Detection of peanut in legume containing food products

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Abstract
Food allergies affect 4% of adults and 8% of children, and each year, about 29,000 cases of anaphylaxis occur in allergic individuals, resulting in roughly 150 deaths. Peanut allergy affects about 0.6% of adults and 0.8% of children in the U.S. is one of the most severe allergies, causing potentially life-threatening reactions. Patients do not generally outgrow peanut allergy, and since there is currently no cure, avoidance of peanut is the only option for the allergic population. The Food Allergen Labeling and Consumer Protection Act of 2004 (FALCPA) mandates that manufacturers label major allergens on food labels. However, inadvertent cross-contact of allergens is still possible, threatening the health of allergic individuals. Peanuts belongs to the legume family and contain homologous proteins to those present in other legume species. In recent years, foods have been recalled due the presence of peanut in legume containing products. Current commercial ELISA methods face challenges in detecting peanut in legume containing products due to cross-reactivity issues. In this study, the limitations of the ELISA methods have been addressed by using orthogonal methods, including the multi-analyte profiling food allergen detection assay (xMAP FADA) and a DNA-based PCR method targeting regions of the peanut chloroplast genome, to detect the presence of peanut in legume containing food products.

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Development and Validation of a Multiplex Real-time PCR Assay to Detect Allergenic Peanut in Complex Food Matrices

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Abstract
Peanut causes some of the most common and most severe allergic reactions to food in the United States. Since there is currently no treatment for food allergy, allergic individuals must rely on accurate labeling avoid offending foods. This requires detection methods that are highly sensitive, specific, and robust in food products. Previous work has demonstrated that real-time PCR is a well-suited and advantageous orthogonal approach to the more standard protein-based allergen detection assays. In this work, we developed and validated a multiplex real-time PCR method for detection of peanut in food products. The assay utilizes three different peanut-specific targets, all derived from the chloroplast genome. Extensive in silico design and evaluation of primers and probes was carried out prior to thorough testing in the laboratory before final assay targets were chosen. Food products were spiked with known quantities of peanut, then subject to thorough homogenization in detergent buffer and DNA extraction according to procedures previously established in our laboratory. PCR conditions were optimized prior to final validation in foods. The method proved to have high reaction efficiency and linearity over 6-7 orders of magnitude, with lower limits of detection at 0.1-1 mg/kg (parts per million) in baked goods, chocolate, and tomato-based products. This is the first peanut detection assay we know of that targets chloroplast markers in a real-time multiplex PCR format and has been thoroughly tested in a variety of food products.

Poster Category: Allergens
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The curation of transcriptomic data for use as a proxy protein database for unsequenced tree nuts

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Abstract
As the apparent incidence of tree nut allergies rises, the development of mass spectrometric methods that accurately identify closely related tree nuts in food is critical. The outcome of LC-MS/MS experiments relies on the availability of protein sequences for database construction, however, these data are often sparse, particularly for non-model species such as tree nuts. We showed previously that translated transcriptomic data can serve as a proxy protein database for the analysis of walnut samples. However, published transcriptomes are not always available, and instead must be re-assembled from archived RNA-Seq reads. Reads typically undergo several pre-assembly processing steps including read quality trimming, error correction, and normalization. The optimal implementation of these steps for high quality, complete transcriptomes is currently under debate, and no information is available regarding their effect on MS/MS search results. Here, we determine the impact of read processing workflows on walnut transcriptome quality as well as downstream peptide identifications. Raw English walnuts, Juglans regia, were extracted and subjected to nLC-MS/MS on an Orbitrap Elite mass spectrometer. Spectra were searched in MASCOT against eight custom transcriptomic databases constructed using stringent versus relaxed read quality trimming, the application of read error correction or not, and two commonly used normalization standard deviation values (100 vs 10,000). A 3-way ANOVA was used to compare the mean number of peptides identified when searching against each database (n=3). Searches of databases constructed using read error correction and/or a standard deviation value of 100 identified a significantly higher mean number of peptides than databases constructed without read error correction and a standard deviation value of 10,000. In addition, we compared the number of peptides identified when searching against a database constructed from nut-specific reads (immature fruit and embryo) versus one made from 16 combined walnut tissues.

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The Selection of Tree Nut Peptide Markers: A Need for Improved Protein Sequences Databases

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Abstract
Mass spectrometry-based detection of allergenic foods relies on the systematic identification of robust and selective peptide markers. The selection of proteotypic peptide markers depends on the availability of high-quality protein sequence information, a bottleneck for the analysis of many plant-based proteomes. The goal of this work was to establish a workflow for the selection of walnut peptide markers, which includes the implementation of a comprehensive protein database and the empirical evaluation of closely-related food ingredients and commercial food commodities. Raw and roasted varieties of common tree nuts (English walnut, black walnut [raw only], pecan and hazelnut) and commercial walnut-containing samples were ground, defatted, and extracted for total protein content. Sample extracts were concentrated and digested using a modified filter-aided sample preparation (FASP) protocol and LC-MS/MS data were evaluated applying a parsimony-driven global proteomics workflow. A two-tier strategy was applied to select candidate peptide markers whereby traditional selection criteria including empirical MS identification, physiochemical properties, and specificity were complimented by differential peptide-based profiling of peptide markers in processed (e.g. roasted) ingredients and peptide presence in commercial food commodities. Candidate peptides (5 peptides per protein family) were selected from priority (high abundance) allergen protein families as the foundation for targeted MS method development. Using walnut as a case study, this work establishes criteria for the selection of candidate peptide markers in tree nut proteomes. Moving forward, improved protein databases will provide a more comprehensive view of tree nut protein and peptide identifications and help establish highly-specific confirmatory workflows for the detection and quantification of allergenic foods.

Poster Category: Allergens
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Western Blot Analysis of Fermented-hydrolyzed Gluten Utilizing Antibodies Employed in a Novel Multiplex-Competitive ELISA

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Abstract
Methods are lacking for the detection and quantitation of gluten in foods subjected to fermentation and other forms of processing that results in hydrolysis. In this study, gluten specific antibodies (G12, R5, 2D4, MIoBS and Skerritt), from nine commercial gluten ELISA test kits, previously utilized in the development of the multiplex-competitive ELISA, were utilized in western blot analyses of 59 fermented-hydrolyzed foods from four food groups (beer, soy-based sauces, vinegar, and sourdough bread). Cluster analysis of the estimated gluten concentration values (based on western blot band intensities relative to intact gluten standards at 2.5 µg/mL and 100 µg/mL) distinguished products among the different categories of fermented-hydrolyzed foods, comparable to what was observed in the multiplex-competitive ELISA. Further, unlike the multiplex-competitive ELISA, the western blot analyses distinguished between the presence of antigenic proteinaceous materials and false positives due to the presence of binding inhibitors (observed with four soy-based sauces and one vinegar). Western blot analysis provides an orthogonal approach that can be used to both confirm the multiplex-competitive ELISA while also providing additional insight into the protein/peptide profile of fermented-hydrolyzed foods. The use of the two complementary approaches should assist in selecting appropriate calibration standards that may be useful in the qualitative and quantitative characterization of gluten in fermented-hydrolyzed food products.

Poster Category: Allergens
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Measurements of Specific Milk Allergens in Baked Food Challenge Materials

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Abstract
Rationale: Oral food challenges (OFC) are considered the 'gold standard' to diagnose a true food allergy. Allergists use baked milk food preparations for OFC under the assumption that they contain decreased allergen levels due to baking. However, the effects of baking on specific allergens has not been thoroughly investigated. The aim was to compare levels of major milk allergens and IgE reactivity in uncooked and baked milk challenge materials currently used in clinical practice.

Methods: Uncooked and baked muffins were prepared using recipes from Mount Sinai (Jaffe Food Allergy Institute) and the UK National Health Service (NHS). Allergen levels were compared using a two-site monoclonal antibody ELISA for beta-lactoglobulin (Bosd5) and for beta-casein (Bosd11). IgE reactivity was assessed using sera from milk-allergic patients in direct binding and inhibition ELISA.

Results: Bosd5 (β-lactoglobulin) concentration decreased from 722µg/g in uncooked muffin mix to 0.18µg/g in baked muffin, representing >99% reduction in Bosd5 allergen. The level of Bosd11 (β-casein) decreased by 32% from 5,640µg/g in uncooked muffin mix to 3,828µg/g in baked muffin. Bosd11 levels in the Mount Sinai muffins (n=35) were higher compared to the NHS muffins (n=15) and varied depending on whether the baked muffin was sampled from the top, middle or bottom. Baked muffins retained ~70% of the IgE reactivity in uncooked muffin mix while baked muffin extracts inhibited IgE antibody binding to uncooked muffin by up to 80%.

Conclusions: The level of major milk allergen Bosd11 remained high in baked muffins used in oral food challenges. These findings emphasize the potential risk for adverse reactions to baked milk challenges, especially in patients who have high anti-casein IgE antibodies. Measurements of specific milk allergens, together with IgE molecular diagnostics, should improve the safety of food products used for OFC and reduce the risks associated with milk challenges in clinical practice.

Poster Category: Allergens
Poster Number: ALGN-06

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Simultaneous Quantification of Major Food Allergens Using a Multiplex Immunoassay

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Abstract
Rationale: Quantification of food allergens is increasingly important for dose assessments of food preparations used in oral food challenges (OFC), food allergy prevention, and monitoring safety in the food industry. Generic immunoassays for 'total protein' do not measure specific allergens. Our aim was to use a molecular approach to food allergy to develop a multiplex immunoassay capable of simultaneously measuring specific allergens, the 'active ingredients', from peanut, cow's milk, shellfish, egg, cashew, soy and hazelnut.
Methods: The multiplex array was developed on the Luminex xMAP system. Microspheres coupled to specific monoclonal antibodies were used for allergen capture. Biotinylated specific monoclonal or polyclonal antibodies were used for detection. Reference standards were formulated from natural or recombinant allergens, with purity established by mass spectrometry. Full method validations were performed to determine parameters of linearity, range, limits of quantification and detection, accuracy and precision of the multiplex food immunoassay.
Results: Method validations were completed for the major food allergens. Standard curves for all analytes allow for quantification over a broad dynamic range. Limits of detection were as low as 0.01ng/ml. Intra- and inter- assay accuracy and precision of three samples assayed in triplicate on four occasions passed acceptance criteria within the range of 70-130% recovery and a coefficient of variation of <15%. Food products and the NIST SRM 2387 Reference Standard were analyzed using the multiplex immunoassay.
Conclusions: A quantitative, accurate and precise multiplex immunoassay was validated for the simultaneous detection of major food allergens. The multiplex array provides a sensitive and efficient tool for measuring specific food allergens, as opposed to generic food source proteins, with potential applications for risk assessment in the food industry and standardization of clinical OFC.

Poster Category: Allergens
Poster Number: ALGN-07

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xMAP FADA: A multiplex method for simultaneous detection of 15 food allergens plus gluten

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Abstract
The Food Allergen Labeling and Consumer Protection Act of 2004 (FALCPA) imposes strict requirements for labeling of foods containing milk, eggs, fish, Crustacean shellfish, tree nuts, peanuts, wheat, or soy. In order to ensure the accuracy of labels, FDA relies on highly sensitive and highly specific allergen detection methods. The xMAP Food Allergen Detection Assay (xMAP FADA) is a unique and powerful analytical method for the simultaneous detection of almond, brazil nut, cashew, coconut, crustacean, egg, gluten, hazelnut, macadamia nut, milk, peanut, pine nut, pistachio, sesame, soy, and walnut in complex matrices. A strength of the xMAP is its redundancy: the use of at least two antibody capture-based assays per target analyte, except for crustacean, provides built-in confirmation of positive responses and the two most commonly employed extraction protocols, buffered-detergent (32-plex) and reduced-denatured conditions (5-plex). The use of multiple antibodies for each allergen permits the calculation of complimentary antibody bead set ratios, thereby allowing results to be interpreted, distinguished from cross-reactivity, and simultaneously confirmed. The high sensitivity of the xMAP technology further enables the use of multi-antibody profiles as an additional form of secondary end-point confirmation. The xMAP FADA is currently being used by the FDA for the analysis of regulatory samples, providing a powerful analytical solution particularly when the presence of multiple food allergens is possible. Additionally, the xMAP FADA has been used to analyze botanicals including dietary supplements and spices. These commodities commonly contain homologous proteins that may cross-react with the antibodies and in ELISA analyses generate false positives. Besides reducing cost and increasing through-put, the xMAP FADA provides a level of definitive detection, identification, and characterization not possible using ELISA technology.

Poster Category: Allergens
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Optimization of a targeted, multi-allergen LC-MS/MS method for the quantification of egg, milk, and peanut in food

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Abstract
Liquid chromatography (LC)-tandem mass spectrometry (MS/MS) can be used as a complimentary analytical technique to immunochemical-based assays for allergen detection. A targeted, multi-allergen LC-MS/MS method has been previously developed for the simultaneous detection and quantification of egg, milk, and peanut. In bakery products, the method provided reliable detection of each allergen at concentrations as low as 5 mg/kg (ppm) incurred allergen ingredient using peptide markers from egg (lysozyme C and ovalbumin), milk (beta-lactoglobulin and alpha-S1 casein), and peanut (Arah1, Arah2, and Arah3). In the absence of established guidelines for MS-based quantification of allergens in food, the goal of this work was to evaluate different modes of quantification, establish acceptance criteria, and demonstrate transparency in the utilization of conversion factors. Allergen fortified, commercial cookie samples were homogenized, defatted, and extracted for total protein content. Sample concentration and trypsin digestion was performed using a modified filter-aided sample preparation (FASP) protocol. Isotopically-labeled synthetic peptides were added as peptide surrogates. The samples were analyzed on multiple LC-MS/MS platforms under both nano- and standard-flow LC conditions using a targeted multiple reaction monitoring (MRM) method on a 6500 QTRAP or nano-LC conditions using a parallel reaction monitoring (PRM) method on a Q Exactive. Method performance including accuracy, precision, limits of detection, and quantification were evaluated for different standards (isotopically-labeled peptide surrogates and chemically analogous protein/peptides), reference materials (synthetic native peptides and allergen standards), and instrument platforms. The suitability of each method platform for routine analysis was evaluated to provide a robust workflow that can be used in support of allergen management within the food industry and the protection of consumers with food allergies.

Poster Category: Allergens
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Workshop

Development of an in vitro Bio-assay using Human Intestinal and Immune Cell-lines to Measure the Immuno-pathogenicity of Food Allergens

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Abstract
Food allergies are a rapidly growing public health problem that affect >15 million Americans. The FDA's Food Allergen Labeling and Consumer Protection Act (FALCPA) requires that foods containing allergenic proteins derived from the eight major food allergens be declared. As strict avoidance is the only option for allergic consumers, accurate methods are needed to ensure correct labeling. The currently used Immunochemical methods (e.g. ELISA) detect IgG antigenic epitopes, and not allergenic elements. Hence, immunochemical methods may not detect antigenic epitopes that are transformed during food processing, while the immuno-pathogenicity could continue to persist. To address this gap in analytics, an in-vitro bio-assay that employs human intestinal epithelial and immune cell-lines to measure the biological effects caused by food allergens was developed. This novel biological activity-based assay compares the allergen-induced immuno-biological responses in Caco-2, HT-29 & T84 intestinal epithelial cells individually, as well as each co-cultured together with THP-1 cells. The goals of this project are to compare the cell signaling and immune modulation induced by different food allergens in a dose-dependent manner, and to further develop an in-vitro bio-assay using these cell lines.

Poster Category: Allergens
Poster Number: ALGN-10

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Characterization and Certification of Milk Proteins as Certified Reference Materials

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Abstract
Milk proteins are important nutritional constituents of milk and important contaminants in allergen testing of food products. Whey proteins, α-lactalbumin, β-lactoglobulin and lactoferrin have physiological activity through moderating gut micro flora, mineral absorption and immune function. Caseins are the main protein constituent in milk and cheese and comprised of related phosphoproteins (αS1, αS2, β, κ). Milk proteins are ingredients of choice in the formulation of modern foods, beverages, infant formula and nutritional supplements. However, milk proteins can also cause severe allergic effects and are subject of contamination control and testing in certain food products. New regulations for infant formula, food and allergen testing as well as advances in quantitation of proteins by LC-MS/MS[1] has led to the need for fully-intact milk protein Certified Reference Materials (CRMs) for accurate quantitation. Research grade proteins do not provide adequate accuracy and consistency in performance. Challenges with development, handling and certification of fully-intact protein CRMs will be presented. Critical factors in CRM development include proper characterization of the protein and its isoforms, certification of protein content, and control of vial-to-vial homogeneity. Research grade whey proteins were purified and lyophilized for development of a CRM. Certification was performed by mass balance incorporating purity by HPLC-UV-MS, water content by Karl Fischer Coulometry, and inorganic content by ICP-MS and residue on ignition. Suitable packaging was developed to preserve protein content. Accelerated stability and real time stability include assessment of water content and purity. Preliminary work on characterization of Casein by LC-UV/MS will also be presented.

Poster Category: Allergens
Poster Number: ALGN-11

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Food allergen reference materials - addressing an unmet need

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Abstract
Walker et al.[1] described the framework required to address problems detecting and quantifying allergenic proteins in foods at clinically relevant concentrations. Part of the solution is the provision of reference materials (RM). As well as two existing peanut allergen QC materials, LGC has recently worked with the University of Manchester (UoM), and Romer Laboratories Ltd to prepare a RM kit which addresses five additional allergens, in the form of skimmed milk powder, egg white powder, almond powder, hazelnut powder and walnut powder. Clinical relevance derives from the matrix and the incurred allergens. The matrix, a cold swelling starch-based chocolate flavoured paste, was used as the challenge vehicle for EuroPrevall food challenge studies. It was later shown to be suitable as an incurred QC material for the analysis of allergens in food. The incurred allergens are clinically relevant as they feature as priority allergens in food labelling law. The RM kit is industrially relevant as a processed food matrix of medium analytical difficulty owing to its polyphenol content with commonly used ingredients. The kit (LGC746-KT) contains the allergenic ingredients, the blank matrix and the incurred matrix containing each allergenic ingredient at 10 mg/kg of the allergenic protein. The allergens have been characterised by UoM by discovery proteomics and LGC-led homogeneity and stability studies (analysis by Romer), which along with in-house LGC work estimates the ELISA derived concentrations of 3 of the allergens. Some limited characterisation by molecular biology has also taken place within LGC. The kit contains instructions for use, indicative ELISA data and clear information on protein conversion factors.
References: [1] Michael Walker et al. 2016, Flawed food allergen analysis–health and supply chain risks and a proposed framework to address urgent analytical needs, Analyst, 141, 24 - 35

Poster Category: Allergens
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Reference materials for food allergen analysis

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Abstract
Effective food allergen risk assessment and food allergen management are important to protect allergic consumers and to comply with allergen labelling regulations. Such approaches require reliable analytical tools for the detection of allergens in food. Both reference methods and reference materials are urgently needed to assure the quality reliability and compatibility of analytical results obtained with different methods. Being an important component of this analytical quality assurance, reference materials contribute to reliable and accurate results. Ensuring the correctness of analytical results is crucial to laboratories, since incorrect results may trigger decisions that can cause economic damage or pose a risk to public health. Validated reference materials/quality control materials and certified reference materials are indispensable for ⇒ Method development ⇒ Method calibration ⇒ Calibration of instruments ⇒ Validation of methods ⇒ Method verification ⇒ Proficiency testing ⇒ Process control and quality assurance in laboratory routine Besides, the use of reference materials is required by ISO/IEC standards.

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