SPECIAL GUEST EDITOR SECTION: FOOD ALLERGENS ORGANIZATIONS AND PROJECTS

Integrating Allergen Analysis Within a Risk Assessment Framework: Approaches to Development of Targeted Mass Spectrometry Methods for Allergen Detection and Quantification in the iFAAM Project

CHIARA NITRIDE, VICTORIA LEE, and IVONA BARICEVIC-JONES
The University of Manchester, Manchester Institute of Biotechnology, Manchester Academic Health Science Centre, Division of Infection, Immunity and Respiratory Medicine, School of Biological Sciences, Oxford Rd, Manchester M13 9PL, United Kingdom
KARINE ADEL-PATIENT
Institut National de la Recherche Agronomique-Commissariat à l'Energie Atomique (CEA), Service de Pharmacologie et d'Immunoanalyse, Laboratoire d'Immuno-Allergie Alimentaire, Bâtiment 136-CEA de Saclay, 91191 Gif-sur-Yvette, France
SABINE BAUMGARTNER
University of Natural Resources and Life Sciences, Department IFA-Tulln, Centre of Analytical Chemistry, Christian Doppler Laboratory for Rapid Test Systems for Allergenic Food Contaminants, Konrad Lorenz Strasse 20, 3430 Tulln, Austria
E.N. CLARE MILLS[1]
The University of Manchester, Manchester Institute of Biotechnology, Division of Infection, Immunity and Respiratory Medicine, Manchester Academic Health Science Centre, School of Biological Sciences, Oxford Rd, Manchester M13 9PL, United Kingdom

Allergen analysis is central to implementing and monitoring food allergen risk assessment and management processes by the food industry, but current methods for the determination of allergens in foods give highly variable results. The European Union-funded "Integrated Approaches to Food Allergen and Allergy Risk Management" (iFAAM) project has been working to address gaps in knowledge regarding food allergen management and analysis, including the development of novel MS and immuno-based allergen determination methods. Common allergenic food ingredients (peanut, hazelnut, walnut, cow's milk [*Bos domesticus*], and hen's egg [*Gallus domesticus*]) and common food matrixes (chocolate dessert and cookie) have been used for both clinical studies and analytical method development to ensure that the new methods are clinically relevant. Allergen molecules have been used as analytical targets and allergenic ingredients incurred into matrixes at levels close to reference doses that may trigger the use of precautionary allergen labeling. An interlaboratory method comparison has been undertaken for the determination of peanut in chocolate dessert using MS and immuno-based methods. The iFAAM approach has highlighted the need for methods to report test results in allergenic protein. This will allow food business operators to use them in risk assessments that are founded on clinical study data in which protein has been used as a measure of allergenic potency.

Self-reported immunoglobulin E (IgE)-mediated allergies are thought to affect around 6% of the European population, although the prevalence of food allergy defined by oral food challenge tests is only 0.9%, with a wide geographic variation in prevalence, with rates being higher in Northern Europe (1). In the United States, the self-reported prevalence of food allergy has been estimated at around 8.0%, with peanut, milk, and seafood, such as crustaceans, being important allergenic foods (2). In the United Kingdom, peanut has been estimated to cause food allergy in around 2% of school-age children (3–5), whereas in Australia, up to 9% of infants have been estimated to suffer from egg allergy (6). With regards to the prevalence of severe and fatal reactions, a recent analysis of fatalities in the United States has shown food anaphylaxis to be the least common cause of fatal anaphylaxis in the United States, with 164 fatalities recorded between 1999 and 2010 (7), and the rate (6.7% of all deaths due to anaphylaxis) is similar to that defined in Australia and the United Kingdom (8, 9).

In the absence of an accepted treatment for food-allergic individuals diagnosed with the condition, these individuals have to practice food avoidance, often life-long, and those thought to be at risk of severe reactions are given medication, such as self-injectable adrenaline (epinephrine) to treat a reaction should they accidentally consume their "problem" food. In order to help patients avoid such problem foods, a list of priority allergenic foods was identified by the Codex Alimentarius Commission, which recommended these allergenic foods should be listed on ingredient labels of prepackaged foods irrespective of the level at which they might be included in a recipe (10). These recommendations have now been implemented into local food-labeling regulations across the world, with an amendment in 2014 in the European Union (EU) bringing certain cereals causing IgE-mediated allergies

Guest edited as a special report on "A Global Reflection on Food Allergen Regulations, Management, and Analysis" by Carmen Diaz-Amigo and Bert Popping.

[1] Corresponding author's e-mail: clare.mills@manchester.ac.uk

This review was generated as part of the European Union "Integrated Approaches to Food Allergen and Allergy Risk Management" project (Grant Agreement No. 312147).
DOI: https://doi.org/10.5740/jaoacint.17-0393

and gluten intolerance (celiac disease) and foods with added phytosterols, phytosterol esters, phytostanols, or phytostanol esters into scope [European Regulation (EU) No. 1169/2011, with amendment No. 78/2014). Although mandatory allergen labeling has helped allergic consumers avoid problem foods, there remain uncertainties with regards to the accidental contamination of foods with allergenic ingredients not declared in the ingredient label, which may occur through, e.g., the use of common food-processing lines. As a result of such unintended allergen presence, precautionary allergen labels (PALs) are often applied to warn consumers of the potential risks such allergens might pose (11).

The philosophy underpinning food allergen management is QA, which spans the food chain, from primary production through to point-of-sale. This means systems are designed and maintained with the aim of ensuring that untoward events do not occur (12). Central to implementing such an approach is the availability of tools able to determine effectively the levels of allergenic food protein(s) to monitor either factory cleaning or ingredients and finished products. However, surveys undertaken in Europe and North America have shown that the way in which PAL has been applied means that the presence of a label is not necessarily related to the level of unintended allergen in a food product, indicating that problems remain with regards to the implementation of effective food allergen management (13–17). Of particular concern are levels of unintended allergens around the threshold dose likely to trigger reactions in 5–10% of the allergic population (18), such data forming the basis for the Voluntary Incidental Trace Allergen Labelling (VITAL) expert group reference doses (19). For example, residues of milk in dark chocolate not carrying any PAL have been found to range between 3 and 3000 mg/kg, the latter sample having the potential to trigger reactions in more than 60% of the milk-allergic population (18). The same issues relate to products carrying positive "free from" claims, with 21% of bakery products containing >3 mg cow's milk protein per serving (20). Such surveys have relied on immunoassay test kit analysis as the best available methodology, with many using more than one test kit and showing divergent results between test kit methodologies (15, 16). Interlaboratory comparisons of five different allergen immunoassay test kits each for egg and milk showed that although all the kits could detect allergen protein at the 3 mg/kg level. ISO 5725-2:1994 criteria were used, which define the general principles for designing interlaboratory experiments were used to allow the numerical estimation of the precision of measurement methods. Using this approach, only one egg kit accurately determined egg protein at the 3 mg/kg level (97% recovery of egg-white protein) and one milk (casein) kit accurately determined milk at the 6 and 15 mg/kg levels (103 and 101% recovery of milk protein, respectively; 21). Such poor analytical performance hampers effective food allergen management, making the validation of food allergen management plans and the monitoring of their implementation an uncertain process. This is further complicated by the known effects of food processing and the food matrix on allergen test kit performance (22), which mean test results for allergen determination in foods can be highly variable and matrix-dependent and the methods themselves have only undergone limited validation.

The EU-funded "Integrated Approaches to Food Allergen and Allergy Risk Management" (iFAAM) project partnership has been working to address many gaps in knowledge with regards to patient-focused food allergy management and food industry-focused issues regarding food allergen management. One aim has been to address shortcomings in allergen analysis by linking the development of effective multianalyte allergen analysis tools to a characterization of how the tools perform with regards to the nature of the allergenic hazard they are attempting to quantify (Figure 1). Analytical methods explored include those potentially suitable for in-factory testing (e.g., immuno-based) and confirmatory in-laboratory analysis (e.g., MS-based). This was implemented for five important allergenic foods that cause many food allergies, including more severe reactions (1, 9), and are frequently involved in food product recalls: peanut and

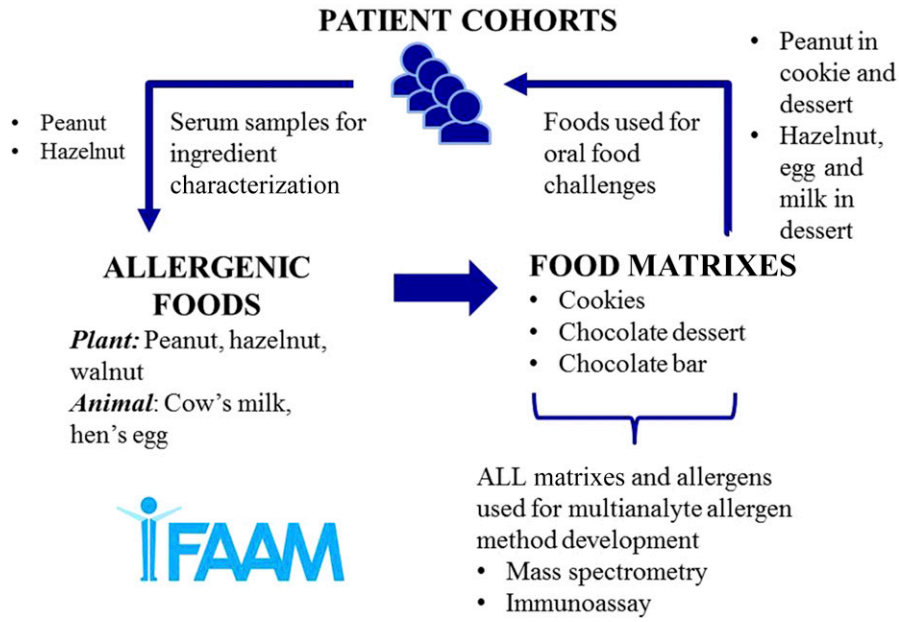

Figure 1. Diagrammatic overview of iFAAM's holistic approach to the development of clinically relevant allergen analysis.

selected tree nuts (e.g., hazelnut and walnut), together with egg and milk (23). The same ingredients used for the development of analytical methodology were also used for clinical studies, including oral food challenges, thus allowing outputs from analytical methods to be more easily related to clinical reactivity (Figure 1).

Raw ingredients in which allergenic proteins are still in their "native" state are relatively easy to analyze using proteomic approaches. However, both manufacture of processed allergenic ingredients and formulated foods in which an allergenic ingredient is included into the food microstructure (also known as the food matrix) can result in complex changes in allergen structure. The physicochemical changes induced in allergens by food-processing procedures (such as protein unfolding, aggregation, and thermally induced chemical modifications, such as Maillard adducts) have the potential to change IgE reactivity and, hence, the potency of a food to cause a reaction in an allergic individual. Similarly, the food matrix may also impair the extraction, detection, and quantification of food allergens, adversely affecting analytical method performance, usually by underestimating the actual concentration of an allergenic ingredient. There is evidence that food processing can affect both clinical reactivity to foods (24–26) and allergen analysis (27), often by modifying allergen extractability (28, 29). However, little is understood regarding the rules governing how food processing (mechanical, thermal, physical, or biochemical) in combination with food composition can modify either the intrinsic allergenicity or detection and quantification of food allergens, making it difficult to predict how different processing conditions and formulations may affect analytical method performance.

In order to address such knowledge gaps and develop robust analytical methodology, the iFAAM project used three food matrixes—chocolate dessert, chocolate bar, and cookie—into which the five main allergenic ingredients were incurred. The chocolate dessert and chocolate bar had previously been used for oral food challenges in allergic patients in the EuroPrevall project (18, 25), whereas the cookie matrix had been used to collect oral food challenge data as part of a clinical matrix comparison study in iFAAM. All three products represent matrixes thought to be difficult to analyze: the chocolate matrixes containing polyphenols and lipids, whereas the cookie is a high-starch, high-fat matrix. The polyphenols are known to interact with proteins, including allergens such as the peanut allergen, Ara h 2, through hydrophobic or hydrophilic interactions (30). The formation of such complexes can affect the bioavailability and digestibility of the allergenic proteins, possibly modulating their allergenic potential, including IgE reactivity (31).

Clinically Relevant Allergen Analysis in iFAAM

Because protein is the hazard that causes allergic reactions, risk assessment methods focus on this component, requiring input data on allergen levels expressed in milligrams of total protein. Therefore, whenever possible, methods should measure protein from an allergenic source and either report in protein units or be converted into protein units (12, 32, 33). Thus, the focus of analytical method development in iFAAM was on allergenic protein molecules in the five allergenic food ingredients. This was essential in order to ensure test results could be interpreted within the context of other iFAAM activities focused on the development of risk models. Such integration enables food allergen management undertaken by the food industry to be more easily understood by patients and health care professionals supporting them in managing their food allergies (Figure 1). The second aspect was to ensure that test methodologies could detect and quantify allergens at or close to reference doses. As a consequence, the food matrixes were incurred with allergen at a range of levels from 2 mg/kg within the different matrixes.

In order to ensure that allergens detected are clinically relevant and represent a risk to allergic consumers, a detailed characterization of the allergens present in the peanut and hazelnut ingredients was undertaken. This was carried out by determining the IgE reactivity of food using serum samples from peanut- and hazelnut-allergic subjects. Complementary methods were combined: IgE immunoblotting, IgE immunoassays (IgE-binding capacity), and in vitro models of elicitation of the allergic reaction previously used in the EuroPrevall (The Prevalence, Cost, and Basis of Food Allergy across Europe) project (34). After ensuring the allergenic activity of the ingredients, the effect of incurring the peanut flour in the three matrixes (chocolate bar, EuroPrevall chocolate dessert, or iFAAM cookie) on the extractability of IgE-reactive proteins was assessed using the same methods.

In addition, the relationship between peanut allergen extractability and detection from the different matrixes used in analytical methods and their bioaccessibility was explored. Bioaccessibility is a property that reflects the impact a food matrix has on the release of a molecule (in this instance, allergens) from the food matrix in the lumen of the gastrointestinal tract. This was modeled using an *in vitro* digestion system that mimics the way in which the body extracts nutrients and subsequently breaks them down to enable them to be absorbed. There are parallels with the way in which food allergens are solubilised and digested in the gastrointestinal tract and the way they are extracted prior to analysis and in MS workflows and digested with gastrointestinal proteases such as pepsin, trypsin, and chymotrypsin.

Development of Multianalyte MS Methods for Five Major Food Allergens

MS methods have much to offer as a complementary method to immunoassay, but are much less well developed. Therefore, much of the iFAAM activity focused on the application of multiplex-targeted MS methods for the selected allergenic foods. Table 1 summarizes the MS-based methods developed for food allergen detection and quantification in baked and chocolate-based products. Because the workhorse instruments of food allergen test laboratories are triple quadrupole mass spectrometers, these were the platform of choice, working in selected-reaction monitoring (SRM) mode. The targeted MS approach monitors proteotypic peptides as a surrogate marker for the precursor protein. This enhances the specificity and sensitivity of a method and provides the opportunity to multiplex the measurement of many analytes in parallel. Such platforms can also deal with protein abundances that may span 4 or 5 orders of magnitude while still maintaining high mass accuracy (27). The approach can also be made quantitative if used with an isotopically labeled form of the target analyte as an internal standard. These labelled peptides are identical in physicochemical structure, chromatographic performance, and ionization efficiency to the corresponding endogenous peptide

Table 1. Summary of different extraction procedures used to develop MS methods for the determination of allergens in foods

Food allergen and food matrix	MS platform	Extraction protocol	Ref.
Raw and roasted peanut (140°C)	Capillary HPLC-nano-QTOF[a]	20 mM TBS buffer, 150 mM NaCl, pH 7.4[b]	(41)
Dark chocolate mixed with Ara h 1	HPLC-QTOF	0.05 M NH_4HCO_3, pH 8, 60°C	(42)
Processed peanut	HPLC-nano-QTOF	20 mM Tris-HCl, 150 mM NaCl, pH 7.4, 4°C	(43)
Milk, egg, soy, peanut, hazelnut, walnut, and almond in bread	HPLC-API-QTRAP[c]	Tris-HCl, pH 8.2, 60°C (Buffer concentration not provided)	(44)
Peanut and peanut-containing food	LC-QQQ[d]	0.2 M Tris-HCl, pH 8.2, 60°C	(45)
Roasted peanuts and different commercial breakfast cereals	LC-LTQ XL linear ion trap instrument	0.2 M Tris-HCl, pH 8.2, 60°C	(46)
Cashew nut, hazelnut, almond, peanut, and walnut in cereals and biscuits	LC-LTQ XL linear ion trap instrument	0.05 M bicarbonate (salt not defined), pH 8.0, 60°C	(47)
Nuts from local markets	HPLC-QTRAP	0.1 M NH_4HCO_3, pH 7.6, 4°C	(48)
Milk in chocolates, cookies, baby foods, frozen desserts, sausages, and ground meats	LC-QTOF	20 mM Tris-HCl and 0.1% (v/v) Tween 20, ambient temperature	(49)
Milk in incurred cookies	LC-QTOF	20 mM Tris-HCl and 0.1% (v/v) Tween 20, pH 10.20, 60°C	(50)
Milk and dairy products from local markets	HPLC-QTRAP	50 mM $NaHCO_3$, pH 9.6, 60°C	(51)
Milk in baby food (puree), infant cereals, and breakfast cereals	HPLC-QQQ	50 mM NH_4HCO_3 and 1 M urea, pH 8.0, 60°C	(52)
Egg in pasta	LC-QTOF	50 mM NH_4HCO_3, pH 7.8	(53)
Milk in baked cookie	HPLC-XCT plus ion trap mass spectrometer	1% (w/v) SDS in NH_4HCO_3 buffer, pH 8.2, 60°C[e]	(54)
Egg, milk, and soy in baked cookies	UHPLC-linear ion trap dual-pressure mass spectrometer[f]	20 mM Tris-HCl, pH 8.2, 60°C	(55)
Milk in baked product	UPLC-QQQ	50 mM NH_4HCO_3	(56)
Milk in home-baked product	UPLC-QTRAP	Predigestion of starch using α-amylase (390 IU), pectinase (22 IU), mannanase (4 IU), and 15% (v/v) acetonitrile in 50 mM NH_4HCO_3, pH 9	(57)
Milk, soy, and gluten in home-baked cookies	UPLC-QTOF[g]	250 mM 2-mercaptoethanol and 2 M guanidine HCl in PBS, 60°C[h]	(58)
Milk, egg, and peanut in cereal bars and muffins	Nano-UPLC-QTRAP	2 M urea and 25 mM DTT in 50 mM TBS, pH 8.0[i]	(27)
Milk, egg, soy, and peanut in cookies; sauce; ice cream; and chocolate	UHPLC-QQQ	200 mM Tris-HCl and 2 M urea, pH 9.2	(59)

[a] QTOF = Quadrupole time-of-flight.

[b] TBS = Tris-buffered saline.

[c] API = Atmospheric pressure ionization.

[d] QQQ = Triple quadrupole.

[e] SDS = Sodium dodecyl sulfate.

[f] UHPLC = Ultra-HPLC.

[g] UPLC = Ultra-performance LC.

[h] PBS = Phosphate buffered saline.

[i] DTT = Dithiothreitol.

despite their difference in mass (5–6 kDa). The isotopically labeled peptides mitigate the effect of instrumental fluctuation, such as interference in SRM ion transition and signal suppression caused by matrix components because we only look at relative measurements (the chromatographic peak area of the natural and the isotopically labeled peptides are integrated and the ratio of the two areas is calculated). The use of stable isotope-labeled peptide internal standards has been widely demonstrated to provide the highest level of detection confidence and precision (35). This is because they coelute with the target analyte and fragment in the same manner, allowing matrix effects that can cause poor spray stability and ion suppression to be taken into account. Advances in peptide chemistry, coupled with increased demand for peptide drugs and the use of peptides in self-structuring materials, are also helping reduce the costs of synthetic peptides (36). QC criteria for peptide standards are being developed in the clinical arena, which are applicable to the application of multiple-reaction monitoring technology in the food arena (37). Purified protein standards have also been used, although these are not readily available for food allergens, making peptide standards cheaper and easier to obtain.

The proteomic pipeline used for method development in iFAAM is summarized in Figure 2. In order to target the allergenic protein hazard, a systematic approach to signature peptide target identification from different foods and food matrixes was undertaken. Initially, peptides were generated via in silico digestion of allergenic proteins selected as markers for the presence of the five allergenic foods. The selection of the proteotypic peptides followed the design criteria originally developed through the MoniQA (Monitoring and Quality Assurance in the Total Food Supply Chain) project (38) and were as follows:

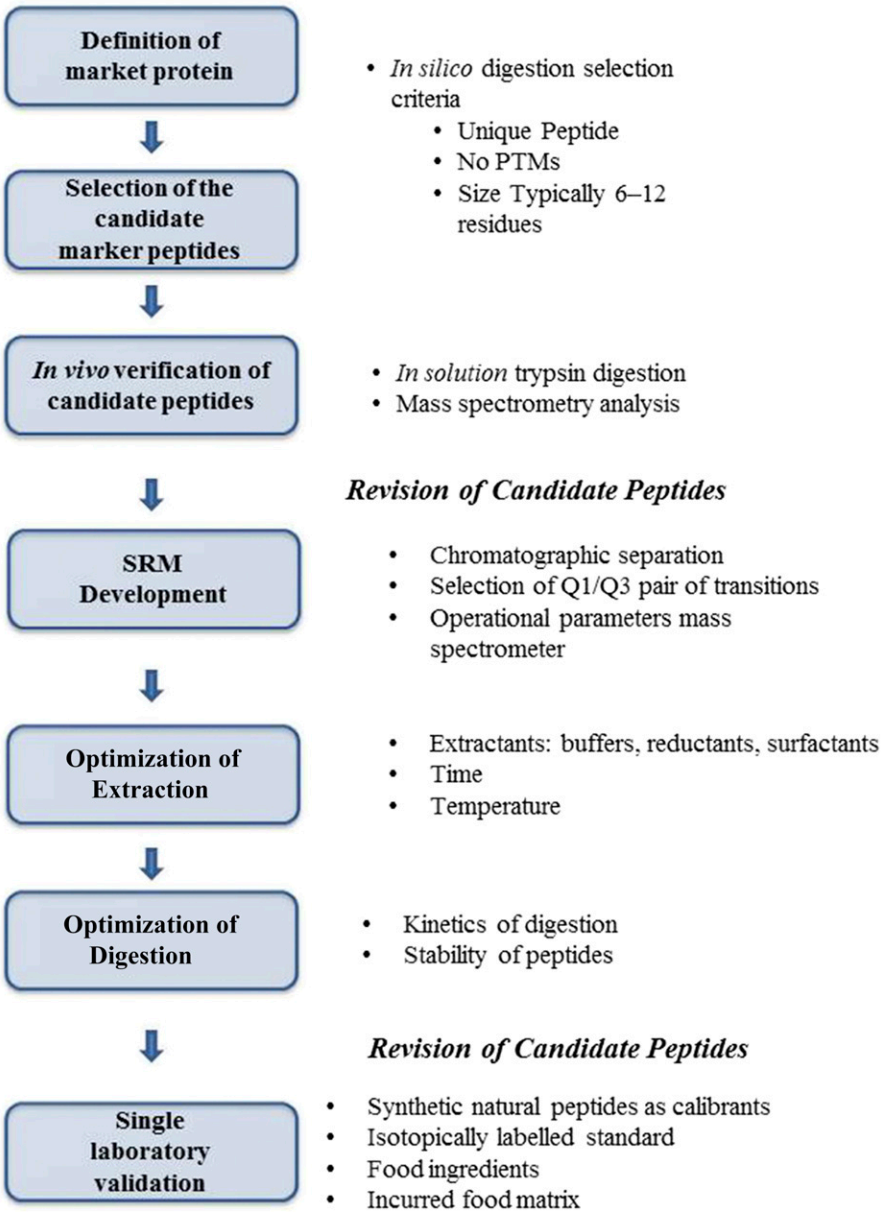

Figure 2. Experimental workflow for the selection of markers and optimization of extraction and digestion for the development of multianalyte allergen MS methods. PTMs = post-translational modifications; SRM = selected-reaction monitoring.

(*1*) Target peptides were identified from allergen protein sequences that are known and available in UniProt.

(*2*) All of the listed allergenic proteins were abundant (e.g., seed storage proteins) and found in widely used food ingredients.

(*3*) In order to increase the specificity of the method, peptides were selected that were preferentially unique to a specific protein or, when the protein exists as different isoforms, to the given protein type (e.g., 7S or 11S seed storage globulins or 2S albumins).

(*4*) Peptides were not subjected to natural post-translational modifications. This was achieved by excluding peptides containing phosphorylation and *N*-glycosylation sites.

(*5*) In general, 2^+ and 3^+ peptide ions were selected because these generally fragment well in MS analysis.

(*6*) Peptides were chosen that ranged from 5 up to 12 amino acids in length.

In order to increase the number of candidate peptides, the search criteria had to be relaxed to accommodate peptides prone to chemical modifications, such as oxidation and deamidation, even though this increases the probability of poor reproducibility. The stability of the peptides during storage and sample processing was also evaluated during the optimization of the digestion steps in the sample workflow and the information fed into the decision-making process for final signature peptide selection.

The success of the protein quantification by MS analysis of peptide targets relies on the efficiency of the extraction and the generation of an equimolar concentration of peptides from the parent protein. The inclusion of peptides that are either not fully released (e.g., as a result of partial cleavage and that might be prone to further degradation) or are subject to chemical modification might lead to an underestimation

of the parent protein concentration. Thus, the generation of candidate signature peptides during digestion with trypsin was confirmed using either purified standard proteins if they were available or proteins extracted from the allergenic ingredients and the processed food matrixes. In addition, the chromatographic behavior and ionization efficiency of the peptides were also determined by MS. This allowed the best performing conditions for protease digestion, chromatographic separation, precursor peptide-to-fragment ion transitions, and the operational parameters of the mass spectrometer to be determined experimentally.

Extraction conditions were optimized for the highest recovery of protein as estimated by total protein analysis and relative peptide quantification using the incurred matrixes and the selected MS conditions. Both aspects were taken into account because protein extraction efficiency alone may be poorly predictive of the peptides released after digestion (29, 30). The conditions evaluated included buffer type, detergent addition, temperature, and time, all of which are known to have a considerable effect on digestion efficiency, peptide yields, and chemical modifications. After optimizing the extraction efficiency an assessment of the in-solution protease digestion step was undertaken using trypsin, an endoprotease widely used in proteomic workflows was used because of its high proteolytic activity and cleavage specificity. Ideally, the signature peptide should be fully released from the parent protein and stable during the entire time of digestion. Thus, digestion kinetics and target peptide stability were evaluated over the entire time course of digestion. The selection of peptides was then revisited based on experimental observations to arrive at a final peptide inclusion list used for multiplex MS method development aimed at providing methods capable of determining the five allergenic foods in each of the incurred matrixes.

Interlaboratory Validation of ELISA and MS Methods for Detection of Peanut: The iFAAM Ring Trial

ELISA-based tests are currently the method of choice for the detection and quantification of allergens in food matrixes. However, these have undergone limited validation and give variable, matrix-dependent results. Multiplexed MS methods are being developed that could provide confirmatory evidence of allergen presence in a given sample. However, validated MS methods for allergen detection in food matrixes are also lacking. Based on the previously published approach for undertaking an interlaboratory comparison of egg and milk determination by ELISA (21), the iFAAM project has undertaken an interlaboratory comparison of targeted MS and ELISA test methods for the quantification of peanut in the chocolate dessert matrix at candidate reference dose levels. The transferability of the extraction, sample preparation, and MS methods among different laboratories using diverse LC-tandem MS instrumentation was studied, and the results compared with those obtained by ELISA. In collaboration with ELISA test kit manufacturers, MS platform vendors, and analytical laboratories from across Europe, North America, Australia, and Japan, the trial has involved more than 20 different organizations, including the National Measurement Institutes and other laboratories, such as public analysts.

Lightly roasted, mechanically defatted peanut flour manufactured by the Golden Peanut Company (Alpharetta, GA) was selected as the peanut ingredient to be incurred into the chocolate dessert matrix for both the ELISA and MS arm of the ring trial. As mentioned previously, peanut flour was chosen as it had been used for undertaking oral food challenges in EuroPrevall (18) and iFAAM and had well-characterized allergen profiles that were known to be stable over many years, as well as demonstrable clinical reactivity (39). Taking the proposed estimated dose that elicits a reaction identified by the VITAL scientific expert panel (0.2 mg protein), the dose series in hydrated chocolate dessert created for the ring trial was 0, 2, 4, 10, and 30 mg peanut protein per kilogram of chocolate dessert (19). The peanut-incurred dessert matrix was shipped to all participating laboratories; for the laboratories undertaking the ELISA and MS arms of the ring trial and for those undertaking the MS arms, an aliquot of the peanut flour was also shipped to be used as a reference control. Those undertaking the MS analysis also received the synthetic peanut peptide mix solutions—one consisting of unlabeled synthetic peanut peptides used as external calibrants and the other consisting of synthetic peanut peptides labeled with ^{13}C and ^{15}N C-terminal arginine or lysine residues, used as internal standards. A set of standard operational procedures was developed to provide guidance to the different laboratories undertaking the study and maximize harmonization of methodology. All activities undertaken were overseen by a steering group responsible for reviewing all the steps of the ring trial. Participants of the ELISA ring trial received kits and protocols directly from the kit manufacturers.

An initial prering trial was undertaken for the ELISA and MS arms in order for participants to familiarize themselves with the materials and protocols. Following completion of the prering trial, protocols were revised in consultation with the steering group and the full ring trial undertaken.

A key aspect for analysis of the full ring trial data is the conversion factors to apply. As was required for the previous egg and milk study (21), it was necessary to apply a conversion factor to data obtained for those kits reporting in peanut to allow comparison of results between kits and with the expected values. Therefore, based on the average peanut seed containing 25% protein (40), kit results reported in milligrams of peanut per kilogram were divided by four to give milligrams per kilogram of peanut protein. For the MS trial, this was more complex because there is a need to convert from peptide to peanut proteins. One approach being applied is to first convert the peptide concentration to a concentration of individual allergens using a set of predefined MWs (27) and to subsequently develop a further conversion factor to arrive at peanut protein based on the relative abundance of each allergen type in the peanut flour sample, determined, e.g., using discovery proteomics data (39). This can give an estimate of total allergen protein, and subsequently, based on extractable protein content, arrive at an estimate of allergenic ingredient protein. In addition to allowing a comparison of the different platforms used in the study, these calculations will also allow comparison of MS-derived measurements with the ELISA test kit results.

Conclusions

Allergen risk assessment and management approaches are being increasingly used by the food industry that are founded on clinical data collected in food-allergic subjects undergoing oral food challenges in which the allergenic component has been defined in the mass of the protein that causes an allergic reaction.

The holistic approach used within the iFAAM project aimed to link the data from clinical studies to the allergen risk assessment and management approaches and the analytical measurement. This has made it apparent that, in order to provide food business operators with meaningful data that can be easily used to implement such risk assessment action plans, the presence of allergens in foods will need to be reported as a protein measurement (e.g., peanut protein), rather than just as a commodity (e.g., peanut).

The iFAAM activities with regards to allergen analysis will help support the development of MS methods that can be deployed in a complementary manner to immuno-based methods such as ELISA. Parallel development of different analytical methodologies accompanied by effective test method validation and interlaboratory comparisons will help improve the quality of allergen analysis and promote best practices in allergen analysis across different analytical laboratories around the world. However, the development of metrologically traceable reference materials and standards remains a priority, as does the definition of relevant allergenic protein markers and conversion factors to allow reporting of analytical test results as milligrams of total allergenic protein. Such resources are time consuming and costly to produce, but are vital if the results of allergen analysis are to be trusted by the food industry, allergic consumers, and regulators alike (11).

References

(1) Nwaru, B.I., Hickstein, L., Panesar, S.S., Roberts, G., Muraro, A., & Sheikh, A. (2014) *Allergy* **69**, 992–1007, doi:10.1111/all.12423

(2) Gupta, R.S., Springston, E.E., Warrier, M.R., Smith, B., Kumar, R., Pongracic, J., & Holl, J.L. (2011) *Pediatrics* **128**, e9–e17, doi:10.1542/peds.2011-0204

(3) Grundy, J., Matthews, S., Bateman, B., Dean, T., & Arshad, S.H. (2002) *J. Allergy Clin. Immunol.* **110**, 784–789, doi:10.1067/mai.2002.128802

(4) Venter, C., Hasan Arshad, S., Grundy, J., Pereira, B., Bernie Clayton, C., Voigt, K., Higgins, B., & Dean, T. (2010) *Allergy* **65**, 103–108, doi:10.1111/j.1398-9995.2009.02176.x

(5) Nicolaou, N., Poorafshar, M., Murray, C., Simpson, A., Winell, H., Kerry, G., Harlin, A., Woodcock, A., Ahlstedt, S., & Custovic, A. (2010) *J. Allergy Clin. Immunol.* **125**, 191–197.e1–13

(6) Osborne, N.J., Koplin, J.J., Martin, P.E., Gurrin, L.C., Lowe, A.J., Matheson, M.C., Ponsonby, A.L., Wake, M., Tang, M.L., Dharmage, S.C., & Allen, K.J. (2011) *J. Allergy Clin. Immunol.* **127**, 668–676 e1–2

(7) Jerschow, E., Lin, R.Y., Scaperotti, M.M., & McGinn, A.P. (2014) *J. Allergy Clin. Immunol.* **134**, 1318–1328.e1317, doi:10.1016/j.jaci.2014.08.018

(8) Liew, W.K., Williamson, E., & Tang, M.L. (2009) *J. Allergy Clin. Immunol.* **123**, 434–442, doi:10.1016/j.jaci.2008.10.049

(9) Turner, P.J., Gowland, M.H., Sharma, V., Ierodiakonou, D., Harper, N., Garcez, T., Pumphrey, R., & Boyle, R.J. (2015) *J. Allergy Clin. Immunol.* **135**, 956–963.e951

(10) Mills, E.N., Valovirta, E., Madsen, C., Taylor, S.L., Vieths, S., Anklam, E., Baumgartner, S., Koch, P., Crevel, R.W., & Frewer, L. (2004) *Allergy* **59**, 1262–1268, doi:10.1111/j.1398-9995.2004.00720.x

(11) DunnGalvin, A., Chan, C.H., Crevel, R., Grimshaw, K., Poms, R., Schnadt, S., Taylor, S.L., Turner, P., Allen, K.J., Austin, M., Baka, A., Baumert, J.L., Baumgartner, S., Beyer, K., Bucchini, L., Fernandez-Rivas, M., Grinter, K., Houben, G.F., Hourihane, J., Kenna, F., Kruizinga, A.G., Lack, G., Madsen, C.B., Clare Mills, E.N., Papadopoulos, N.G., Alldrick, A., Regent, L., Sherlock, R., Wal, J.M., & Roberts, G. (2015) *Allergy* **70**, 1039–1051, doi:10.1111/all.12614

(12) Hattersley, S., Ward, R., Baka, A., & Crevel, R.W. (2014) *Food Chem. Toxicol.* **67**, 255–261, doi:10.1016/j.fct.2014.01.036

(13) Pele, M., Brohee, M., Anklam, E., & Van Hengel, A.J. (2007) *Food Addit. Contam.* **24**, 1334–1344, doi:10.1080/02652030701458113

(14) Remington, B.C., Baumert, J.L., Blom, W.M., Houben, G.F., Taylor, S.L., & Kruizinga, A.G. (2015) *Allergy* **70**, 813–819, doi:10.1111/all.12625

(15) Khuda, S.E., Sharma, G.M., Gaines, D., Do, A.B., Pereira, M., Chang, M., Ferguson, M., & Williams, K.M. (2016) *Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess.* **33**, 1265–1273

(16) Khuda, S.E., Sharma, G.M., Gaines, D., Do, A.B., Pereira, M., Chang, M., Ferguson, M., & Williams, K.M. (2016) *Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess.* **33**, 1274–1282

(17) Robertson, O.N., Hourihane, J.O., Remington, B.C., Baumert, J.L., & Taylor, S.L. (2013) *Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess.* **30**, 1467–1472, doi:10.1080/19440049.2013.804953

(18) Ballmer-Weber, B.K., Fernandez-Rivas, M., Beyer, K., Defernez, M., Sperrin, M., Mackie, A.R., Salt, L.J., Hourihane, J.O., Asero, R., Belohlavkova, S., Kowalski, M., de Blay, F., Papadopoulos, N.G., Clausen, M., Knulst, A.C., Roberts, G., Popov, T., Sprikkelman, A.B., Dubakiene, R., Vieths, S., van Ree, R., Crevel, R., & Mills, E.N.C. (2015) *J. Allergy Clin. Immunol.* **135**, 964–971, doi:10.1016/j.jaci.2014.10.047

(19) Taylor, S.L., Baumert, J.L., Kruizinga, A.G., Remington, B.C., Crevel, R.W., Brooke-Taylor, S., Allen, K.J., & Houben, G. (2014) *Food Chem. Toxicol.* **63**, 9–17, doi:10.1016/j.fct.2013.10.032

(20) Trendelenburg, V., Enzian, N., Bellach, J., Schnadt, S., Niggemann, B., & Beyer, K. (2015) *Allergy* **70**, 591–597, doi:10.1111/all.12588

(21) Johnson, P.E., Rigby, N.M., Dainty, J.R., Mackie, A.R., Immer, U.U., Rogers, A., Titchener, P., Shoji, M., Ryan, A., Mata, L., Brown, H., Holzhauser, T., Dumont, V., Wykes, J.A., Walker, M., Griffin, J., White, J., Taylor, G., Popping, B., Crevel, R., Miguel, S., Lutter, P., Gaskin, F., Koerner, T.B., Clarke, D., Sherlock, R., Flanagan, A., Chan, C.H., & Mills, E.N.C. (2014) *Food Chem.* **148**, 30–36, doi:10.1016/j.foodchem.2013.09.115

(22) Poms, R.E., Capelletti, C., & Anklam, E. (2004) *Mol. Nutr. Food Res.* **48**, 459–464, doi:10.1002/mnfr.200400052

(23) Bucchini, L., Guzzon, A., Poms, R., & Senyuva, H. (2016) *Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk. Assess.* **33**, 760–771

(24) Grimshaw, K.E., King, R.M., Nordlee, J.A., Hefle, S.L., Warner, J.O., & Hourihane, J.O. (2003) *Clin. Exp. Allergy* **33**, 1581–1585, doi:10.1046/j.1365-2222.2003.01795.x

(25) Mackie, A., Knulst, A., Le, T.M., Bures, P., Salt, L., Mills, E.N.C., Malcolm, P., Andreou, A., & Ballmer-Weber, B.K. (2012) *Mol. Nutr. Food Res.* **56**, 1708–1714, doi:10.1002/mnfr.201200330

(26) Turner, P.J., Mehr, S., Sayers, R., Wong, M., Shamji, M.H., Campbell, D.E., & Mills, E.N. (2014) *J. Allergy Clin. Immunol.* **134**, 751–753, doi:10.1016/j.jaci.2014.06.016

(27) Parker, C.H., Khuda, S.E., Pereira, M., Ross, M.M., Fu, T.J., Fan, X., Wu, Y., Williams, K.M., DeVries, J., Pulvermacher, B., Bedford, B., Zhang, X., & Jackson, L.S. (2015) *J. Agric. Food Chem.* **63**, 10669–10680, doi:10.1021/acs.jafc.5b04287

(28) Maleki, S.J., Casillas, A.M., Kaza, U., Wilson, B.A., Nesbit, J.B., Reimoneqnue, C., Cheng, H., & Bahna, S.L. (2010)

Ann. Allergy Asthma Immunol. **105**, 451–457, doi:10.1016/j.anai.2010.09.025
(29) Sayers, R.L., Johnson, P.E., Marsh, J.T., Barran, P., Brown, H., & Mills, E.N. (2016) *Analyst* **141**, 4130–4141
(30) Vesic, J., Stambolic, I., Apostolovic, D., Milcic, M., Stanic-Vucinic, D., & Cirkovic Velickovic, T. (2015) *Food Chem.* **185**, 309–317, doi:10.1016/j.foodchem.2015.04.001
(31) Plundrich, N.J., White, B.L., Dean, L.L., Davis, J.P., Foegeding, E.A., & Lila, M.A. (2015) *Food Funct.* **6**, 2145–2154, doi:10.1039/C5FO00162E
(32) Spanjersberg, M.Q., Kruizinga, A.G., Rennen, M.A., & Houben, G.F. (2007) *Food Chem. Toxicol.* **45**, 49–54, doi:10.1016/j.fct.2006.07.018
(33) Madsen, C.B., Hattersley, S., Buck, J., Gendel, S.M., Houben, G.F., Hourihane, J.O., Mackie, A., Mills, E.N., Norhede, P., Taylor, S.L., & Crevel, R.W. (2009) *Food Chem. Toxicol.* **47**, 480–489, doi:10.1016/j.fct.2008.12.001
(34) Vissers, Y.M., Blanc, F., Skov, P.S., Johnson, P.E., Rigby, N.M., Przybylski-Nicaise, L., Bernard, H., Wal, J.-M., Ballmer-Weber, B., Zuidmeer-Jongejan, L., Szepfalusi, Z., Ruinemans-Koerts, J., Jansen, A.P.H., Savelkoul, H.F.J., Wichers, H.J., Mackie, A.R., Mills, E.N.C., & Adel-Patient, K. (2011) *PLoS One* **6**, e23998, doi:10.1371/journal.pone.0023998
(35) Carr, S.A., Abbatiello, S.E., Ackermann, B.L., Borchers, C., Domon, B., Deutsch, E.W., Grant, R.P., Hoofnagle, A.N., Huttenhain, R., Koomen, J.M., Liebler, D.C., Liu, T., MacLean, B., Mani, D.R., Mansfield, E., Neubert, H., Paulovich, A.G., Reiter, L., Vitek, O., Aebersold, R., Anderson, L., Bethem, R., Blonder, J., Boja, E., Botelho, J., Boyne, M., Bradshaw, R.A., Burlingame, A.L., Chan, D., Keshishian, H., Kuhn, E., Kinsinger, C., Lee, J.S., Lee, S.W., Moritz, R., Oses-Prieto, J., Rifai, N., Ritchie, J., Rodriguez, H., Srinivas, P.R., Townsend, R.R., Van Eyk, J., Whiteley, G., Wiita, A., & Weintraub, S. (2014) *Mol. Cell. Proteomics* **13**, 907–917, doi:10.1074/mcp.M113.036095
(36) Behrendt, R., White, P., & Offer, J. (2016) *J. Pept. Sci.* **22**, 4–27, doi:10.1002/psc.2836
(37) Hoofnagle, A.N., Whiteaker, J.R., Carr, S.A., Kuhn, E., Liu, T., Massoni, S.A., Thomas, S.N., Townsend, R.R., Zimmerman, L.J., Boja, E., Chen, J., Crimmins, D.L., Davies, S.R., Gao, Y., Hiltke, T.R., Ketchum, K.A., Kinsinger, C.R., Mesri, M., Meyer, M.R., Qian, W.J., Schoenherr, R.M., Scott, M.G., Shi, T., Whiteley, G.R., Wrobel, J.A., Wu, C., Ackermann, B.L., Aebersold, R., Barnidge, D.R., Bunk, D.M., Clarke, N., Fishman, J.B., Grant, R.P., Kusebauch, U., Kushnir, M.M., Lowenthal, M.S., Moritz, R.L., Neubert, H., Patterson, S.D., Rockwood, A.L., Rogers, J., Singh, R.J., Van Eyk, J.E., Wong, S.H., Zhang, S., Chan, D.W., Chen, X., Ellis, M.J., Liebler, D.C., Rodland, K.D., Rodriguez, H., Smith, R.D., Zhang, Z., Zhang, H., & Paulovich, A.G. (2016) *Clin. Chem.* **62**, 48–69
(38) Johnson, P.E., Baumgartner, S., Aldick, T., Bessant, C., Giosafatto, V., Heick, J., Mamone, G., O'Connor, G., Poms, R., Popping, B., Reuter, A., Ulberth, F., Watson, A., Monaci, L., & Mills, E.N. (2011) *J. AOAC Int.* **94**, 1026–1033
(39) Johnson, P.E., Sayers, R.L., Gethings, L.A., Balasundaram, A., Marsh, J.T., Langridge, J.I., & Mills, E.N.C. (2016) *Anal. Chem.* **88**, 5689–5695, doi:10.1021/acs.analchem.5b04466
(40) Pancholy, S.K., Deshpande, A.S., & Krall, S. (1978), Proceedings of the American Peanut Research and Education Association, **Vol. 10**, Gainesville, FL, pp 30–37
(41) Chassaigne, H., Nørgaard, J.V., & van Hengel, A.J. (2007) *J. Agric. Food Chem.* **55**, 4461–4473, doi:10.1021/jf063630e
(42) Shefcheck, K.J., Callahan, J.H., & Musser, S.M. (2006) *J. Agric. Food Chem.* **54**, 7953–7959, doi:10.1021/jf060714e
(43) Pedreschi, R., Nørgaard, J.V., & Maquet, A. (2012) *Nutrients* **4**, 132–150, doi:10.3390/nu4020132
(44) Heick, J., Fischer, M., & Pöpping, B. (2011) *J. AOAC Int.* **94**, 938–943
(45) Careri, M., Costa, A., Elviri, L., Lagos, J.B., Mangia, A., Terenghi, M., Cereti, A., & Perono Garoffo, L. (2007) *Anal. Bioanal. Chem.* **389**, 1901–1907, doi:10.1007/s00216-007-1595-2
(46) Careri, M., Elviri, L., Lagos, J.B., Mangia, A., Speroni, F., & Terenghi, M. (2008) *J. Chromatogr. A* **1206**, 89–94, doi:10.1016/j.chroma.2008.07.091
(47) Bignardi, C., Elviri, L., Penna, A., Careri, M., & Mangia, A. (2010) *J. Chromatogr. A* **1217**, 7579–7585, doi:10.1016/j.chroma.2010.10.037
(48) Ansari, P., Stoppacher, N., & Baumgartner, S. (2011) *Anal. Bioanal. Chem.* **402**, 2607–2615, doi:10.1007/s00216-011-5218-6
(49) Weber, D., Raymond, P., Ben-Rejeb, S., & Lau, B. (2006) *J. Agric. Food Chem.* **54**, 1604–1610, doi: 10.1021/jf052464s
(50) Monaci, L., Nørgaard, J.V., & van Hengel, A.J. (2010) *Anal. Methods* **2**, 967–972, doi:10.1039/c0ay00151a
(51) Ansari, P., Stoppacher, N., Rudolf, J., Schuhmacher, R., & Baumgartner, A. (2011) *Anal. Bioanal. Chem.* **399**, 1105–1115, doi:10.1007/s00216-010-4422-0
(52) Lutter, P., Parisod, V., & Weymuth, H. (2013) *J. AOAC Int.* **94**, 1043–1059
(53) Azarnia, S., Boye, J.I., Mongeon, V., & Sabik, H. (2013) *Food Res. Int.* **52**, 526–534, doi:10.1016/j.foodres.2013.02.039
(54) Lamberti, C., Acquadro, E., Corpillo, D., Giribaldi, M., Decastelli, L., Garino, C., Arlorio, M., Ricciardi, C., Cavallarin, L., & Giuffrida, M.G. (2016) *Food Chem.* **199**, 119–127, doi:10.1016/j.foodchem.2015.11.130
(55) Monaci, L., Pilolli, R., De Angelis, E., Godula, M., & Visconti, A. (2014) *J. Chromatogr. A* **1358**, 136–144, doi:10.1016/j.chroma.2014.06.092
(56) Chen, Q., Zhang, J.S., Ke, X., Lai, S.Y., Tao, B.H., Yang, J.C., Mo, W.M., & Ren, Y.P. (2015) *Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess.* **32**, 25–34
(57) Newsome, G.A., & Scholl, P.F. (2012) *J. Agric. Food Chem.* **61**, 5659–5668, doi:10.1021/jf3015238
(58) Gomaa, A., & Boye, J. (2015) *Food Chem.* **175**, 585–592, doi:10.1016/j.foodchem.2014.12.017
(59) Planque, M., Arnould, T., Dieu, M., Delahaut, P., Renard, P., & Gillard, N. (2016) *J. Chromatogr. A* **1464**, 115–123, doi:10.1016/j.chroma.2016.08.033