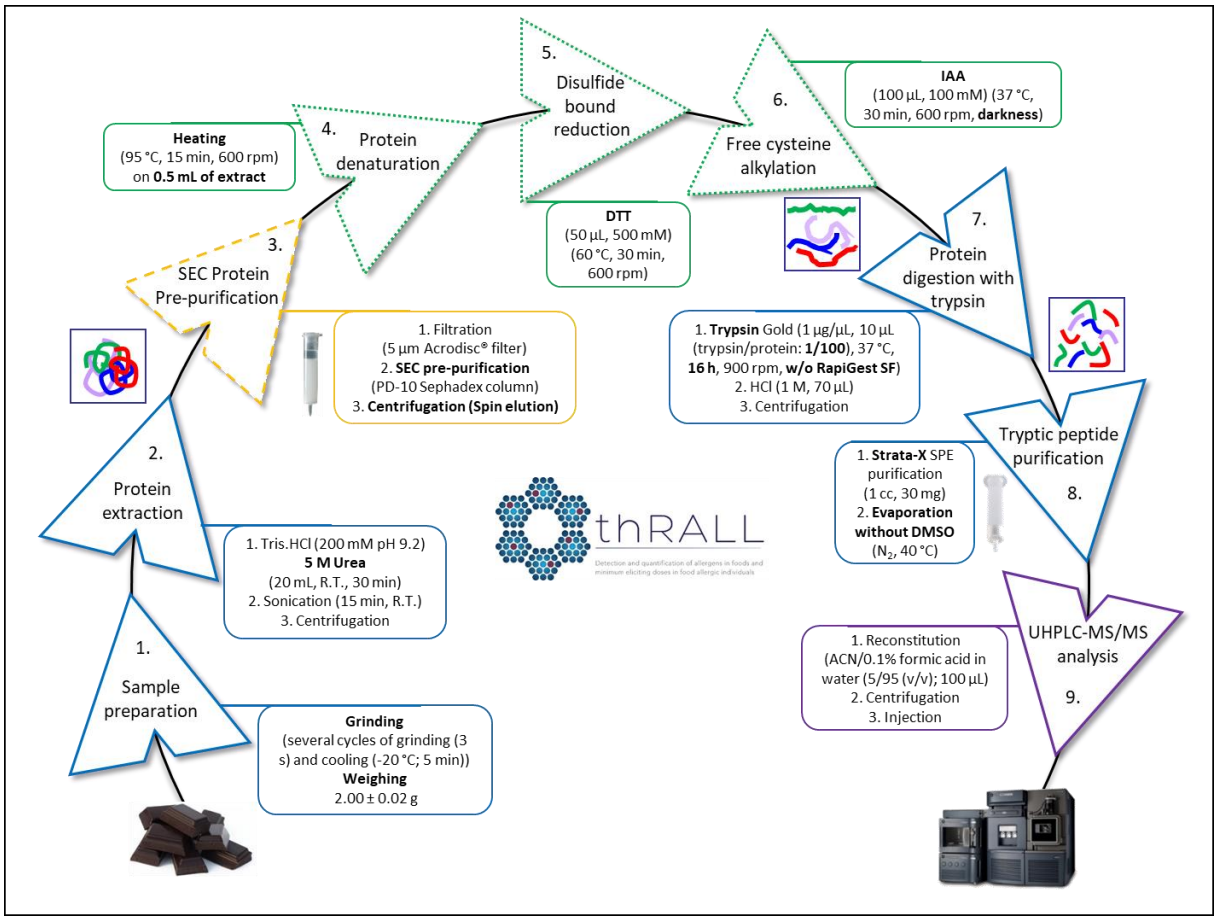

Figure 2: Optimized harmonized reference protocol for use in sample preparation for multi-allergen detection by UHPLC-MS/MS analysis. From (Henrottin et al., 2023). Reprinted from Food Control, Vol. 143, Henrottin et al. "Optimization of a sample preparation workflow based on UHPLC-MS/MS method for multi-allergen detection in chocolate: An outcome of the ThRAll project", Article number 109256, Copyright 2023, with permission from Elsevier.

3.1.4.2 In-house validation of the method

The method sensitivity was assessed for all six allergenic ingredients and all but two of the markers were detected across the concentration range of the matrix matched calibration curves (0.5-50 fmol/µL). The exceptions were two marker peptides for ovalbumin, ISQAVHAAHAEINEAGR and GGLEPINFQTAADQAR, which were only detected from a starting concentration of 2 fmol/µL and 1.5 fmol/µL respectively. All the matrix matched calibration

curves presented a good linearity of the response, with a R^2>0.99 for each quantitative transition. The ratio between transitions was stable and the detected retention time was reproducible for all the markers (relative standard deviation below 5%). LOD and LOQ values were calculated with different approaches as previously described, and differences between them have been discussed among the consortium partners.

Discovery proteomic analyses was also performed on the six allergenic ingredients to calculate experimental conversion factors for proper reporting units. Raw data were processed by MaxQuant software for protein relative quantification and the resulting percentage have been applied to covert peptide concentrations from fmol/µL to ppm of total protein of the allergenic ingredient per g of matrix. These "experimentally" determined conversion factors have been compared with previous results from the literature, whenever available (Parker et al., 2015; Boo et al., 2018; Sayers et al., 2018; Martinez-Esteso et al., 2020; Monaci et al., 2020; Nelis et al., 2022).

The allergenic ingredients were detected (at least two transitions quantified for at least two peptides) in the incurred chocolate samples (0, 2, 4, 10, 40 mg allergenic ingredient protein/kg chocolate), although method sensitivity varied, depending on the ingredient. Quantitative analysis of the incurred samples was carried out for all samples whose concentration resulted above the LOQ values. The detection repeatability and the intermediate precision resulted to be always below 20% for the two best reporting transitions of each marker.

3.1.5 Inter-laboratory comparison of the prototype MS method

Four laboratories participated in the ring-trial and returned test results for all six allergens incurred into the chocolate bar samples at different levels. All laboratories returned the light/heavy peptide peak area ratios for all monitored transitions described in the method in the data return sheets (Henrottin et al., 2023) and data reliability confirmed by assessing the stability of transitions in the data sets. Data were then analysed to determine the regression line over seven point calibration curve running from 0-50 fmol peptide/µL and the following determined for each laboratory:

i) the equation of the regression line y=a+bx, where b= slope and a= intercept;

ii) R^2 (the coefficient of determination);

iii) the limits of detection (LOD) and quantification (LOQ) calculated over 5 data points in the lower range of calibration using the following equations:

$$LOD_{LCR} = (3.3 * S_a)/b$$

$$LOQ_{LCR} = (10 * S_a)/b$$

S_a= standard error over the intercept and b= slope

Only the light/heavy peptide peak area ratios determining values above the LOQ were used for the quantification of the chocolate bar test samples. The LOQ in matrix ranged in between

0.2 and 5 fmol/µL, depending on the peptide marker analysed. Theoretical (Martinez-Esteso et al., 2020) and experimental (Parker et al., 2015) conversion factors were applied to convert from fmol of peptide/µL to mg of total allergenic protein per kg of chocolate bar, experimental conversion factors being calculated from data collected in Task 1.4.2 for the specific allergenic ingredients under investigation.

This analysis is summarised in Table 5 and shows that Laboratory 1 failed to quantify five of the peptide markers (milk casein - FFVAPFPEVFGK; egg yolk vitellogenin - ATAVSLLEWQR; peanut Ara h 3 – TANDLNLLILR; soybean glycinin - VLIVPQNFVVAAR; almond 11S globulin - ALPDDVLANAFQISR) whilst Laboratory 2 failed to quantify the reporter peptides for milk. A review of data from Laboratory 2 indicated errors in the internal standard were the cause of these problems.

Table 5. Summary table of the limits of quantification and determination for the different allergenic protein peptide reporters.

Protein	Peptide reporter	Lab no	Incurred allergenic ingredient mg protein/kg chocolate			
			2	4	10	40
COW'S MILK						
Bos d 9 αS1-casein	FFVAPFPEVFGK	1	ND	ND	ND	ND
		2	> LOD	>LOQ	>LOQ	>LOQ
		3	ND	ND	ND	ND
		4	> LOD	>LOQ	>LOQ	>LOQ
Bos d 10 αS2-casein	NAVPITPTLNR	1	< LOD	< LOD	< LOD	>LOQ
		2	< LOD	> LOD	> LOD*	>LOQ
		3	ND	ND	ND	ND
		4	< LOD	< LOD	> LOD*	>LOQ
Bos d 5 - β-Lactoglobulin	IDALNENK	1	> LOD	> LOD	>LOQ	>LOQ
		2	< LOD	< LOD	> LOD*	>LOQ
		3	ND	ND	ND	ND
		4	> LOD	> LOD	> LOD	> LOD
	VLVLDTDYK	1	< LOD	< LOD	< LOD	>LOQ
		2	< LOD	< LOD	< LOD	> LOD*
		3	ND	ND	ND	ND
		4	< LOD	< LOD	< LOD	< LOD
HEN'S EGG						
Egg yolk Vitellogenin-1	ATAVSLLEWQR	1	ND	ND	ND	ND
		2	< LOD	< LOD	< LOD	> LOD
		3	< LOD	< LOD	< LOD	< LOD
		4	< LOD	< LOD	< LOD	> LOD
Egg yolk Vitellogenin-2	NIGELGVEK	1	< LOD	< LOD	< LOD	< LOD
		2	< LOD	< LOD	< LOD	< LOD
		3	< LOD	< LOD	< LOD	< LOD

Protein	Peptide reporter	Lab no	Incurred allergenic ingredient mg protein/kg chocolate			
			2	4	10	40
Egg white Gal d 2 - Ovalbumin	ISQAVHAAHAEINEAGR	4	< LOD	< LOD	< LOD	< LOD
		1	< LOD	< LOD	> LOD	>LOQ
		2	> LOD*	>LOQ*	>LOQ	>LOQ
		3	< LOD	< LOD	> LOD	>LOQ
		4	< LOD	< LOD	< LOD	< LOD
Egg white Gal d 2 - Ovalbumin	GGLEPINFQTAADQAR	1	< LOD	< LOD	< LOD	>LOQ
		2	< LOD	>LOQ*	>LOQ*	>LOQ
		3	< LOD	< LOD	< LOD	>LOQ
		4	< LOD	< LOD	< LOD	>LOQ
PEANUT						
Ara h 3, 11S Seed Storage Globulin	*SPDIYNPQAGSLK*	1	< LOD	< LOD	> LOD*	>LOQ
		2	< LOD	< LOD	> LOD*	>LOQ
		3	< LOD	< LOD	> LOD	>LOQ
		4	< LOD	< LOD	< LOD	>LOQ*
	TANDLNLLILR	1	< LOD	< LOD	< LOD	>LOQ
		2	< LOD	< LOD	> LOD*	>LOQ
		3	< LOD	> LOD	> LOD	>LOQ
		4	< LOD	< LOD	< LOD	>LOQ
SOYBEAN						
Gly m 6 – Glycinin, 11S Seed Storage Globulin	*VFDGELQEGR*	1	> LOD	> LOD	>LOQ	>LOQ
		2	< LOD	< LOD	> LOD	>LOQ
		3	< LOD	< LOD	> LOD	>LOQ
		4	< LOD	< LOD	< LOD	>LOQ
	VLIVPQNFVVAAR	1	ND	ND	ND	ND
		2	>LOQ*	>LOQ*	>LOQ	>LOQ
		3	> LOD	> LOD	>LOQ	>LOQ
		4	< LOD	< LOD	< LOD	>LOQ
ALMOND						
Pru du 6 Prunin, 11S Seed Storage Globulin	*ADIFSPR*	1	>LOQ	>LOQ	>LOQ	>LOQ
		2	> LOD*	>LOQ*	>LOQ	>LOQ
		3	>LOQ	>LOQ	>LOQ	>LOQ
		4	< LOD	> LOD	>LOQ	>LOQ
	TEENAFINTLAGR	1	>LOQ	>LOQ	>LOQ	>LOQ
		2	> LOD	>LOQ*	>LOQ	>LOQ
		3	> LOD	>LOQ	>LOQ	>LOQ
		4	< LOD	>LOQ	>LOQ	>LOQ
HAZELNUT						
	ADIYTEQVGR	1	> LOD	> LOD	>LOQ	>LOQ
		2	< LOD	> LOD*	>LOQ*	>LOQ

Protein	Peptide reporter	Lab no	Incurred allergenic ingredient mg protein/kg chocolate			
			2	4	10	40
Cor a 9 - 11S Seed Storage Globulin	*ALPDDVLANAFQISR*	3	> LOD	>LOQ	>LOQ	>LOQ
		4	< LOD	< LOD	> LOD	>LOQ
		1	ND	ND	ND	ND
		2	> LOD*	>LOQ*	>LOQ	>LOQ
		3	> LOD	>LOQ	>LOQ	>LOQ
		4	> LOD	> LOD	>LOQ	>LOQ

>LOQ, green; > LOQ, yellow; <LOD, red; ND – not determined.

* indicates that a single biological sample reported passed the acceptance criteria**.**

The sensitivity of the method varied between the different laboratories was as follows:

Milk: Of the milk reporter peptides FFVAPFPEVFGK being the best performing reporter for quantification of milk whilst for the whey protein, β- lactoglobulin, one (IDALNENK) was only quantified by two laboratories, and the second (VLVLDTDYK) by only a single laboratory.

Egg: One egg yolk peptide marker could be detected by two laboratories incurred. Both of the peptide markers reporters for the ovalbumin, ISQAVHAAHAEINEAGR and GGLEPINFQTAADQAR, could be quantified but neither of the yolk protein peptide markers, ATAVSLLEWQR and NIGELGVEK, could quantified by any laboratory.

Legumes: All of the peanut and soybean reporter peptides could be quantified at the highest level of 40 mg of total protein per kg of chocolate whilst the soybean reporters could also be quantified at 10 ppm by two laboratories.

Tree nuts: Hazelnut peptides could quantify hazelnut protein at the 40 and 10 mg of total protein per kg of chocolate. Reporter peptides for almond were among the best performing makers and could be quantified by all laboratories down to a level of 4 mg of total protein per kg of chocolate and one laboratory could quantify the protein at a level of 2 mg of total protein per kg of chocolate.

In most cases, the allergenic ingredient concentration determined in the incurred chocolate samples was comparable across laboratories, and consistent with the data collected during the in-house validation (Task 1.4.2). This demonstrates the feasibility of the prototype reference method, although further refinements in sensitivity will be required for determination of whey proteins and peanut.

3.2 OBJECTIVE 2

3.2.1 Development of harmonised protocols for collection of threshold data in food allergic individuals

For the clinical evaluation and diagnosis of food allergy well designed food allergy testing is essential. Oral food challenges (OFC) are well recognised as the most suitable method to

confirm suspected allergic reactions to food in observational and intervention studies, the gold standard being double-blind placebo controlled food challenges (DBPCFC).

The DBPCFC consists of one placebo day and one active day where the patient is given 7-9 escalating doses of a challenge food in 20-30 minute intervals. The challenge physician records signs and symptoms in relation to the last consumed dose, specifying the time and onset of skin, respiratory, gastrointestinal, neurological or cardiovascular symptoms. The challenge is usually stopped upon predetermined criteria.

However, the documentation used across different settings, particularly in research, are not adequately standardised to allow valid comparisons between studies. Therefore a standardised framework for coding and harmonising data on diagnosis of food allergies in population-based and clinical research were be developed. This was achieved by mapping CRFs from the iFAAM and TRACE studies. Both CRFs are generic forms which capture details about the severity and timing of symptoms observed during an OFC, such as attributing a grade for the signs and symptoms of food allergic reactions.

iFAAM: The iFAAM CRF uses a simple ordinal severity scoring system to support scoring of symptoms in a DBPCFC (Fernández-Rivas et al., 2022). This scoring system breaks down the symptoms into mild, moderate, and severe which are in turn aligned with an ordinal score. A DBPCFC is discontinued if a patient develops ≥1 objective clinical manifestation of grading that is sufficient to stop challenge. For all subjective symptoms a patient will be asked to mark the severity of that symptom on a scale from 0 to 10 on Visual Analogue Scale (VAS) sheet after each dose level if relevant.

TRACE: The TRACE CRF also breaks down the symptoms into mild, moderate, and severe in a similar way (Dua et al., 2019). TRACE derived the scoring system for scoring and stopping DBPCFCs from the Practall guidelines, which is widely accepted from US and European allergists. The scoring system is designed to indicate symptoms and signs that may warrant caution (repeating a dose, delaying a dose, consideration for stopping) or are clear enough to warrant stopping a challenge and declaring the result positive. As with the iFAAM guidelines, challenges are stopped and considered positive when objective symptoms occur. However, some mild objective symptoms may be considered insufficient to stop a challenge (e.g., one or two transient urticarial lesions, perioral hives from contact with the food or one episode of vomiting in a patient with anxiety and a distaste for the challenge substance).

In some cases, the TRACE protocol will allow for a challenge to be stopped in response to subjective symptoms, such as, by having repetitive symptoms or multiple subjective symptoms in several organ systems. However, it should be noted that this may increase the risk of a false positive test result. Investigators report the reasons for stopping a challenge in detail and how the symptoms were assessed to determine a positive, negative, or inconclusive result.

Both the iFAAM and TRACE studies applied grading systems for symptoms and severity which were also mapped, breaking down the symptoms recorded in each of the following categories: skin, respiratory, gastrointestinal, cardiovascular or neurological symptoms. Mild, moderate, and severe symptoms were colour coded into yellow, orange, and red accordingly. Red

symptoms are indicative of a symptom severity score high enough to warrant stopping an OFC (as seen in the figure below).

A number of differences were noted between the two study CRFs which are summarised below.

Respiratory symptoms

- Extra symptoms are noted in iFAAM, "itching in ear canal" and "throat tingling/ altered sensation in throat" both are scored green in severity.
- In iFAAM "persistent rhinorrhea" is graded yellow in severity and in TRACE "long bursts, persistent rhinorrhea OR continuous rubbing" is graded red.
- Additionally, iFAAM breaks down the respiratory symptoms into two categories, upper and lower respiratory symptoms. The lower respiratory symptoms noted in iFAAM (chest tightness) are not mentioned in the TRACE CRF, bar wheezing.

Gastrointestinal symptoms

- In the iFAAM CRF "oral itching" is green and in the TRACE CRF "itchy mouth" is yellow.
- In the iFAAM CRF abdominal pain is broken down into both a yellow and green grading, "persistent abdominal pain" (green) and "transient abdominal pain" (yellow). TRACE only categorises "complaints of nausea OR abdominal pain" as yellow.
- TRACE mentions levels of activities related to nausea and abdominal pain (as this was a co-factor studied in the TRACE study.

Cardiovascular symptoms:

- iFAAM uses slightly different terminology as follows: "weak/ dizzy or tachycardia" whereas TRACE only mentions tachycardia.
- Also, slight differences in the following, Red classified symptoms: iFAAM- "drop in BP and/ or 20% from baseline" whereas TRACE only states ">20% drop in BP". Also, "Cardiovascular collapse/ signs of impaired circulation" in IFAAM compared to "cardiovascular collapse" in TRACE.

Neurological symptoms:

- IFAAM only has one category "altered level of consciousness" (red). TRACE has 3 as follows: "subjective response (e.g., weak, dizzy)" (yellow) and "significant change in mental status", and "loss of consciousness" (both red).

Overall, slight differences in the terminology and symptom classification were used between the two studies. Also, neither of the CRFs have a clear space for "other" symptoms.

Such forms allow for the interpretation of DBPCFC, therefore, common terminology for communicating outcomes is required for consistency between studies and reduce the influence of subjective judgment of supervising physicians. At the Berlin ThRAll meeting an

additional symptom/organ system was identified that should be included in future food challenge eCRFs were uterine cramps/female reproductive system. The workshop also identified the need to include scales to characterise the severity of rhinitis experienced during a challenge would be useful.

Based on this mapping an assessment was made of different systems for coding the food allergy symptom data and the allergenic foods. This identified two coding systems:

- Clinical terminology: SNOMED CT was identified for coding patient data. It is a structured clinical vocabulary which can be used in coding data in eCRFs. It is considered to be the most comprehensive approach to providing precise and controlled terminology in the world (https://www.snomed.org/).
- Food terminology: Using FoodEx2 developed by EFSA

The approach developed in ThRAll has been taken forward in the UK Food Standards Agency Prevalence of Adult Food Allergy (PAFA) study (FS101174) and is being further developed and extended to include non-IgE mediated adverse reactions to foods. The coding system developed for the ThRAll project can be found at https://figshare.manchester.ac.uk/ with the DOI 10.48420/21688199.

3.2.2 Population and curation of database with historic and published data

Using the consensus approach for coding food allergy data a database was constructed in REDCap and used for collation of oral food challenge data. The final ThRAll database comprised 557 records representing challenges to thirteen different foods undertaken in six studies spanning fourteen different countries (Figure 3) and can be found at https://figshare.manchester.ac.uk/ with the DOI 10.48420/21688199.

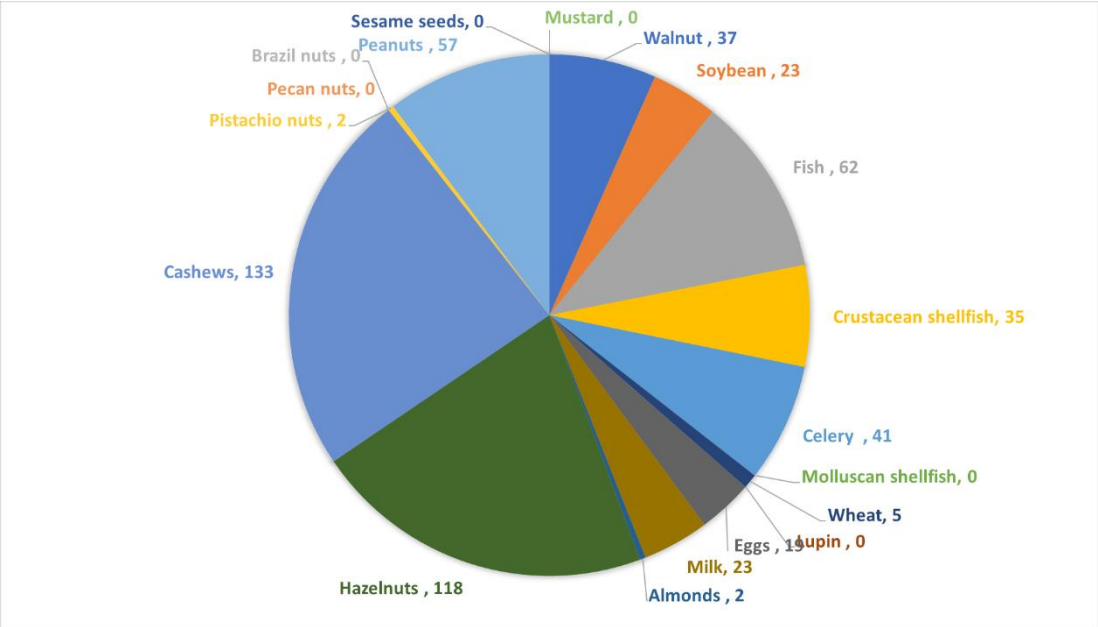

Figure 3: Summary of thrall database entries by food. The numbers are the number of patient records included. The data collated was also available in the public domain through peer reviewed publications (Ballmer-Weber et al., 2015; van der Valk et al., 2016)

An approach to harmonisation of data was undertaken using fish as an example, collating data from the EuroPrevall study [7] with that published by Sørensen and co-workers (Sørensen et al., 2017). Using data from reactive challenges allowed the NOAEL and LOAELs to be identified for 30 participants from the EroPrevall study and 19 from the Sørensen study to give a total of 62 (Table 6). These data illustrate the benefits of having challenge sets comprising greater than 60 study subjects in narrowing confidence intervals described by others (Klein Entink et al., 2014a). A comparison of the ED values calculated for fish based on the combined data set are similar to the cumultative ED01 calculated using the model averaging approach which identified ED05 and ED10 values of 1.3, 15.6 and 45.6 mg of protein (Houben et al., 2020) compared to 1.1, 7.3 and 20.2 mg of protein identified using a log-normal dose distribution model. It also shows the differences in the ED values calculated using the different dose distribution modelling approaches which have been observed previously [7] although single dose challenges for peanut and milk have largely validated the ED05 values identified using a log-normal distribution (Hourihane et al., 2017; Turner et al., 2021). A mixed model approach has been developed which employs a Bayesian model averaging technique (Wheeler et al., 2021); its use was explored in the scope of the ThRAll project but although the modelling code is in the public domain it has been configured for a particular data set. The revision required to make it suitable for the ThRAll data sets was beyond the scope of this project.

Table 6: Summary of cumulative elicting doses (ED) for a population calculated for fish using interval censoring survival analysis and three different types of non-paramatric curve fitting analysis. Modelling is based on objective symptome lowest observed effect levels identified from oral food challenge data from EuroPrevall (Ballmer-Weber et al., 2015) and the Sorensen study (Sorensen et al., 2017) individually and as a combined data set. ED values are expressed as my of protein.

Eliciting dose (ED)	Study	Dose distribution type								
		Log normal			Logistic			Weibull		
		ED	lower CI	upper CI	ED	lower CI	upper CI	ED	lower CI	upper CI
ED01	EuroPrevall	0.9	0.1	10.6	0.4	0.01	8.6	0.08	0.002	4.4
	Sorensen	2.7	0.5	15.7	1.4	0.2	12.5	0.5	0.03	9.1
	Combined data set	1.1	0.2	5.3	0.4	0.06	3.2	0.06	0.004	0.9
ED05										
	EuroPrevall	7.9	1.2	51.6	7.3	0.8	64.6	3.9	0.3	55.7
	Sorensen	10.9	2.9	41.3	9.7	2.1	44.5	6.5	0.9	44.7
	Combined data set	7.3	2.2	24.4	6.2	1.5	24.7	2.5	0.4	15.3
ED10										
	EuroPrevall	25.3	5.1	125.1	28.9	4.9	168.9	21.9	2.723	176.6
	Sorensen	22.6	7.2	70.9	23.4	6.7	81.5	20.0	4.332	92.0
	Combined data set	20.2	7.2	56.4	20.6	6.6	64.1	13.0	3.1	54.4

Data gaps were identified for certain foods on Annex II of FIR including certain tree nuts (such as Brazil nut, Macadamia), molluscan shellfish and lupin having no threshold data available. There were many foods for which few data were identified, and which were below the 60 data points identified as being required for best practice modelling (Klein Entink et al., 2014b). Many of the foods for which threshold data are lacking also represent less prevalent food allergies which makes it more difficult for clinical studies to identify many patients to include in any threshold study. For example, in the EuroPrevall surveys in school-age children and adults the prevalence of allergy to soybean was very low in adults approximating to zero in many countries and estimated to be 0.08% [0.07-0.93 95%CI] only in Switzerland but was a little higher in school-age children at 0.31 [0.01-1.1495%CI] in Poland. Sesame being similar with a prevalence of 0.03% [0.07-0.93 95%CI] in Switzerland but approximated to zero in other centres and in school-age children as did mustard allergy (Lyons et al., 2019; Lyons et al., 2020). For other foods, notably walnut and pecan and cashew and pistachio allergy, the concordance of food allergies is very high (Elizur et al., 2018; Brough et al., 2020) and hence threshold doses for the key tree nuts – walnut and cashew – are likely more relevant, as indicated by the FAO-WHO expert consultation (FAO-WHO, 2022b, a).

Collating data from the literature can be difficult and especially when arising from older studies prior to publication of the PRACTALL guidance (Sampson et al., 2012). This is illustrated for mustard below where three publications could be identified, one with fourteen patients from Spain (Figueroa et al., 2005), and 19 patients from France from two different publications (Rancé et al., 2001; Morisset et al., 2003). For the Spanish group only four of the 14 subjects having positive DBPCFC challenges reacted with objective symptoms (Figueroa et al., 2005). For one of the French studies (Morisset et al., 2003) three of the seven positive challenges were single blind challenges and one was considered positive based on "minor eczema exacerbation" which occurred 8h after challenge; this would not be a qualifying symptom using the approaches adopted in EuroPrevall and iFAAM since it is a delayed reaction (Ballmer-Weber et al., 2015; Grabenhenrich et al., 2017). In the second study fifteen out of 36 subjects with a positive DBPCFC reacted with objective symptoms but no data is provided on eliciting dose beyond a general observation that it varied from 1-936mg (Rancé et al., 2000). These data have been used for dose distribution modelling employing model averaged interval-censored survival analysis but gave wide confidence intervals as might be expected from a small data set (Houben et al., 2020; Remington et al., 2020).

4 Conclusions

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

Building on the outputs of previous EU and nationally funded projects (iFAAM, MANOE, Safe&Smart, Allersens, MoniQA) two incurred matrices were prepared for the project based on a chocolate bar and a powdered soup. These were used to develop a harmonised quantitative MS-based prototype reference method for the detection of six foods allergens

(cow's milk, hen's egg, peanut, soya, hazelnut and almond). These included reference materials developed through the UK FSA call FS101206 Development of Quality Control Materials for Food Allergen Analysis). Analysis of these materials, coupled with a systematic review of the literature allowed a suite of peptide markers to be identified for the six allergenic foods that met quality criteria for use in a prototype reference method.

An assessment of ddPCR-based methods showed they were not suitable for use with such complex incurred matrices whilst analysis using commercial ELISA test kits showed the broth powder to be very highly processed with many allergens poorly detected. Consequently, further MS based methods development and validation was undertaken using allergens incurred into the chocolate bar matrix. Around fifty peptide markers were taken forward into a test method optimisation using multiple reaction monitoring experiments executed on a triple quadrupole MS platform. From this exercise key method parameters were defined, and a subset of the peptide markers identified for method validation. Stable isotope-labelled forms of the peptides were synthesised for use as external calibrants. 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. It is capable of providing accurate quantification of the six allergenic food ingredients, in a hard-to analyse chocolate matrix. The test method proved to be transferable between laboratories and has the sensitivity required to quantify the allergens from egg, milk, almond and hazelnut and can perform in line with the test method performance requirements identified for these allergenic foods by the recent FAO-WHO expert consultation (FAO-WHO, 2022a). Further refinement to improve the sensitivity by ~3-fold will be required to enable the method to be fully deployed for analysis of whey proteins and peanut in line with the FAO-WHO expert consultation recommendations for test method performance. There is also a need to confirm the allergenic activity of highly processed food matrices, such as the broth powder, especially given data that food processing procedures, such as boiling, appears to reduce the allergenicity of foods such as peanuts (Turner et al., 2014).

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

A harmonised approach for coding of food allergy data was developed that will allow the collation of good quality data on MED's from low-dose oral food challenges undertaken in food allergic patients. This was implemented in a REDCap electronic record to which data were uploaded or entered directly from the literature, the EU-funded project EuroPrevall, and nationally-funded projects in the Netherlands. Data gaps identified included the lack of challenge data for foods such as Brazil nut, macadamia nut, molluscan shellfish and lupin. Many foods for which few data were identified which were below the 60 data points identified as being required for best practice modelling (Klein Entink et al., 2014b). Many of the foods for which threshold data are lacking also represent less prevalent food allergies which makes it more difficult for clinical studies to identify many patients to include in any threshold study. There is also a need to collate data on the impact of processing on eliciting doses given the extensive changes to the allergenic ingredients incurred in the broth powder, especially given

data indicating that processes, such as boiling, reduce the allergenic activity of foods such as peanut (Beyer et al., 2001; Mondoulet et al., 2005; Turner et al., 2014).

Further data collation and curation is required and the REDCap eCRF provides a framework for development of such as data repository, akin to platforms developed for curation of nutritional data (such as EuroFIR). Working with the clinical community (e.g. Ga^{2}len-Anacare, EAACI) an approach should be explored whereby authors of publications reporting oral food challenge data should be encouraged to deposit them in such a repository, in the same way that databases, such as PRIDE, have been developed for sharing of proteomic data sets.

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Abbreviations

CRF	Clinical record forms
DNA	Deoxyribose nucleic acid
ddPCR	Digital droplet polymerase chain reaction
DBPCFC	Double blind placebo controlled food challenges
DMSO	Dimethylsulphoxide
DTT	Dithiothreitol
eCRF	Clinical record forms
ELISA	Enzyme-linked immunosorbent assay
EuroPrevall	Patterns and prevalence of food allergies across Europe (EuroPrevall) GA514000
FAO	Food and Agriculture Organisation
iFAAM	Integrated approaches to food allergy and allergen management GA 312147
PAFA	Prevalence of Adult Food Allergy Study
LC-MS	Liquid chromatography -Mass spectrometry
LOD	Limit of detection
LOAEL	lowest observed adverse effect level
LOQ	Limit of quantification
MED	Minimum Eliciting Doses
MOED	Minimum Observed Eliciting Doses
MS	Mass spectrometry
NOAEL	and no observed adverse effect level
PCR	Polymerase chain reaction
REDCap	Research Electronic Data Capture
SD	Standard deviation
SEC	Size exclusion chromatography
SOP	Standard operation procedure
SPE	Solid phase extraction

ThRAll	Detection and Quantification of Allergens in Foods and Minimum Eliciting Doses in Food-Allergic Individuals EFSA project GP/EFSA/AFSCO/2017/03
UHPLC	Ultra high performance liquid chromatography
WHO	World health Organisation

Appendix A – Project Scientific Output

Appendix A.1 Detection and Quantification of Allergens in Foods and Minimum Eliciting Doses in Food-Allergic Individuals (ThRAll).

Publication type: Special report on "Mass Spectrometry: Status Quo in Food Allergen and Food Authenticity Applications" by Bert Popping and Carmen Diaz-Amigo.

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doi: 10.5740/jaoacint.19-0063. Open Access

Abstract

Risk-based approaches to managing allergens in foods are being developed by the food industry and regulatory authorities to support food-allergic consumers to avoid ingestion of their problem food, especially in relation to the traces of unintended allergens. The application of such approaches requires access to good quality data from clinical studies to support identification of levels of allergens in foods that are generally safe for most food-allergic consumers as well as analytical tools that are able to quantify allergenic food protein. The ThRAll project aims to support the application of risk-based approaches to food-allergen management in two ways. First, a harmonized quantitative MS-based prototype reference method will be developed for the detection of multiple food allergens in standardized incurred food matrices. This will be undertaken for cow's milk, hen's egg, peanut, soybean, hazelnut, and almond incurred into two highly processed food matrices, chocolate and broth powder. This activity is complemented by a second objective to support the development and curation of data on oral food challenges, which are used to define thresholds and minimum eliciting doses. This will be achieved through the development of common protocols for collection and curation of data that will be applied to allergenic foods for which there are currently data gaps.

Appendix A.2 - Development of incurred chocolate bars and broth powder with six fully characterised food allergens as test materials for food allergen analysis.

Publication type: Paper in Forefront

Authors: Huet, A. C., Paulus, M., Henrottin, J., Brossard, C., Tranquet, O., Bernard, H., Pilolli, R., Nitride, C., Larré, C., Adel-Patient, K., Monaci, L., Mills, E. N. C., De Loose, M., Gillard, N. & Van Poucke, C.

References: Anal Bioanal Chem, 2022, 414, 2553-2570.

Doi: 10.1007/s00216-022-03912-z

Abstract

The design and production of incurred test materials are critical for the development and validation of methods for food allergen analysis. This is because production and processing conditions, together with the food matrix, can modify allergens affecting their structure, extractability and detectability. For the ThRAll project, which aims to develop a mass spectrometry-based reference method for the simultaneous accurate quantification of six allergenic ingredients in two hard to analyse matrices. Two highly processed matrices, chocolate bars and broth powder, were selected to incur with six allergenic ingredients (egg, milk, peanut, soy, hazelnut and almond) at 2, 4, 10 and 40 mg total allergenic protein/kg food matrix using a pilot-scale food manufacturing plant. The allergenic activity of the ingredients incurred was verified using

food-allergic patient serum/plasma IgE, the homogeneity of the incurred matrices verified and their stability at 4 °C assessed over at least 30-month storage using appropriate enzyme-linked immunosorbent assays (ELISA). Allergens were found at all levels from the chocolate bar and were homogenously distributed, apart from peanut and soy which could only be determined above 4 mg total allergenic ingredient protein/kg. The homogeneity assessment was restricted to analysis of soy, milk and peanut for the broth powder but nevertheless demonstrated that the allergens were homogeneously distributed. All the allergens tested were found to be stable in the incurred matrices for at least 30 months demonstrating they are suitable for method development.

Appendix A.3 Critical review on proteotypic peptide marker tracing for six allergenic ingredients in incurred foods by mass spectrometry.

Publication type: Review Paper

Authors: Pilolli R, Nitride C, Gillard N, Huet AC, van Poucke C, de Loose M, Tranquet O., Larr[CO., Adel-Patient K., Bernard H., Mills E.N.C., Monaci L.

References: Food Research International, 2020, 128, 108747.

Doi: 10.1016/j.foodres.2019.108747.

Abstract

Peptide marker identification is one of the most important steps in the development of a mass spectrometry (MS) based method for allergen detection, since the robustness and sensitivity of the overall analytical method will strictly depend on the reliability of the proteotypic peptides tracing for each allergen. The European legislation in place issues the mandatory labelling of fourteen allergenic ingredients whenever used in different food formulations. Among these, six allergenic ingredients, namely milk, egg, peanut, soybean, hazelnut and almond, can be prioritized in light of their higher occurrence in food recalls for undeclared presence with serious risk decision.

In this work, we described the results of a comprehensive evaluation of the current literature on MS-based allergen detection aiming at collecting all available information about proteins and peptide markers validated in independent studies for the six allergenic ingredients of interest. The main features of the targeted proteins were commented reviewing all details available about known isoforms and sequence homology particularly in plant-derived allergens. Several critical aspects affecting peptide markers reliability were discussed and according to this evaluation a final short-list of candidate markers was compiled likely to be standardized and implemented in MS methods for allergen analysis.

Appendix A.4 Discovery based high resolution MS/MS analysis for selection of allergen markers in chocolate and broth powder matrices.

Publication type: Research Paper

Authors: Rosa Pilolli, Christof Van Poucke, Elisabetta De Angelis, Chiara Nitride, Marc de Loose, Nathalie Gillard, Anne-Catherine Huet, Olivier Tranquet, Colette Larré, Karine Adel-Patient, Hervé Bernard, E.N.Clare Mills, Linda Monaci

References: Food chemistry 2021, 343, 128533.

Doi: 10.1016/j.foodchem.2020.128533

Abstract

Peptide marker identification is an important step in development of a mass spectrometry method for multiple allergen detection, since specificity, robustness and sensitivity of the overall analytical method will depend on the reliability of the proteotypic peptides. As part of the development of a multi-analyte

reference method, discovery analysis of two incurred food matrices has been undertaken to select the most reliable peptide markers. Six allergenic ingredients (milk, egg, peanut, soybean, hazelnut, and almond) were incurred into either chocolate or broth powder matrix. Different conditions of protein extraction and purification were tested and the tryptic peptide pools were analysed by untargeted high resolution tandem mass spectrometry and the resulting fragmentation spectra were processed via a commercial software for sequence identification. The analysis performed on incurred foods provides both a prototype effective and straightforward sample preparation protocol and delivers reliable peptides to be included in a standardized selected reaction monitoring method.

Appendix A.5 Optimization of a sample preparation workflow based on UHPLC-MS/MS method for multi-allergen detection in chocolate: An outcome of the ThRAll project.

Publication type: Research Paper

Authors: Henrottin, J., Pilolli, R., Huet, A.-C., Van Poucke, C., Nitride, C., De Loose, M., Tranquet, O., Larré, C., Adel-Patient, K., Bernard, H., Mills, E. N. C., Gillard, N. & Monaci, L.

References: Food Control, 2023, 143, 109256**.**

Doi: 10.1016/j.foodcont.2022.109256

Abstract

Developing reliable methodologies for detecting and quantifying allergens in processed food commodities is crucial to support food business operators in allergen risk assessment and properly implementing precautionary allergen labels whenever required to safeguard the health of allergic consumers. Multiple Mass Spectrometry (MS) methods have been developed so far and applied for single and multi-allergen detection in foods, generating a heterogeneous literature on this topic, with little attention paid to the extraction and the digestion steps, crucial in delivering accurate allergen measurements. This investigation carried out within an international consortium specifically built up to convey a prototype MS based reference method, reports on the first part of the method development, namely the optimization of the sample preparation protocol for six allergens detection (cow's milk, hen's egg, soy, peanut, hazelnut, and almond) in chocolate. The latter was chosen as model complex food matrix, having a high lipid and polyphenol content. Different steps of the sample preparation protocol have been taken into consideration: (i) sampling, (ii) composition of the extraction buffer, (iii) protein purification, (iv) protein enzymatic digestion, (v) peptide purification and pre-concentration, and some experiments were carried out by two independent laboratories and two different MS platforms to provide a first assessment of the robustness of the method under development. Fifty target peptides were monitored in multiple reaction monitoring mode and validated in different laboratories to trace the six allergenic ingredients in the incurred chocolate and the best performing protocol for sample preparation was identified. This work paves the way of the forthcoming full analytical validation of a prototype reference method for MS-based allergen quantification.

Appendix A.6 Abstracts presented in national and international conferences.

- **May 7-9, 2018 10th Workshop on Food Allergens Methodologies, Toronto, Canada** Invited talk Calibrants and reporting units – iFAAM approaches to getting usable test results in allergen analysis. E. N. Clare Mills.
- **7-8 June 2018 Food Fraud Prevention and Effective Food Allergen Management, Vienna, Austria.** Invited talk "Free-from foods – what does it mean for allergens?" E. N. Clare Mills.

- **18 Ottobre 2018, Bruxelles (Belgio), International Symposium Food Allergens: regulation, management and detection, 2nd edition**. Invited Keynote: «The ThRAll-EFSA project: detection and quantification of allergens in food and minimum eliciting doses in food allergic individuals» L. Monaci.
- **7-8th November 2018. China International Food Safety & Quality Conference** Invited Talk Food Allergen Analysis in a Risk Assessment Context EN Clare Mills
- **1-3 April 2019, Amsterdam, The Netherlands; 2nd International Conference, Food Allergy Forum.** Poster Communication: «Production of well characterised incurred chocolate bars for the development of a harmonised quantitative MS reference method" C. Van Poucke, K. Coudijzer, D. De Paepe, O. Tranquet, C. Larré, K. Adel-Patient, H. Bernard, N. Gillard, A.-C. Huet, R. Pilolli, C. Nitride, L. Monaci, M. De Loose and E.N.C. Mills.
- **13th-16th May 2019 3rd Food Allergen Management Symposium, Melbourne, Australia.** Invited Talk. From reference material to reference methods – ways of harmonizing clinically-relevant allergen determination in food E. N. Clare Mills.
- **03-05 September 2019, Manchester, UK; The 40th BMSS annual meeting.** Poster Communication: «DETECTION AND QUANTIFICATION OF ALLERGENS IN FOODS AND MINIMUM ELICITING DOSES IN FOOD-ALLERGIC INDIVIDUALS (ThRAll)». R. Pilolli, L. Monaci, C. van Poucke, M. de Loose, N. Gillard, A.-C. Huet, O. Tranquet, C. Larré, K. Adel-Patient, H. Bernard, C. Nitride, E.N.C. Mills.
- **30 October 2019-01 November 2019, Rockville, MD, USA; 3rd MoniQA Symposium - Food Fraud Prevention and Effective Food Allergen Management.** Poster Communication «DETECTION AND QUANTIFICATION OF ALLERGENS IN FOODS AND MINIMUM ELICITING DOSES IN FOOD-ALLERGIC INDIVIDUALS (ThRAll)» R. Pilolli, L. Monaci, C. van Poucke, M. de Loose, N. Gillard, A.-C. Huet, O. Tranquet, C. Larré, K. Adel-Patient, H. Bernard, C. Nitride, E.N.C. Mills. Book of Abstract published on Quality Assurance and Safety of Crops and Foods, 2019, 11 (Supplement 1) (Print ISSN: 1757-8361, Online ISSN: 1757-837X). https://doi.org/10.3920/qas2019.s1
- **30 October 2019-01 November 2019, Rockville, MD, USA; 3rd MoniQA Symposium - Food Fraud Prevention and Effective Food Allergen Management.** Invited Talk Identifying, curating and harmonising clinical data to identify minimum eliciting doses for food allergens in the ThRAll project. E. N. Clare Mills. Book of Abstract published on Quality Assurance and Safety of Crops and Foods, 2019, 11 (Supplement 1) (Print ISSN: 1757-8361, Online ISSN: 1757-837X). https://doi.org/10.3920/qas2019.s1
- **Division of Infection, Immunity and Respiratory Medicine, School of Biological Sciences, Manchester Institute of Biotechnology, The University of Manchester, UK 5-8 November 2019, Prague, Czech Republic; 9th International Symposium on RECENT ADVANCES IN FOOD ANALYSIS, RAFA2019.** Poster Communication: «IDENTIFICATION OF PROTEOTYPIC PEPTIDES TRACING FOR MULTIPLE ALLERGENIC INGREDIENTS IN INCURRED MATRICES» R. Pilolli, C. van Poucke, M. de Loose, N. Gillard, A.-C. Huet, O. Tranquet, C. Larré, K. Adel-Patient, H. Bernard, C. Nitride, E.N.C. Mills, L. Monaci. Book of Abstract ISBN: 978-80-7592-055-3, p. 234.
- **5-8 November 2019, Prague, Czech Republic; 9th International Symposium on RECENT ADVANCES IN FOOD ANALYSIS, RAFA2019.** Poster Communication: «Mass-spectrometry-based analysis of multiple allergenic ingredients in incurred matrices: optimization of sample preparation» N. Gillard, R. Pilolli, C. van Poucke, M. de Loose, A.-C. Huet, O. Tranquet, C. Larré, K. Adel-Patient, H. Bernard, C. Nitride, E.N.C. Mills, L. Monaci. Book of Abstract ISBN: 978-80-7592-055-3, p. 232.
- **25-27 September 2019, Camerino, Italy; 6th MS FOOD DAY.** Oral Communication: «SELECTION OF PROTEOTYPIC PEPTIDE MARKERS TRACING FOR SIX ALLERGENIC INGREDIENTS

IN INCURRED CHOCOLATE BAR» R. Pilolli, C. van Poucke, M. de Loose, N. Gillard, A.-C. Huet, O. Tranquet, C. Larré, K. Adel-Patient, H. Bernard, C. Nitride, E.N.C. Mills, L. Monaci.

- **16-18 July 2020; WAO International Scientific Conference (WISC 2020), Rome, Italy.** Poster Communication (Abstract ID 6758): «Scouting for reliable allergen markers in complex food models produced at pilot scale in the ThRAll project» R. Pilolli, C. Van Poucke, C. Nitride, M. De Loose, N. Gillard, A. Huet, O. Tranquet, C. Larrè, K. Adel-Patient, B. Hervé, E.N.C. Mills, L. Monaci.
- **8th-24th September 2020 2020 AOAC INTERNATIONAL Annual Meeting & Exposition** Invited Talk: Detection and Quantification of Allergens in Foods and Minimum Eliciting Doses in Food-Allergic Individuals (ThRAll). Linda Monaci1, Christof van Poucke2, Nathalie Gillard3, Olivier Tranquet4, and **E. N. Clare Mills**
- **16-17th October 2020 FAAM-EUROBAT Digital**. Poster Communication Classification and coding of information about IgE mediated food allergy C.E. French, B. Green, B. Hall, S. Tovey Lawson, M. Morisset, A. Knulst , T-M Le, A. Simpson, S. Dua, N. de Jong, B. Ballmer Weber, M. Fernandez-Rivas, K. Beyer, E.N. C. Mills, P. Couch.
- **16-17th October 2020 FAAM-EUROBAT Digital**. Oral Poster Communication Compiling a consensus database for ThRAll C.E. French, S. Tovey Lawson, B. Hall, P. Couch, B. Javed, K. Wang, M. Morisset, O.Tranquet, A. Knulst , T-M Le, A. Simpson, S. Dua, N. de Jong, B. H. Wichers, A. Muraro, A. Ballin, Alice Toniolo, K. Beyer, M. Fernandez-Rivas, Ballmer Weber, E.N. C. Mills.
- **22-24 November 2021, On-line Conference; XXI EuroFoodChem.** Oral Communication: «In-house validation of a prototype reference method for six allergens detection in chocolate by HPLC-MS/MS analysis» R. Pilolli, C. Nitride, C. Van Poucke, M. de Loose, N. Gillard, J. Henrottin, A.-C. Huet, O. Tranquet, C. Larré, E.N.C. Mills, L. Monaci. Book of Abstract ISBN 978-989-8124-34-0.