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Sara Schlange
University of Nebraska-Lincoln, sschlange2@huskers.unl.edu

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DEVELOPMENT OF A TARGETED MASS SPECTROMETRY METHOD FOR THE DETECTION AND QUANTIFICATION OF PEANUT PROTEIN IN INCURRED FOOD MATRICES

by

Sara K. Schlange

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DEVELOPMENT OF A TARGETED MASS SPECTROMETRY METHOD FOR THE DETECTION AND QUANTIFICATION OF PEANUT PROTEIN IN INCURRED FOOD MATRICES

Sara K. Schlange, M.S.

University of Nebraska, 2022

Advisors: Philip E. Johnson and Melanie L. Downs

The unintentional presence of peanut in food products through allergen cross-contact is a considerable safety concern for peanut-allergic individuals. The food industry monitors for this contamination using immunoassays; however, these detection methods demonstrate issues with recovery and accurate quantification of allergenic protein when analyzing processed, complex food matrices. Of particular concern is the deficit in immunoassay-based detection and quantification of peanut in cookie and dark chocolate matrices, as the unintentional presence of peanut has been observed in these food products. A liquid chromatography with tandem mass spectrometry (LC-MS/MS) method for the detection and quantification of peanut protein in cookie and dark chocolate was developed to overcome the issues plaguing immunoassays in analysis of peanut in these matrices.

Peanut-incurred cookie and dark chocolate matrices were generated at various concentrations of peanut. Untargeted MS analysis of incurred matrices identified and quantified peanut peptides. Peptides were subjected to selection criteria, based on abundance and robustness in matrix, to determine 32 (cookie) and 67 (dark chocolate) candidate target peptides for the method. Candidate peptides were filtered to determine

robust and sensitive target peptides in each matrix using iterative rounds of targeted MS. Six (cookie) and seven (dark chocolate) final peptides were determined. This resulted in nine unique peanut peptides for the method.

A quantitative strategy was developed based on stable isotope labeled (SIL) peptides and an external calibration to peanut flour (PF). Quantification was reported in parts per million (ppm) peanut protein. Optimization of various aspects of the method, including instrument parameters, LC, and sample preparation, improved the method's sensitivity and variability. The LC-MS/MS method was evaluated with incurred matrices and demonstrated highly sensitive and reliable detection, even at low concentrations of peanut protein (1.24 ppm peanut protein in cookie and 2 ppm peanut protein in dark chocolate). This sensitivity is sufficient to detect peanut concentrations relevant for the most sensitive peanut-allergic individuals.

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I. INTRODUCTION

Food, though essential to human life, can elicit a number of adverse reactions following its consumption. There are a wide variety of such negative reactions, however one of the most prominent examples is food allergy. A food allergy is an individualistic hypersensitivity reaction to food proteins, which can result in a range of symptoms, from mild to life threatening[1, 2]. One important allergenic food source is peanut, which effects approximately 2% of US individuals and elicits particularly severe reactions[3-5]. In order to protect allergic consumers in the United States (US), there are regulatory requirements for the food industry in their labeling of allergens on food products and in their management of food allergens throughout food processing[6, 7]. To comply with the US governance of allergens, the food industry has adopted several detection methods for the purposes of identifying and quantifying the presence of food allergens in products. Detection methods face a wide variety of issues in detecting and accurately quantifying peanut protein in certain food matrices. One approach to the detection and quantification of food allergens that shows promise for overcoming some of the issues plaguing current detection methods is mass spectrometry (MS).

II. FOOD ALLERGY

Adverse reactions to food can be mediated by an individual's immune system or non-immune mediated. Reactions which are not facilitated by the immune system can have a metabolic, pharmacologic, toxic, or undefined mechanism[8]. Metabolic reactions include intolerances, such as the malabsorption of lactose due to the enzymatic deficiency

implicated in lactose intolerance[9]. Pharmacologic reactions result from a biochemical or physiological effect of food ingredients, for example the effect of caffeine[10]. When food is contaminated with a particular dose of chemical compounds, like fertilizers, or natural compounds, like mycotoxins, toxic reactions to food can occur[11]. There are also reactions to food additives, like sulfites, which have an undefined or poorly understood mechanism[8]. These adverse reactions to food are not considered food allergies.

Immune-mediated mechanisms include a wide variety of reactions which can be further classified as immunoglobulin E (IgE)-mediated, non-IgE-mediated, cell-mediated, or mixed and non-IgE mediated reactions[10]. Food allergy is an immune-mediated reaction of particular importance and is considered a global health concern due to its prevalence and implications for individuals affected[12].

Food allergy is defined as an individualistic hypersensitivity reaction to a food protein facilitated by the immune system[1]. A hallmark characteristic of food allergy is the reproducibility of the reaction in allergic individuals each time the implicated food is consumed[13]. Food allergy can elicit an extensive array of symptoms and can be caused by, in theory, any protein found in food. There are two important types of allergic reactions to food proteins including non-IgE-mediated food allergy and IgE-mediated food allergy.

i. Non-IgE-Mediated Food Allergy

Non-IgE-mediated food allergies are adverse reactions to a food protein which are characterized by the absence of IgE antibodies in the induction of this allergic reaction, rather cell components cause the clinical allergenic response[14]. Though mechanisms of

non-IgE-mediated food allergy are variable by disorder and not fully understood, there is evidence that immune cells, like T cells, B cells, macrophages, and eosinophils, plus their resulting cytokines are involved in the inflammatory response to the food protein[15, 16]. T cells may play a particularly important role in these reactions as food-specific T cells have been isolated and identified in individuals with non-IgE-mediated food allergies[17].

Non-IgE-mediated food allergies are known as delayed hypersensitivity reactions, meaning symptoms present a number of hours after the food has been ingested[18]. This onset time of symptoms can vary from six hours to over 48 hours following the consumption of the implicated food[1]. The most prominent disorders which are classified as non-IgE-mediated food allergies are food protein-induced enterocolitis, food protein-induced proctitis, food-protein induced enteropathy, and celiac disease[19]. The gastrointestinal tract or the skin is the primary location of symptoms resulting from these reactions. In fact, the type of T cell implicated in the reaction varies based on this location, T helper 1 (Th 1) cells are involved in gastrointestinal reactions and cutaneous lymphocyte antigen (CTA+) cells in skin-based reactions[17]. As a result, some of the most common symptoms associated with non-IgE-mediated food allergy are vomiting, atopic eczema, nausea, and diarrhea[13, 15]. Individuals diagnosed with a non-IgE-mediated allergy typically adopt a diet which avoids any consumption of the allergenic food[20]. Though non-IgE-mediated food allergies are important, much of the focus for the food industry, in the literature, and for research is on IgE-mediated food allergies, as these are the more common cause of food allergy[15].

ii. IgE-Mediated Food Allergy

IgE-mediated food allergies are designated as such based on the involvement of food-specific IgE antibodies in the allergic reaction[14]. These hypersensitivity reactions are the food allergy reactions which are most common, best characterized, and most well-known[15, 21]. Though IgE plays a beneficial role in host defense against parasites and venoms, it is an antibody type that is deleterious to humans in the case of food allergy[22]. IgE antibodies play a crucial role in both steps of a food allergy – sensitization and elicitation.

The development of an IgE-mediated food allergy begins with sensitization, a mechanism in which the exposure to allergenic food proteins initiates the production of allergen-specific IgE antibodies which bind to mast cells or basophils[23]. B cells are responsible for the production of these specific IgE antibodies[24]. The binding of an IgE antibody to these effector cells is facilitated by the high-affinity IgE receptor (FcεRI) or the low-affinity IgE receptor (FcεRII)[25]. After specific IgE antibodies are bound to mast cells or basophils, the individual is considered primed for an allergic reaction and sensitized to the allergenic protein. It is important to clarify that sensitization alone does not equate to allergy as the presence of specific IgE in a patient's serum does not confirm the individual will react to the implicated food[13].

Upon subsequent exposure to an allergenic protein in a sensitized individual, elicitation of an allergic reaction occurs. The allergenic protein crosslinks the IgE antibodies bound to sensitized cells, which triggers the release of mediators, like histamine, from the cells into the bloodstream to initiate a wide variety of physiological

responses[1]. The release of mediators from mast cells or basophils is known as degranulation. In addition to histamine, other mediating molecules can be released, including heparin, tryptase, chymase, prostaglandins, leukotrienes, and other cytokines[25]. These released molecules then mediate the hypersensitivity reaction by inducing vasodilation, increasing vascular permeability, stimulating nerves and muscles, and activating the complement system[23]. This results in the immediate onset of the symptoms typically manifested in an IgE- mediated allergic reaction.

Food allergy is thought to be caused by a variety of factors, including genetic influences such as loss of function mutations or polymorphisms in tolerance-associated genes, and environmental factors like reduced microbial exposure or vitamin D deficiency[26]. Additionally, the dual allergen hypothesis suggests that early oral exposure to food allergens is more likely to induce tolerance, whereas epicutaneous exposure from the environment is more likely to result in sensitization to the allergen[27].

a. Prevalence of Food Allergy

The prevalence of IgE-mediated food allergy in children and adults has been continually increasing in recent decades and is now deemed a major health concern for many westernized nations[2, 28]. In the US, it is estimated that around 32 million individuals have food allergies, including 5% of children and 3-4% of the adult population[26, 28, 29]. Over 90% of all food allergic reactions in the US are due to the presence of eight allergenic foods which are known as the "Big Eight"[30]. These eight allergenic foods are wheat, peanuts, tree nuts, eggs, soy, crustacean shellfish, fish, and milk. In children, the most common food allergy is peanut (2.2%), followed by milk (1.9%), shellfish (1.3%),

and tree nut (1.2%)[3]. Among adults, shellfish (2.9%), milk (1.9%), peanut (1.8%), and tree nut (1.2%) are the most common allergenic foods[31]. Of individuals with food allergy, including children and adults, approximately 40-45% report having multiple food allergies[3, 31]. Of the US population, 1.3% of individuals are allergic to more than one food[32]. In children, 2.4-3.4% have multiple food allergies[32, 33]. The prevalence of individuals who are allergic to more than one food follows a clear trend of decreasing as the individual ages[32]. For children 1-5 years old, 3.4% have multiple food allergies, for individuals over 60 years old the prevalence decreases to 0.6%[32]. Individuals with multiple food allergies are required to avoid multiple foods in an elimination diet and this may affect growth in children and nutrition in adults[34]. One phenomenon associated with food allergy prevalence data is that in studies which require individuals to self-report their food allergy, the prevalence is commonly over-estimated compared to the true prevalence of food allergy which emphasizes the importance of objective diagnostic methods and criteria[8]. Additionally, the prevalence of food allergy has been correlated with certain factors of individuals including age, race, income, and geographic region[33].

b. General Food Allergy Symptoms

As these reactions are IgE-mediated, symptoms are immediate and typically present within minutes to one or two hours after ingestion[13]. Symptoms of allergic reactions can present in multiple different organ systems and can range in severity from mild to life-threatening[2]. The cutaneous, gastrointestinal, respiratory, cardiovascular, and neurological system can all exhibit symptoms following elicitation[13]. Some of the most common symptoms include nausea, urticaria, pruritis, abdominal cramping, rhinitis, and

swelling[35]. The most severe symptom is anaphylaxis, a potentially fatal reaction which can include a drop in blood pressure, difficulty breathing, or shock[36]. Symptoms of food allergy and their severity may vary based on the allergen, form of the allergen, amount of allergen ingested, and sensitivity of the individual[1].

c. Diagnosis of Food Allergy

An accurate diagnosis of food allergy is critical, not only for the safety of the individual, but also because a diagnosis comes with economic, social, and emotional costs to the diagnosed. The first step in diagnosis of a food allergy is evaluation of the medical history of the patient[37]. After food allergy is suspected as the condition affecting the patient, the next step is typically a skin prick test (SPT). In an SPT, extracts from allergenic foods are injected into the epidermis to bind and detect IgE to the allergenic proteins[38]. Wheal size resulting from SPT is used to confirm sensitization but does not confirm allergy[5]. Food-specific serum IgE (sIgE) testing is also used to diagnosis food allergy, as an increase in sIgE is associated with increased probability of allergic reaction[5]. The most preferred method for diagnosis of food allergy is the oral food challenge (OFC). OFCs require the patient to consume incremental amounts of the suspected allergenic food and any symptoms are recorded and used for diagnosis[37]. A double-blind, placebo-controlled, food challenge (DBPCFC) is the gold standard for the diagnosis of allergy, but due to the cost, time required, and potential for severe reactions, many clinicians rely on skin SPT, sIgE testing, and convincing patient reaction history[39].

d. Food Allergy Treatment

Currently there is no cure for food allergy, instead individuals must simply manage their hypersensitivity. To manage a food allergy, individuals adopt an avoidance diet in which they eliminate any consumption of the implicated allergenic food from their dietary intake. However, an avoidance diet is not entirely effective as approximately 10% of allergic individuals experience an allergic reaction each year though attempting to avoid their allergen[40]. In the case that a severe allergic reaction does occur, the recommended treatment is administration of epinephrine injected intramuscularly to reduce and reverse the symptoms of allergy[41]. One treatment option, growing in popularity over recent years, is immunotherapy. Immunotherapy attempts to induce desensitization in an allergic individual by repeatedly and incrementally exposing the individual to a dose of their allergen. This is thought to cause the individual to be less reactive to the allergen and increase their tolerance through elevated interleukin-10 and IgG4 production, which suppress the allergic response[42]. While currently viewed as a valid treatment option for some allergic individuals, the long-term efficacy of immunotherapy is uncertain[43].

e. Resolution of Food Allergy

As opposed to the desensitization induced by immunotherapy, some individuals will spontaneously outgrow their food allergy through what is termed as resolution[42]. Though the exact mechanism is unknown, those with resolved food allergy have shown increased populations of T regulatory cells in individuals and an increase in secretion of interleukin-10[44]. Rates of food allergy are typically consistent throughout early

childhood, but the prevalence decreases by approximately 2.3% by age 10 due to the resolution of allergies in this time frame[45]. There are certain food allergies which are more likely to resolve compared to other food allergies. For example, allergies to milk or egg are more likely to resolve, while peanut or tree nut allergies are more likely to persist[46]. Persistence of a food allergy has been associated with a number of factors, including more severe symptomology, low threshold dose, and the presence of other atopic diseases[46].

f. Cost of Food Allergy to Stakeholders

Food allergy comes at a significant cost to many stakeholders, including the individual diagnosed, family members, food manufacturers, and other members of the food industry. In fact, the overall economic cost of food allergy in children alone is estimated to be over $24 billion annually[47]. The cost of food allergy is not only economic, but also has social and emotional effects on allergic individuals and their families. Food allergy can cause anxiety for a number of reasons, including uncertainty surrounding diagnosis and fear of reaction[48]. Parents of children with food allergy report an emotional impact of having a child with food allergy, as well as a restriction in activities the family or individual participates in[49]. Food allergy has also been linked to growth impairment in children with food allergy and nutritional deficits in allergic individuals[50].

III. PEANUT AND PEANUT PROTEINS

Peanut, *Arachis hypogaea,* is a tetraploid organism belonging to the *Fabaceae* family, which is commonly referred to as the legume family[51]. Peanut or “groundnut” is a

legume and oilseed by classification, not a nut, and produces its seeds underground, yet flowers above the soil[52]. Peanut seeds, which are round or oblong, develop within the plant's underground pods in two-seeded or three-seeded varieties[53]. *A. hypogea* is divided into two subspecies *hypogea* and *fastigiata*, each having several varieties[52]. Peanuts have a long history of evolution, cultivation, and use as a food product. Due to their macromolecular composition, peanuts are considered a valuable crop, ingredient, and food product.

i. Origin of Peanut

Domesticated peanut, which is widely cultivated and used today, is the result of an ancient evolutionary hybridization event followed by selection and cultivation of the species by man[54]. The exact date of hybridization is not known, but this cross is estimated to have occurred over 4,500 years ago[55]. The origin of *A. hypogea* occurred in South America, specifically in the regions of southern Bolivia and northern Argentina[56]. In the hybridization event, two diploid progenitors crossed and formed the tetraploid organism, an organism containing four copies of each chromosome. The crossing organisms have been determined by cytogenetic analysis to be *A. duranensis* and *A. ipaensis*[56]. Because this hybridization and ensuing polyploidy occurred between two different species within the *Arachis* genus, *A. hypogea* is an allotetraploid[52]. Following hybridization, ancient peoples began cultivation and the early stages of domestication.

ii. Peanut Cultivation

Human cultivation of tetraploid peanut is suggested to have occurred as early as 1200-1500 B.C. in Peru, according to palaeobotanical artifacts[56]. Over time, the

cultivation of peanuts expanded across Central and South America[57]. By the time of Spanish exploration of the region, peanut agricultural activity had spread to many areas, including Mexico, Brazil, Argentina, Paraguay, and Bolivia, as documented by early explorers[58]. After European discovery of peanut, the plant was spread across the globe and was grown in Europe, Africa, Asia, and the US[58].

A. hypogea is now widely cultivated in the modern world. The US produces over 3.1 million tons of peanuts each year[59]. Peanuts are typically grown in tropical, sub-tropical, or temperate climates[52]. Peanuts are harvested around 120-140 days after planting, and the seeds are allowed to dry before storage or further processing[53]. In the US there are three primary growing regions for peanut; Southeast (Alabama, Florida, Georgia), Southwest (New Mexico, Oklahoma, Texas), and Virginia-Carolina (North Carolina, South Carolina, and Virginia[60]. There are four market types of peanuts which are produced in the US, including Runner, Virginia, Spanish, and Valencia. The predominant market types grown in the country are Runner and Virginia, and Runner types account for approximately 80% of all peanuts in US[60, 61]. These market types are characterized by different physical features, including kernel size and skin color.

iii. Peanut Consumption

Peanut seeds are widely consumed in the US. Per capita consumption for the US is estimated at approximately 7.1 pounds of shelled peanut annually[62]. Peanut products can take a variety of forms, some of the most common include peanut oil, peanut butter, roasted snack nuts and mixes, and peanut flour[63]. In the US, the most popular consumption of peanuts is through peanut butter products, which account for 50% of the

peanuts consumed in the country[53, 64]. Peanut oil is produced by extracting oil from shelled and crushed peanuts and is often used for cooking or frying[65]. Peanut butter production begins with roasting and grinding the peanut seeds, followed by the addition of additives such as sugar and salt[66]. Roasted peanut snacks are typically produced by frying the nut and coating the outside with sweet or salty flavor components[63]. Peanut flour is obtained by processing raw or roasted peanuts with a heat treatment, removal of oil, and drying[65]. Peanut flour is commonly used in confections, nutritional bars, and baked goods and is often added for its protein content as it is approximately 50% protein[65].

iv. Composition of Peanut

Peanuts are recognized as a nutritionally dense food due to their macromolecular composition. Raw peanuts are comprised of 49.2% lipids, 25.8% protein, and 16.1% carbohydrates, as well as low amounts of water and ash[67]. The lipid fraction of peanuts, predominantly triacylglycerols, is high in unsaturated fatty acids and is often extracted for peanut oil products[53, 67]. The lipid content of a peanut seed can vary from 36-54% depending on peanut type and cultivar[68]. Peanuts have comparable protein contents to other legumes, including chickpea, cowpea, lentil, and green pea which are approximately 24-26% protein[69]. When compared to nuts, including almond, cashew nut, hazelnut, walnut and others, peanut has a greater protein content than all the edible nut seeds[70]. Peanut protein content is known to vary by market type and cultivar[67]. The major carbohydrates in peanut seed are oligosaccharides, including sucrose and stachyose, and starch[71]. Peanuts also include micronutrients such as vitamins and minerals[71].

v. Peanut Protein

As plant seeds, peanuts contain numerous types of proteins that have varying biological functions for the organism. Proteins in plant seeds serve different roles including metabolic, structural, nutrient storage, or defense roles[51, 72]. The primary protein types in peanuts are cupins, prolamins, oleosins, defensins, profilins, and pathogenesis-related proteins[51]. Cupins function as seed storage proteins and the main two types of cupins in peanuts include vicilins and legumins[73]. A seed storage protein is a protein which serves as a deposit of amino acids for later use by the plant during germination and growth[72]. Prolamins can also be divided into two types, 2S albumins and nonspecific lipid transfer proteins (nsLTP)[51]. 2S albumins are seed storage proteins and also act as trypsin inhibitors[51, 74]. Cell wall organization, membrane stability, and signal transduction are roles of nsLTPs[75]. Oleosins function to bind to and cover oil bodies within peanuts for the purpose of stabilization and integrity during seed rehydration[73]. Defensin proteins' primary function is to protect the plant through an innate immune response, mainly against plant pathogens and fungi[76]. Profilins are responsible for binding actin and regulating polymerization[77]. Plant pathogenesis-related proteins have varying enzymatic activities, depending on the protein, that are induced under stress or infection[73].

Peanuts contain all nine essential amino acids, though at low levels compared to the recommendations for intake[78]. The major amino acids in peanuts, based on abundance, are aspartic acid, glutamic acid, and arginine, while the limiting amino acid is

methionine[79]. The amino acids found in peanut protein have been determined to be approximately 39% hydrophobic, 10.5% hydrophilic, 33% acidic, and 17.5% basic[70].

vi. Peanut Allergens

Due to the proteinaceous nature of peanuts and their high consumption, the potential for allergenic proteins is evident. The World Health Organization and International Union of Immunological Studies (WHO/IUIS) has classified 17 peanut proteins as allergenic proteins[80]. Of the 17 peanut allergens, nine show more prominent clinical relevance in the allergic population[81]. Nomenclature for these allergens follows the typical allergen naming in which the first three letters from the genus (Ara), the first letter from the species (h), and a number is used to designate allergens[80]. For example, one allergenic protein from peanut is designated as Ara h 1. Of the 17 identified peanut allergens, four are considered major allergens. These four allergens include two cupins, Ara h 1 and Ara h 3, as well as two prolamins, Ara h 2 and Ara h 6[51, 82]. Ara h 2 and Ara h 6 are specifically 2S albumins, which have been demonstrated as allergenic proteins with high risk for severe reactions[83]. Depending on market type, Ara h 1, 2, 3, and 6 comprise approximately 17.1%, 6.2%, 70.6%, and 5.8% of the total protein in peanut, respectively[84]. Minor allergens include Ara h 7 (2S albumin), Ara h 9, Ara h 16, and Ara h 17 (nsLTPs), Ara h 10, Ara h 11, Ara h 14, and Ara h 15 (oleosins), Ara h 12 and Ara h 13 (defensins), Ara h 5 (profilin), and Ara h 8 (pathogenesis-related protein)[51]. Ara h 4 was once considered to be a separate allergen but is now deemed an isoform of Ara h 3 due to its sequence and amino acid homologies[85]. A protein isoform is a different form of

a protein which can originate from multiple genes or a singular gene which has been alternatively spliced[86].

Several factors are thought to contribute to the allergenicity of these peanut proteins, namely abundance, multiple IgE-binding epitopes, resistance to digestion and processing, and the structure of the allergen[87]. The four major peanut allergens are known to be the most abundant proteins in peanuts[84]. Additionally, Ara h 2 and Ara h 6 have been found to be considerably resistant to digestion by pepsin, a major stomach enzyme[88]. Furthermore, linear epitopes have been well mapped for the major peanut allergens; 21 epitopes have been identified for Ara h 1, 8-10 for Ara h 2, 4 per Ara h 3 monomer, and 7 for Ara h 6[74, 89].

IV. PEANUT ALLERGY

Due to the proteinaceous nature of peanut and its widespread consumption, the potential for allergy to peanut is evident. In the US, peanut allergies are one of the most common food allergies and have been increasing in prevalence over recent decades[31, 90]. Allergy to peanut is characterized by its reaction severity, low threshold doses, and low resolution rates[28, 91, 92].

i. Prevalence of Peanut Allergy

Current estimates of peanut allergy prevalence for individuals in the US are approximately 2.2% for children and 1.8-2.0% in adults[3, 4]. The type of prevalence study and requirements for diagnosis impacts the estimated prevalence of peanut allergy. In a study that used peanut-specific IgE testing and corresponding clinical criteria, peanut allergy prevalence was determined to be 1.3% overall, with age group determinations of

1.8% for ages 1-5, 2.7% for ages 6-19, 0.9% for ages 20-59, and 0.3% ages 60 and above[32]. Using self-reported symptoms and surveyor determinations of probable allergy, another study found peanut allergy prevalence to be 1.4% in children and 0.8% overall[93]. Prevalence enumeration based on peanut allergy diagnosis codes and healthcare databases was used in one study which found prevalence to be 2.2% for children[94]. Among children with food allergy, peanut allergy is the most common as 25.2% of children with food allergy are allergic to peanut[33]. For adults, peanut allergy is the third most common allergenic food, only shellfish and milk allergies have higher prevalence[31]. With respect to multiple food allergies, 25-50% of peanut allergic individuals are also allergic to tree nuts[9]. Both peanut allergy prevalence and incidence is known to be increasing in recent decades[94]. For children, the self-reported prevalence of peanut allergy increased from 0.4% in 1997 to 0.8% in 2002[93]. With current estimates of peanut allergy near 2% for children, there is sufficient evidence that peanut allergy is increasing in prevalence.

ii. Peanut Allergy Symptoms

Peanuts are one of the allergenic foods that are known for severe symptomology[91]. Because a peanut allergy is an IgE-mediated reaction, its symptoms are characterized by acute onset after consumption of peanut protein, typically within seconds or minutes[15, 95]. In fact, the median time elapsed between exposure to peanut and elicitation of symptoms in peanut allergic individuals is three minutes[96]. Exposure to peanut can occur through one of three different routes, ingestion, skin contact, or inhalation, but reactions predominantly occur through ingestion[97]. A majority of peanut-

allergic individuals begin to experience symptoms of allergy within the first two years of life and symptoms are observed after the first known consumption of peanut in 72% of children[9, 96].

Reactions to peanut are particularly severe when compared to allergic reactions elicited by other food sources. For reactions in children with any food allergy, 38.7% of reactions in children are considered severe, however for peanut-allergic and tree nut-allergic individuals, more than 50% have histories of severe reactions[33]. In adults with food allergy, peanut allergy has the greatest proportion of individuals with histories of severe reactions, 67.8%, compared to other food allergies[31]. In a study of allergic reactions to peanut, 54% of reactions involved one organ system, while two organ systems were involved in 32% of reactions, three for 13% of reactions, and four organ systems were implicated in only 1% of reactions to peanut[49]. Symptoms relating to the skin are the most prominent for peanut allergy and are found in 89% of reactions to peanut[49]. These include rashes, urticaria, and angioedema[95]. Respiratory symptoms, such as laryngeal edema, coughing, and asthma, are found in 42% of peanut reactions and 26% of reactions include gastrointestinal symptoms like abdominal pain, diarrhea, and vomiting[49, 95]. Additionally, cardiovascular symptoms, like hypotension and arrhythmias, are present in 4% of peanut reactions[49, 95]. The most severe symptoms involved in a peanut allergic reaction are anaphylaxis and anaphylactic shock. Approximately 5% of all allergic reactions to peanut involve anaphylaxis[97]. Anaphylactic reactions to peanut can result in a wide variety of symptoms, including cardiovascular collapse, severe abdominal pain, trouble swallowing, difficulty breathing, wheezing, hypotension, and

systemic shock[98]. In the US, fatal food-induced anaphylaxis rates are estimated at 0.04 deaths per million individuals each year, and over half of these deaths are attributed to peanut-induced anaphylaxis[99, 100]. In fact, peanuts and tree nuts are the allergenic food sources with the highest fatal anaphylaxis rates, resulting in 55-87% of food-triggered anaphylactic deaths[101]. The mortality of peanut allergy is estimated at 2.13 per million person years, which is higher than any other food allergy[102].

One factor that contributes to the severity of peanut allergy is its characteristically low threshold doses for many individuals. The amount of peanut needed to elicit a reaction in peanut-allergic individuals may be as little as 0.2 mg of peanut protein[92]. Considering a single peanut contains approximately 300 mg of peanut protein, the most sensitive allergic individuals can react severely to trace amounts of peanut[103].

iii. Diagnosis of Peanut Allergy

The diagnosis of peanut allergy is analogous to the diagnosis of other food allergies. With regard to diagnosis, it is important to consider the onset of peanut allergy. Peanut allergy can present at any life stage, including in adulthood; however, the average age of presentation is 14-18 months[46, 49]. Diagnosis, as with other food allergy, is highly reliant on a convincing patient history. In fact, a history of reaction to peanut is often considered the most important "test"[104]. In addition to patient history, SPT, sIgE, component-resolved diagnostics (CRD), and OFC are the most prominently used diagnostic methods for peanut allergy. SPT can be used in early stages of diagnosis of peanut allergy, but only indicates sensitivity and does not directly correlate with clinical reactivity[81]. In SPT, the size of the wheal can be used to predict reactivity. A wheal size

larger than 8 mm is over 95% predictive of clinical reactivity to peanut[105]. Like SPT, sIgE testing only indicates sensitivity to the allergenic food, not clinical reactivity and certain sIgE results may indicate different degrees of reaction severity[81].

CRD is another diagnostic method that is commercially available and is used for peanut allergy diagnosis[106]. This approach is a type of sIgE testing; however, it tests for specific food proteins or epitopes of food proteins[106]. In theory, this differentiates IgE binding which is clinically relevant from binding that would not elicit a reaction[106]. When sIgE testing is used for peanut components, 70-90% of individuals with confirmed peanut allergy have sIgE to Ara h 1 and Ara h 2[103, 107]. Between 45-95% of allergic individuals have sIgE to Ara h 3[108]. However, sIgE to Ara h 2 and Ara h 6 is known to have the highest diagnostic ability for peanut allergy, and it can give insight into reaction severity[106, 109]. Sensitization to Ara h 8 alone is a phenomenon observed in some tested individuals and this usually indicates tolerance for peanut, not reactivity[110].

Though the aforementioned tests are commonly used in diagnosing peanut allergy, the gold standard is DBPCFC, as for other food allergies. When physician-supervised, an OFC is considered the most preferred and definitive test for peanut allergy[104]. However, DBPCFCs are expensive, require significant challenge time, and have the potential to cause dangerous reactions[104].

iv. Peanut Allergy Treatment

The treatment of peanut allergy, much like that of other food allergies, consists primarily of adoption of an avoidance diet. Though individuals seek to avoid exposure to peanut through elimination diets, about 10% of peanut-allergic individuals report an

allergic reaction each year due to an accidental exposure[40]. Because individuals still experience allergic reactions, additional treatment is needed. One principal component of peanut allergy treatment is the use of epinephrine upon accidental exposure to the allergen. Due to the severity of allergic reactions to peanut, the administration of epinephrine, antihistamines, and bronchodilators are recommended to treat allergic individuals who have been exposed to peanut[111]. The use of these treatment medications is typically based on reaction severity. For severe reactions and anaphylaxis, intramuscular injection of epinephrine is administered and for less severe reactions antihistamines and bronchodilators are recommended[111]. Epinephrine is critical in controlling a severe reaction to peanut and potential anaphylaxis because it can reverse any symptom of the allergic reaction, while antihistamines are not as effective for respiratory and cardiovascular symptoms[112]. The actions of epinephrine include decreasing mast cell and basophil release of mediators, vasoconstriction, bronchodilation, and overall increased blood flow to counter an allergic reaction[113]. There are risks in using epinephrine to treat anaphylaxis and those primarily stem from recognition of a reaction and improper use of epinephrine injectors[112]. Unfortunately, it is common for epinephrine administration to be too late, at the incorrect or insufficient dose, or simply erroneous due to lack of knowledge regarding administration[114].

Another option to manage peanut allergy is oral immunotherapy (OIT), which has been increasing in interest from both patient and research perspectives. The goal of peanut OIT is to increase an individual's threshold of tolerance to peanut in order to reduce their reaction risk upon an accidental or trace exposure. In a peanut OIT sequence,

the initial phase of immunotherapy involves rapidly increasing doses of peanut, followed by the build up phase with a more gradual increase of peanut protein[111]. Next, the immunotherapy transitions to a maintenance phase, which consists of a therapeutic dose of peanut for an extended period[111]. One study of peanut OIT in children found 71% of children receiving immunotherapy achieved desensitization[115]. However, another study found OIT to be effective in children, but non-statistically significant desensitization rates were observed between adults in OIT-treated groups (41.5%) and placebo groups (14.3%)[116]. While there is some evidence of OIT promoting desensitization for peanut-allergic individuals, specifically for children, long term studies and follow up is needed to assess real tolerance[117]. OIT may be a promising management option for peanut-allergic individuals, but immunotherapy-induced dosing reactions occur which may deter certain individuals[111]. There is one peanut OIT product, Palforzia, that was approved by the US Food and Drug Administration for use in peanut-allergic children in 2020[118].

v. Resolution of Peanut Allergy

In contrast to desensitization promoted through OIT, a subset of peanut allergic individuals will naturally outgrow their peanut allergy through a process known as resolution. Resolution is typically marked by an increase in T regulatory (T_{REG}) cells, a decrease in peanut-specific IgE, and the absence of clinical reactivity when exposed to peanut[117]. Unlike other food allergies, including allergy to egg and milk, peanut allergies are much less likely to resolve as an individual develops[28]. In fact, approximately 20% of peanut allergic children will outgrow their allergy by adulthood[117]. Resolution is more likely before the age of six years old and occurs at a lower rate after the individual is 10

years of age[119]. Wheal sizes resulting from SPT analysis are thought to be a predictor of tolerance or persistence. Decreasing wheal size indicates a higher probability that the individual will resolve their peanut allergy, while larger and increasing wheal sizes are more indicative of persistence of peanut allergy[120].

vi. Cost of Peanut Allergy to Stakeholders

Peanut allergy is accompanied by a wide variety of costs, including financial and psychological, to many stakeholders, such as patients, caregivers, clinicians, and the food industry. Financial costs associated with peanut allergy include both direct and indirect costs[91]. Peanut allergy increases annual medical costs for the allergic individual or the family of the individual. Direct costs include general physician visits, allergist visits, emergency department (ED) visits, antihistamine medication, epinephrine autoinjectors, and more[47]. In fact, peanut is the most common allergenic food to cause ED visits and 23% of peanut-allergic children and 20% of peanut-allergic adults report a visit to the ED each year[3, 31, 121]. Lifetime costs associated with anaphylaxis treatment and epinephrine autoinjectors for peanut-allergic individuals are estimated at $25,228[122]. Furthermore, there are indirect costs associated with peanut allergy, including special diets for allergic individuals, allergen-free foods, changes in childcare, lost wages due to doctor appointments, and more[91]. The financial burden of all childhood food allergy in the US is approximately $24.8 billion[47].

In addition to the financial costs, there are substantial psychological and emotional costs associated with peanut allergy[91]. The severity of allergic reactions to peanut and the fear of accidental exposures leads to the observed psychological impact of

peanut allergy, which most commonly manifests as sadness, depression, embarrassment, nervousness, and anxiety[91]. For peanut-allergic children, bullying from peers contributes negatively to social and emotional health[112]. Additionally, it is well understood that peanut allergy reduces the overall quality of life (QoL) for patients, family members, and caregivers[91, 123]. The restrictions on daily life and activities, especially with school, restaurants, and travel, have a negative impact on QoL[91]. Modifications to diet, avoidance diets, and restrictive eating can also have a negative effect on peanut-allergic individuals health-related QoL[124].

V. ALLERGEN REGULATION

Due to the danger associated with IgE-mediated allergic reactions, the need to regulate allergenic foods is imperative in order to increase the safety of food products for consumers. In the US, regulation of the presence of allergenic foods occurs for packaged foods through labeling requirements and mandated allergen control plans to ensure good manufacturing practices and preventive controls for cross-contact prevention. There are two primary legislative acts that govern food allergen regulation in the US, the Food Allergen Labeling and Consumer and Protection Act and the Food Safety Modernization Act. In addition to regulated allergen labeling, precautionary allergen labeling is a voluntary allergen statement used to communicate the potential risk of allergen presence in a food product, although it is recognized by stakeholders as misleading or confusing to consumers. The purpose of these regulations, both mandated and voluntary, is to protect allergic individuals from consuming products which certainly or may contain an allergenic food, so that an avoidance diet can be followed.

i. Food Allergen Labeling and Consumer Protection Act

As allergic individuals must adopt an elimination diet to avoid any consumption of the allergenic food, consumers must be able to discern whether or not their allergen is present in packaged food products. In 2004, the US Congress enacted the Food Allergen Labeling and Consumer Protection Act (FALCPA)[6]. FALCPA requires plain-language labeling of the allergenic foods which have been intentionally added to a food product[6]. The act requires "major allergens" to be declared, and this list is what is known as the "Big Eight" allergenic foods (milk, eggs, fish, shellfish, tree nuts, peanuts, wheat, and soy)[6, 30]. Plain-language labeling requires ingredients to be listed by their common name, even if a derivative of the allergenic food source has been used in the food product[125]. For example, if casein is used in a formulation the label must declare the presence of "milk" in the food product [126]. The declaration of the presence of allergens can be listed within the ingredients statement or recorded in a separate, typically adjacent, statement which lists any and all allergenic foods present in the food[127]. FALCPA does not include labeling requirements for highly refined oils because protein residues, if present, should be at trace levels which may not cause allergic reactions in most allergic individuals[125]. Additionally, FALCPA does not apply to made-to-order food products or food prepared in restaurants[112].

ii. Food Safety Modernization Act

Because allergic consumers adopt avoidance diets to minimize the potential for reactions, the unintentional addition of food allergens to food products is also a concern. This unintentional presence of allergen residues in foods is known as cross-contact and is

a hazard which may pose a risk to allergic consumers. In fact, the leading cause of food recalls in the US is the presence of undeclared food allergens in food products[126]. In attempt to mitigate this risk and protect allergic consumers, the Food Safety Modernization Act (FSMA) was passed by the US Congress in 2011[7]. FSMA requires food manufacturers to develop and implement a food allergen control plan (ACP) to prevent allergen cross-contact during food production and processing. This aspect of FSMA's guidance, requires a food hazard analysis in which allergens are classified as a chemical hazard[128]. The motivation behind ACPs is to mandate the diligence of food manufacturers in preventing allergen cross-contact within their production and packaging processes[129]. ACPs and current good manufacturing practices (cGMPs) for allergen management typically include dedicated equipment, cleaning and sanitation protocols, supplier ingredient controls, product sequencing, and labeling controls[127].

iii. Precautionary Allergen Labeling for Allergens

Precautionary allergen labeling (PAL) is a voluntary statement which can be used by food companies to communicate to consumers the possible presence of an allergen in a food product as a result of cross-contact[92, 130]. PAL statements are intended to denote the risk of the unintended presence of an allergen in a food product, however they are only slightly regulated in the US. PAL statements must be truthful and not misleading, plus cannot be used as a substitute for cGMPs[131, 132].

This results in inconsistencies of PAL statement phrasing and consumer confusion about the actual risk associated with these labels[133]. Phrases which are considered PAL include, but are not limited to, "may contain (allergenic food)," "may contain traces of

(allergenic food)," "processed in a facility that manufactures (allergenic food)," "packaged in a shared facility," and others[40, 106].

The issue with inconsistencies of PAL statements is that consumers interpret PAL statements differently based on wording; however, these phrases do not denote varying degrees of risk. For example, one study found that consumers with peanut allergy were more likely to purchase products with PAL statements containing "shared facility" rather than a "may contain peanuts" statement[134]. Consumers may even ignore PAL statements, especially if previous consumption of a product did not result in an allergic reaction[135]. Because PAL is not regulated, food companies may use these labels in order to avoid liability, regardless of the risk[136]. Non-risk-based and widespread use of PAL statements can be advantageous to food manufacturers because consumers who have allergic reactions to foods with PAL may be unable to litigate regarding these reactions[136].

The primary consideration for PAL statements is whether the warning label is effectively communicating a risk of allergic reaction to consumers. For peanut contamination specifically, one study investigated allergen contamination in products with and without PAL statements. Peanut was found in 4.5% of products with peanut PAL statements and in 0% (of 120 analyzed products) with no declaration of peanut presence[137]. Another study found detectable levels of peanut in 7% of products containing a peanut PAL statement[134]. Company size may play a role in the likelihood that a product with a peanut PAL statement tests positive for peanut contamination[137]. Products from small companies had 7.7% of PAL statement products contain peanut, while large companies tested positive for peanut only 1.7% of the time[137]. While the frequency of peanut detected in

products labeled with PAL statements may be low as determined by some studies, these results do reflect the expected sporadic nature of allergen cross-contact[138].

iv. Other Voluntary Labeling

In addition to PAL, there are other unregulated voluntary allergen labels that have been observed on packaged food products. This subcategory of voluntary allergen claims is referred to as "free-from" claims. Examples of these claims include, "allergen-free" or naming a specific allergen that is supposedly absent from the product, such as "peanut-free"[139, 140]. These types of claims are currently not regulated in the US by FALCPA or FSMA and have no quantitative basis[6, 7, 141]. There is one exception for these unregulated claims and that is a "gluten-free" claim which is indeed regulated and has a quantitative limit of 20 ppm gluten[142].

v. Food Allergen Risk Assessment

A critical component of allergen control and food allergen labeling considerations is risk assessment. Food allergens are unique in the risk that they pose to allergic consumers and therefore require a specific risk assessment strategy[143]. There are four components of a food allergen risk assessment, hazard identification, hazard characterization, exposure assessment, and risk characterization[143]. In the first step of food allergen risk assessment, the hazard identified is generally the potential of causing an allergic reaction in allergic individuals[143]. Hazard characterization, the second step of risk assessment, consists of a determination of the possibility of an adverse effect following exposure to the hazard[144]. This characterization is primarily based on a minimum dose or threshold concentration below which reactions are less likely to

occur[144]. For food allergens, reference doses can be used. The Voluntary Incidental Trace Allergen Labeling (VITAL) program established reference doses for several allergenic foods[92]. These were calculated using distribution models of no-observed adverse effect levels (NOAELs) and lowest-observed adverse effect levels (LOAELs) from clinical challenge data to deduce an eliciting dose for the most sensitive 5% (ED_{05}) or 1% (ED_{01}) of allergic individuals[92]. EDs, eliciting doses, refer to the dose that would elicit an allergic response in x% of the allergic population[145]. The VITAL reference dose for peanut is 0.2 milligrams (mg) of peanut protein and was established based on the ED_{01}[92]. In short, an eating occasion that contains a total of 0.2 mg of peanut protein would be expected to elicit an allergic reaction in the most sensitive 1% of peanut-allergic individuals. The third step of a risk assessment for a food allergen hazard is an exposure assessment. In an exposure assessment, the concentration of the food allergen in the food and the intake amount of food are multiplied to deduce the total exposure to the allergenic protein in a single eating occasion[144]. The fourth and final step of food allergen risk assessment is risk characterization, in which all previous steps are considered to calculate the probability or frequency of an adverse allergic reaction to the presence of allergens in the food at certain population levels[146].

vi. Food Allergen Recalls

One important aspect of food allergen regulation in the US is the recall of products found to contain undeclared allergens. FALCPA requires declaration of the major allergenic foods on a food label; therefore, products containing a major allergenic food without appropriate labeling are considered “misbranded”[147]. Upon inspection or

detection of undeclared allergens in a food product, firms and the Food and Drug Administration (FDA) or USDA Food Safety and Inspection Service (FSIS) work to recall products and inform consumers[148]. In the US, the most common cause of food recalls by the FDA is related to food allergen labeling[147]. The products recalled at the highest frequency include bakery products, followed by snacks and candies[147]. Additionally, the most common allergenic foods which prompt a recall are milk, wheat, and soy[147].

VI. ALLERGEN DETECTION METHODS

As a result of regulatory measures and for the purpose of risk reduction, the ability to detect and quantify the presence of protein from allergenic food sources in food products is of significant value to the food industry. Current analytical allergen detection methods for food products include enzyme-linked immunosorbent assays (ELISA), lateral flow devices (LFD), polymerase chain reaction (PCR), and liquid chromatography tandem mass spectrometry (LC-MS/MS)[149]. ELISA and LFD detection methods have an immunological basis, while PCR and LC-MS/MS are non-immunologic and detect nucleic acids or proteins, respectively.

i. Immunoassay Overview

ELISA and LFD are both immunoassays that are commonly used as food allergen detection methods in the food industry. ELISA and LFD are immunological assays that, at the most basic level, function through binding of an antigen to antibodies, typically immunoglobulin G (IgG) antibodies, which have been raised against the target antigen[150]. With respect to antibodies for immunoassays, there are two types that are utilized by kits.

Polyclonal antibodies are generated from different B cell clones, while monoclonal antibodies originate from a single B cell[150]. The result of raising antibodies from different B cell clones compared to a single B cell is that polyclonal antibodies will bind different epitopes, while monoclonal antibodies will recognize a single epitope[150].

ii. Enzyme-Linked Immunosorbent Assays (ELISA)

a. Principles of ELISA

ELISA detection methods are the most commonly used food allergen detection methods in the food industry primarily due to their reasonable sensitivity and simplicity for routine monitoring of food allergens[35, 150, 151]. There are both commercial and noncommercial ELISA assays. Companies may manufacture a kit to be sold commercially or laboratories may develop their own assays for use in house. Because it is an immunoassay, the fundamental concept of an ELISA is the binding of kit antibodies to a target antigen, the allergenic protein in the food[35]. There are multiple types of ELISA kits, including sandwich ELISA assays and competitive ELISA assays. The most common ELISA format used for food allergen detection is the sandwich ELISA[152]. Furthermore, kits can be crafted in order to detect a specific allergenic protein, and some can detect multiple proteins[152]. In a sandwich ELISA, capture antibodies are coated in the sample wells to bind any antigen present in the sample[150]. Second, a detection antibody binds to any antigen which has been retained by the capture antibody. The detection antibody is often conjugated with an enzyme to create a detectable signal[150]. Quantification of the allergen or allergenic food in the sample is then based on the

interpolation of sample absorbance to a standard curve. For a sandwich ELISA, the absorbance is directly proportional to the concentration of the target analyte[153].

b. Advantages of ELISA Methods

Overall, food allergen detection by ELISA is considered an effective and valuable tool for use in the food industry for multiple reasons. It is widely accepted that these kits have sensitives or detection limits at ranges which would generally ensure safety for food-allergic consumers[149]. Commonly, kits have detection limits in the low parts per million (ppm) range. Reagents and materials used in the kits are also conducive to food processing facilities[149]. Another advantage of food allergen detection by ELISA is that these analyses are generally quick and do not require great expertise for completion of the analysis or expensive, specialized laboratory equipment[150]. Instrumentation for colorimetric measurements, such as a plate reader, is a necessary, but is considered a reasonable expense for most laboratories or facilities[152].

c. Limitations of ELISA

There are several limitations that exist for ELISA methods when used for food allergen detection. One prominent issue with ELISA detection methods is the ability of the extraction process to effectively extract the target protein from the food that is being analyzed[150]. For food allergen detection, one issue contributing to an inefficient extraction is the food matrix and its interference with the target protein[150]. To overcome this issue, many extractions for ELISA may utilize an extraction additive to assist in protein extraction[154].

ELISA detection methods can be limited in their ability to detect allergens after the food has been processed due to changes in proteins' structures and properties, which may impact binding[155]. Various processing effects of the food matrix may change the accessibility or structure of conformational epitopes for detection by kit antibodies[156]. There are a wide range of changes to target proteins that can occur during food processing, which may impact the ability of ELISA kits to detect the analyte. Examples of these changes include aggregation, denaturation, hydrolysis, degradation, reduction, and oligomerization of proteins[151]. Furthermore, food matrix components can interact with proteins and impact their solubility and thus, their extractability by the kit buffer[151]. On the other hand, these assays may be cross-reactive with components of a food matrix, prompting over-recovery and inaccurate quantification by over-estimation of the allergen[155]. These sorts of changes may impact the ability of the kit antibody to capture the intended target protein in the food matrix and thus impact the kit's efficacy to detect food allergens precisely and accurately in food matrices. Another limitation of ELISAs is that because the assay hinges on antibody binding, there are varying epitopes that will be recognized and to different extents, thus there is the possibility of variable quantification due to binding affinities[157]. Due to uncertainties with ELISA kits' ability to effectively detect and quantify food allergens in specific matrices, it may be necessary to confirm the results of an ELISA analysis with a non-immunological detection method.

d. Peanut ELISA Methods

There are a large number of ELISA methods that have been developed commercially for the detection of peanut, which target different proteins and result in

different sensitivities in certain food matrices. Some of these commonly used assays include the Veratox® for peanut allergen (Neogen®) with a range of quantitation of 2.5-25 ppm peanut, the BioKits Peanut Assay Kit (Neogen) with a range of quantitation of 1-20 ppm peanut, the AgraQuant® Peanut Kit (Romer Labs®) with a range of quantitation from 1-40 ppm peanut, the Ridascreen® Peanut Kit (R-Biopharm®) with a quantitation limit of 0.75 ppm peanut, and the Peanut Protein ELISA Kit II (Morinaga Inc.) with a quantitation range of 0.31-20 ppm peanut protein according to each respective manufacturer. However, a factor in an ELISA kit's recovery and quantification is often the food matrix which is analyzed.

One study of six commonly used assays determined that all kits reported reasonable recoveries of peanut protein[158]. The same study characterized the antibodies of the ELISA kits, reporting which peanut proteins are predominately recognized by the kit antibodies[158]. Five of the six kits predominately recognized Ara h 3, the most abundant peanut protein, and the other, the Morinaga kit, recognized primarily Ara h 2 and Ara h 6. In a study comparing the recovery of peanut protein in processed pastry samples by the Morinaga Peanut ELISA kit and the Veratox for peanut allergen (Neogen) using different extraction buffers, the Morinaga kit outperformed the Veratox kit in recovery of peanut in samples that were extensively processed[159]. However, with minimally processed pastry samples or in the pastry dough, peanut detection was greater with the Veratox kit[159]. This suggested an effect of the protein targeted by the kits' antibodies because Veratox kits target mainly Ara h 3[159]. Though Ara h 3 is abundant, it was more susceptible to heat processing and therefore, recovered to a greater extent with less thermal processing[159].

In another study of ELISA recovery of peanut protein, food matrices were treated with moist heat and dry heat and then analyzed by the Veratox kit and the BioKits assay[160]. The results indicated a lower level of peanut recoveries for all heat-treated samples for both kits, though to different extents[160]. Overall, the study concluded that certain ELISA kits may not be suitable for analysis or result in accurate quantitation of food matrices that have been thermally processed, due to the induced structural and conformation changes of the proteins[160]. In a study comparing the peanut recoveries of two ELISA kits (BioKits from Neogen and Peanut Residue ELISA kit from ELISA Systems) from peanut-incurred cookies, the results showed a dominant effect of thermal processing, as all four samples were detected in the 10 ppm peanut cookie that was baked for 11 min but in only one 10 ppm peanut cookie sample after 16 minutes of baking[161]. In a study of spiked dark chocolate and incurred cookies, five commercially available ELISA kits were used to detect peanut in various levels of the food matrices[162]. In the cookie, the average recovery ranged from 72.9-190.9% and for the dark chocolate, 43.7-151.8%[162]. The study concluded that all five kits performed well in the 5-10 ppm peanut range but struggled in the lower concentration ranges and had variations of 44-191% across all kits[162]. There is an observed issue with the detection of trace levels of peanut protein when using ELISA detection methods to analyze cookie and dark chocolate matrices.

There are also noncommercial assays for peanut detection in food products which have demonstrated some efficacy in a number of food matrices. One polyclonal antibody assay evaluated different matrices (oil, ice cream, cookies, chips, chocolate candy, pasta

sauces) and reported recoveries of peanut protein ranging from 45-100%[163]. Another noncommercial ELISA was developed and used to analyze various spiked matrices (milk, chocolate candies, cereals) and found recoveries of peanut protein ranging from 99-126%, with an overall LOD of 2 ppm peanut protein in matrix[164]. An ELISA assay, developed with a calculated LOD of 0.07 ppm peanut protein and LOQ of 0.15 ppm peanut protein, was used to analyze spiked chocolate and effectively detected the 10 ppm peanut chocolate[165]. Another noncommercial ELISA was developed and detected peanut in ice cream at approximately 40 ppm peanut protein[166]. A common acknowledgement of ELISA method developers is that a greater understanding of the impact that the food matrix imparts on target proteins and their detection, if solved, would improve the sensitivity of ELISA assays[166]. One sandwich ELISA was developed to detect peanut in complex food matrices (spiked dark chocolate and ice cream) and reported LODs ranging from 0.2-1.2 ppm peanut[167]. One important consideration for peanut ELISAs, whether commercial or noncommercial, is that many underestimate (approximately 3.5-fold) peanut when the peanut material has been roasted or thermally processed[168]. On the other hand, these ELISA methods can overestimate raw peanut by a factor of 3.9[168].

iii. Lateral Flow Devices (LFD)

a. Principles of LFD

LFDs are another common immunoassay used in the food industry for food allergen detection. LFDs are also referred to as dipsticks, strip tests, or lateral flow assays[169]. An LFD functions like an ELISA detection kit in that it relies on antibody-antigen binding to indicate the presence of a food allergen, however an LFD yields only a

qualitative or a semi-quantitative result[152]. Most LFDs are designed with four main parts, sample pad, conjugate pad, membrane, and absorbent pad[170]. The sample is added to the sample zone with a membrane containing specific antibodies raised for the target analyte[150, 169]. These antibodies are not bound to the membrane, but bind to target protein, if present. The antigen-antibody complex then progresses through the LFD to the test zone[169]. The test zone contains a second target-specific antibody that will bind the antigen-antibody complex to form the test line, indicating a positive result[169]. The visual indicators of the LFD tests are created using colored particles coated with antibody[171]. These devices also use a control line that will display a band, whether or not any analyte was detected, to confirm the test was run properly[171].

b. Advantages for LFD Use

The primary advantages of LFDs are their speed and ease-of-use for routine analysis in food processing facilities[150]. LFDs are fast, relatively inexpensive, and do not require any additional instrumentation or equipment[157]. It is widely accepted that LFDs are valuable food allergen detection methods for verifying the efficacy of sanitation and cleaning protocols for the food industry[149].

c. Limitations of LFD

The most prominent limitation of LFDs for use as a food allergen detection method is that they are not capable of accurate quantification of the detected analyze. LFDs commonly have limits of detection (LOD) near 0.5-5 ppm but have limited efficacy in quantifying detected analyte[171]. For risk assessment purposes, it is much more valuable to obtain a quantitative amount for the food allergen present in order to calculate

exposure dose[143]. LFDs typically determine the presence or absence of a food allergen in a sample and, at best, can only be considered semi-quantitative or qualitative[152]. The semi-quantitative consideration comes from the fact that the intensity of the test band is correlated to the amount of the target antigen in the sample[35].

Because LFDs function fundamentally similar to an ELISA, they face many of the same matrix-based issues in their detection of food allergens. As a result of food processing, the target proteins of LFDs may have endured conformational or solubility changes which may negatively affect their detection by the strip test[169]. As with ELISA methods, potential cross-reactivity of the test's antibody with matrix and other proteins is considered a drawback for LFD[172]. Another issue with LFDs is the possibility for false-negative reactions which is called the prozone phenomenon or hook effect[173]. This effect occurs when there are high amounts of the allergenic protein present in the test sample, so much so that it is greater than the antibody binding capacity of the test and the result is no band formation at the test line[174]. This is an overloading effect that results in a false-negative test result. This is a significant limitation of this detection method with respect to the protection of allergic consumers and food safety. Some LFD manufacturers utilize an overload line to address the issue of false negative results due to the hook effect[169].

d. Peanut LFD Methods

There are several LFDs that have been developed for the detection of peanut, both for uses in sanitation and cleaning validation or analysis of food materials. Some developed LFDs have shown comparable sensitivity to ELISA methods. One lateral flow immunoassay developed using Ara h 1 monoclonal antibodies accomplished sensitive

detection of peanut in peanut meal (0.5 ppm peanut)[175]. Another LFD analyzed peanut in food matrices (chocolate milk, cereal bar, chocolate candy, and others) and reported LODs near 1 ppm peanut protein in food sample[176]. However, like ELISA methods, LFDs struggle when analyzing peanut in complex or processed food matrices. For example, in a study of two commercially available LFDs, peanut-containing cookies were analyzed, and several false-negative results were reported in this matrix at concentrations less than 21 ppm peanut in cookie[177]. Additionally, three commercially available peanut LFDs were used to evaluate spiked cookie dough and chocolates, and two LFDs detected at 1 ppm peanut in both matrices, while the other LFD detected only at 14.2 ppm peanut in the chocolate and 4 ppm peanut in the cookie dough[178]. These LODs in cookie and chocolate matrices are not necessarily sensitive enough to ensure food safety for allergic consumers.

iv. Polymerase Chain Reaction (PCR)

a. Principles of PCR

PCR is a deoxyribonucleic acid (DNA)-based method that can be used to detect DNA from an allergenic food source. PCR methods function by targeting and amplifying specific DNA sequences that are unique to the species from which the allergen originates[179]. Generally, PCR methods can be divided into three steps. First, DNA is extracted and purified from the food matrix that is to be analyzed for allergenic material[180]. Next, the specific DNA target sequence is amplified[150]. The amplification process is completed by a thermostable DNA polymerase and begins with melting or denaturation of the DNA strands[179]. Next, DNA primers, which are oligonucleotides

flanking the target DNA, bind to the denatured DNA strand[174]. Lastly, DNA polymerase extends the primer DNA and synthesizes a new strand or copy of the target DNA sequence[181]. These three steps of amplification are controlled by temperature and are usually completed in multiple (25-45) thermal cycles until a detectable amount of copy DNA has been generated[179]. Following amplification, the third step of PCR is that the amplified DNA is detected which can be done via several methods[180].

There are several types of PCR, but most of the PCR methods that are commonly used for indirect food allergen detection are general PCR or real-time PCR[150]. Though there are multiple different types of PCR methodologies, real-time PCR is considered especially beneficial as an allergen detection method because it can quantify the initial amount of DNA present in the sample prior to amplification[180]. For real-time PCR, quantification can be completed using the number of thermal cycles needed to produce detectable product DNA to determine initial target DNA concentration[182]. On the other hand, end-point PCR is considered only qualitative[179].

b. Advantages of PCR Use

The target DNA for the PCR method can originate from a gene for an allergenic protein or simply originate from the allergenic food[183]. The key to target DNA selection is the specificity of the target sequence to the source of the allergenic material. In theory, a target DNA sequence for a PCR method may be more specific than an amino acid sequence for a peptide or protein-based detection method[183]. This is because there are three nucleotides to encode each codon for a specific amino acid and for most amino acids there are multiple codons[180]. Therefore, one advantage of PCR is that a DNA

sequence is, in theory, more specific than an amino acid sequence that might be used in a peptide or protein-based detection method[180]. Additionally, nucleic acids are more stable than proteins so PCR methods can, theoretically, utilize harsher extractions to analyze food that has been extensively processed[150].

c. Limitations of PCR Methods

One limitation of using a DNA-based method to indicate the presence of food allergens is that the quantitative correlation between DNA and a specific protein amount is not well understood[184]. The allergen-encoding DNA is not always linearly related to the presence of an allergen in the sample[157]. However, the amount of DNA in an organism is considered more stable than the protein amounts, since proteins can vary across individual organisms, species, and varieties[150]. Even so, many allergenic food sources have much less DNA by composition compared to protein[149]. Another limitation of DNA-based detection methods is the effect of the food matrix and processing on target DNA. First, processing of the food matrix has the potential to alter the DNA and its structure, which will decrease its detection[157]. Further, DNA can be degraded during food processing, which can significantly decrease its detection by a PCR method[185]. Additionally, food matrix components like salt, lipids, and proteins, are known to interfere with DNA amplification and result in a decrease in the amplification efficiency and result in lower sensitivity or false negative results[179]. PCR also requires more expensive instrumentation compared to immunoassays[150].

The main limitation of using PCR to indicate the presence of food allergens is based on the principle of the detection method, in that it is not detecting proteins from the

allergen source or surrogates of allergenic proteins. The issue is that PCR detects a class of molecules, nucleic acids, which are not the causative agent of food allergy[186]. In short, PCR an indirect detection method. Additionally, the processing of food differently impacts nucleic acids and proteins because they are two separate types of molecules, plus DNA and protein can be separated during food processing techniques like protein concentration[152, 186]. Therefore, detection of DNA to indicate the presence of allergenic food proteins is flawed in its principle as a detection method.

d. Peanut PCR Methods

There are many real-time PCR methods which have been developed to detect peanut. Several qualitative methods for the detection of peanut in foods by PCR have been developed and appear to be generally robust and relatively sensitive in their detection, with LODs less than 10 ppm peanut[165, 187, 188]. Semi-quantitative and quantitative PCR methods for peanut have also been established, though they recognize their deficit in being able to accurately quantify allergens in some food matrices[165]. One PCR method developed with an Ara h 1 primer target with an absolute LOD of 5 picograms (pg) of peanut was used to analyze chocolate material with peanuts and reported a positive detection, even in a complex matrix[189]. One study used real-time PCR of a mat K chloroplast marker to detect peanut protein at 1 ppm peanut, with an absolute LOD of 2.5 pg of DNA and to detect peanut in commercial matrices (cereal bar and chocolate bar) with a broad quantification range of $1\text{-}10^5$ ppm peanut[190]. A comparative study of ELISA and real-time PCR in their detection and quantification of peanut-containing food matrices indicated that heat treatments resulted in a decrease in recovery

in both detection methods, in a similar manner[161]. It is often observed that peanut PCR methods demonstrate high variation between replicates due to the exponential component of amplification[191].

v. Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

a. Principles of MS

MS-based allergen detection methods have been developed for an extensive list of allergenic foods. The principle of these detection methods is that MS is used for the detection of specific mass-to-charge (m/z) ratios corresponding to specific peptide sequences to indicate the presence of protein in a sample[192]. These methods can also be developed to be quantitative using internal or external calibration techniques[192].

A critical component of understanding food allergen detection and quantification by MS is the basic principles of MS. There are three basic components of an MS instrument, the ion source, mass analyzer, and detector. The ion source is responsible for ionizing and volatilizing the particles to be analyzed[193]. Ion sources for protein or peptide methods are usually matrix-assisted laser desorption ionization (MALDI) or electrospray ionization (ESI)[194].For ESI, the ionization is completed using electric voltage, and this ion source is compatible with an upstream LC system[193].

The next part of MS instrumentation is the mass analyzer. The role of the mass analyzer is to determine the m/z of the ions[195]. There are five commonly used mass analyzer types. These are quadrupoles (Q), ion traps (IT), time-of-flight (TOF) analyzers, Fourier transform ion cyclotron resonance (FTICR) analyzers, and Orbitraps[193]. Each of these mass analyzers, have varying sensitivities, as well as differences in mass range,

resolution, and accuracy[155]. A quadrupole allows ions of certain m/z values to pass based on radio frequency and voltage potentials[193]. Quadrupoles have limited resolution and mass accuracy[193]. Orbitraps are able to separate ions using an oscillating electric field and are known for their accuracy and resolution[193].

Instruments can have a single mass analyzer or can have two mass analyzers in sequence, which is known as tandem MS (MS/MS). The coupling of mass analyzers in tandem for MS increases the sensitivity of detection, as well as allows for higher resolution and mass accuracy[149]. Before the second mass analyzer, fragmentation of precursor ions is completed to generate product ions[196]. Product ions are important for the identification of the target analyte or target peptide because product ions increase specificity or confidence in detection[196]. Most commonly, fragmentation is completed by collision gas, which breaks up the molecular bonds that are the most labile[193]. In peptide analysis, this is most commonly the cleavage of the CO-NH bond of the peptide backbone[193]. This results in y and b ions[193].

The next step within in MS is the detection of ions. There are several types of ion detectors, including electron multipliers, Faraday cups, photographic plates, scintillation counters, and others[197]. The ion detector does not measure mass, but instead generates a signal any time an ion is detected. Results of the detector are shown as spectra of relative ion abundance as a function of the m/z [197]. MS spectra are then used to discern sequence information using software to compare with protein sequence databases to identify the amino acid sequences of detected m/z values[193, 198].

MS analysis benefits from the use of analyte separation and this often occurs in the form of liquid chromatography (LC). A common MS set up for the detection of food allergens or other analytes consists of an LC coupled to an MS instrument containing two mass analyzers (tandem MS), abbreviated as LC-MS/MS. High pressure LC (HPLC) is the most commonly used separation technique in the field of proteomics, specifically reverse phase HPLC (RPLC)[199, 200]. It is also the superior method for separation, as it is markedly reproducible and has a large dynamic range[193]. The theory behind using RPLC is after an ion-pairing agent is added to sample peptides, they become positively charged and can be separated using the mobile phase, typically acetonitirile[201]. This method separates peptide based on their hydrophobicity interaction with the column material, or stationary phase[200]. An important aspect of RPLC for a quantitative MS method is that the retention time (RT) of a peptide, the time it elutes from the column into the instrument, is consistent across similar chromatographic conditions because the elution is based on the peptide's composition and physiochemical properties[202, 203]. One benefit to using LC is that it has a very high dynamic range, which helps when analyzing analytes that may be in low abundance, like food allergens[150].

There are two broad categories for types of MS methods, untargeted and targeted analyses, which each have several varieties within them. In untargeted proteomics strategies, also referred to as discovery proteomics, the signal intensity of precursor ions determines their selection for fragmentation for MS2[204]. There is no user determination of which precursor ions to select. In contrast, targeted proteomics workflows allow for the user to predetermine precursor ions for isolation in MS1 and fragmentation for MS2[205].

There are multiple kinds of targeted MS methods, including selected reaction monitoring (SRM, also referred to as multiple reaction monitoring or MRM), and parallel reaction monitoring (PRM). In SRM, both precursor and product ions are selected, whereas in PRM methods only the precursor is predetermined, and all product ions are measured[205]. PRM is a valuable approach on a high-resolution MS (HRMS) instrument, specifically with a hybrid quadruple Orbitrap mass analyzer and has been shown to have superior quantification performance compared to SRM methods[157].

In addition to untargeted and targeted MS strategies, there are also two different approaches to using MS for proteomics. These include top down proteomics and bottom up proteomics[193]. Top down proteomics is the measurement and fragmentation of intact proteins by MS, without prior enzymatic digestion. These methods may involve separation strategies for proteins and specific fragmentation techniques[193]. Bottom up proteomics involves enzymatic digestion of proteins in a sample extract, which are then separated by chromatography and evaluated by MS[193].

Of particular value for MS-based allergen detection methods is their quantitative potential. Targeted MS methods can be designed for absolute quantification methods in which the goal is quantification of protein or peptide present in the analyzed sample[206]. Quantification of allergenic protein in food samples assumes a proportional relationship between the signal from the MS and the amount of the target analyte in the sample[193]. Targeted MS-based detection methods, when appropriately developed and applied, are considered to be accurate and reproducible[157]. One common approach to quantification is to include a known amount of an internal standard, typically isotopically labeled versions

of target peptides[193]. These internal standards will behave similarly to the peptide analytes as they are molecularly identical except for the mass difference due to the isotopic nuclei (C, N, H)[207]. This strategy guarantees nearly identical behavior of the two versions of the peptide through LC and MS-MS methodologies and instrumentation[193]. The ratio between the "light" peptide (the analyte) and the "heavy" peptide (the isotope labeled peptide) can be used for quantitation through a light-to-heavy ratio since a molar amount for the heavy peptide is known[207].

There are also strategies for quantifying allergenic protein via external calibration using calibration curves. In this approach, absolute quantification of proteins and peptides can be determined using a correlation of the target analyte signal and the measured standard curve of samples with known concentrations[193]. MS-based methods boast LOQs that may be as low as in the femtomole or attomole range[151]. Additionally, it is important to consider the reporting units generated by the quantification for these methods so that they can be used for comparison with reference doses for risk assessment[149]. Thus, it is advisable to create methods with reporting units of total protein from the allergenic food source.

One key to appropriate quantitation for these targeted methods is target selection, which is a very important component of method development[205]. Target selection should consider a number of factors, but primarily target peptides should be sensitive, reproducible, and specific[193]. Furthermore, target peptides must be present and detectable in the native food matrix and in the processed food matrix[193].

b. Advantage of MS-Based Methods

There are several advantages to using MS as a food allergen detection method. First, the advantage over DNA based detection methods is that MS is directly detecting the protein responsible for food allergy, whereas DNA-based methods only indirectly indicate the presence of allergenic protein. MS-based detection methods also have the ability to overcome the effects of a food matrix, an issue demonstrated in immunoassay detection methods. This is because MS only relies on m/z values of selected peptides instead of antibody recognition of an epitope[151]. LC-MS/MS methods have an advantage over ELISA in processed food products because the conformation of the protein does not need to be maintained for detection of peptide sequences by MS[208]. LODs and LOQs for MS-based methods can be in the femtomole or attomole range for peptide on column, which is a comparable level to immunoassays[151]. With targeted MS methods, sensitivities for the detection of food allergens can be achieved with respect to consumer safety[157]. MS methods can also be multiplexed, in which target peptides for multiple allergens can be included in a single analysis[149]. This results in the possibility of a more time and resource efficient allergen analysis method.

c. Limitations of MS

Though MS-based allergen detection methods have advantages over most current detection methods, there are still some limitations to using MS for food allergen detection and quantification. The first is that peptide identifications are highly dependent on the availability of protein sequence databases[209]. Furthermore, the quality of the MS data and resulting quantitation is dependent on the accuracy of protein sequence databases used, as

well as the search algorithms and software utilized for the identifications[150]. Second, while MS may be advantageous for food matrix analyses as it does not need to preserve protein conformation, processing or matrix-induced modifications change the m/z of the target peptide and therefore may impact detection and quantification. Another limitation of MS is that quantification of allergens by MS relies heavily on the initial extractability of proteins containing the target peptides. Food processing can improve or diminish the extractability of proteins which can greatly altern the allergen concentration of extracts compared to the starting material[151, 152]. This increases the importance of accurate calibration, either internal or external, for MS methods. Another broadly recognized limitation of MS-based detection methods is the cost and expertise required for these analyses. Equipment, materials, and instrumentation for these detection methods are expensive and require costly maintenance[150]. Additionally, MS workflows and data analysis often require personnel with great expertise[150]. Because of cost and expertise requirements, MS methods are typically not used for routine analysis but have value as confirmatory methods upon unexpected results with other detection methods, like ELISA[149].

d. Peanut MS Methods

There are numerous LC-MS/MS methods that have been developed for the detection and quantification of peanut protein. Methods may target different peptides from different proteins, approach quantification in various ways, and be established for detection in a wide range of matrices. Some work has been completed for the selection of target peptides that perform well in a wide variety of matrices (fermented, thermally

processed, complex, and fatty matrices), and one study identified 16 quality target peptides that are sensitive and robust across the aforementioned matrix types[210]. These targets selected also considered the isoforms of major allergens, which promotes accurate quantitation of peanut[210]. In some methods, peptide markers from more than one protein are used to encompass different expression levels of proteins across different varieties of peanut and peanut products[211].

An LC-MS/MS method for analysis of peanut in a complex mixture of nuts (hazelnuts, pistachios, almonds, and walnuts) achieved an LOD of 26 ppm peanut in matrix (6.5 ppm peanut protein)[212]. Methods have been developed for the detection and quantification of peanut in complex matrices, like chocolate dessert and chocolate bars but struggled to robustly detect below 10 or 50 ppm peanut protein, depending on target peptide[213]. One MS method was able to detect incurred dark chocolate material at levels as low as 2 ppm peanut protein using two tryptic peptides from Ara h 1[214]. An LC-MS/MS method used to analyze rice crispy and chocolate snacks obtained LODs of 5 ppm peanut protein in matrix for Ara h 2 and 1 ppm peanut protein in matrix for Ara h 3[211]. An SRM method targeting two tryptic peptides from Ara h 3 was developed and was successful in its detection of peanut at levels as low as 10 ppm peanut (2.5 ppm peanut protein) in enriched cookies[215]. Detections as low as 2.5 ppm peanut protein were achieved in an MRM method analyzing contaminated chocolate, cookie, ice cream, and tomato sauce[216]. For the four peptide markers included in that method, the LOQs ranged from 2.5-50 ppm peanut protein in matrix[216].

Though MS methods may overcome several matrix and processing issues observed by ELISA, target peptides for MS methods from Ara h 1 and Ara h 3 (cupins) appear to be more susceptible to processing effects than Ara h 2, Ara h 6, and Ara h 7 (2S albumins)[217]. Even so, an MRM method to detect peanut peptides in thermally processed samples (boiling, roasting, frying) utilized peptides that achieved sensitivities of 0.1-30 attomoles on-column[217]. Furthermore, an MRM method developed to detect peanut in raw and cooked wheat matrices using two Ara h 1 peptides showed greater quantitative accuracy than five analyzed ELISA kits, as the ELISAs reported overestimations of peanut in the wheat matrix[218].

VII. SUMMARY

Food allergy is an important food safety consideration for both the food industry and allergic consumers. Peanut allergy is of particular concern due to its prevalence, reaction severity, low threshold dose, and common presence in a variety of food products in the US. Because of regulatory measures, the food industry must have the ability to detect peanut protein in processed foods for the purposes of consumer protection and government compliance in the US. Current peanut allergen detection methods, specifically ELISA, PCR, and LFD struggle in their detection and accurate quantification of peanut protein in certain processed food matrices. MS-based peanut detection methods may have potential for effective quantification of peanut protein, even in complex and processed food matrices.

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CHAPTER II: THE SELECTION AND FILTRATION OF TARGET PEPTIDES USING UNTARGETED AND TARGETED MS ANALYSES OF INCURRED COOKIE AND DARK CHOCOLATE MATRICES

I. ABSTRACT

Peanut allergy has prevalence rates near 2% for individuals in the United States (US) and is associated with particularly severe reactions[1-3]. The US requires ingredient labeling of peanut to protect peanut-allergic consumers[4]. Thus, the detection of peanut protein in food matrices is a critical component of food safety for the food industry and is used to monitor allergen cross-contact. Immunochemical detection methods for peanut have demonstrated deficits in accurate quantitation in processed and complex matrices, including cookie and dark chocolate[5, 6]. Mass spectrometry (MS)-based detection methods may provide an alternative approach. In this work, target peptides were identified for an MS method that detects and quantifies peanut in cookie and dark chocolate matrices.

A discovery-based approach to target selection was utilized. Incurred cookie and dark chocolate matrices were generated at various concentrations of peanut protein. Incurred matrices and peanut flour (PF) samples were analyzed using untargeted MS proteomic techniques. Peptide identification was completed using a peptide sequence database search through a proteomics software for peanut (*Arachis hypogea*) and for respective matrix components. Selection criteria, designed to identify abundant peptides which are the least affected by the matrix, were applied to identified peanut peptides to

classify a subset of peptides as candidate target peptides. In the matrices, 32 (cookie) and 67 (dark chocolate) candidate target peptides were determined.

Following identification of candidates, iterative rounds of targeted MS were completed to empirically discern underperforming target peptides. Six (cookie) and seven (dark chocolate) target peptides persisted through all targeted MS rounds. Combining the filtered target peptides for both matrices, resulted in a list of nine unique peanut peptides. The nine final target peptides demonstrated promising sensitivity, between 10-50 ppm PF prior to any method optimization. Many of the nine final target peptides have been included in other published quantitative MS methods for peanut.

II. INTRODUCTION

Peanut is considered one of the "Big Eight" allergenic foods and allergy to peanut affects approximately 2% of individuals in the US[1, 2]. The Food Allergen Labeling and Consumer Protection Act (FALCPA) requires a plain-language declaration of peanut and peanut-derived ingredients in food products in order to protect allergic consumers[4]. To comply with US regulation and increase consumer safety, the food industry has adopted various detection methods for peanut, of which ELISA-based methods are most prominently used. A food matrix, which can be defined as the assembly of, and complex interactions between, physical and chemical components of a food, often complicates the detection of peanut contamination by immunoassay detection methods[5, 7]. Undeclared peanut has been detected in commercially available cookie and dark chocolate products and ELISA methods have demonstrated issues in accurately quantifying this contamination due to changes in target analytes in the matrix or after processing[5, 6, 8]. In an interlaboratory study that investigated five commercially available ELISA kits, peanut in cookie was consistently overestimated by the kits with average percent recoveries greater than 100% for four of the five kits[6]. For dark chocolate, recoveries ranged from 43.7-151.8%, depending on the kit used[6]. Other studies have reported concerning low recoveries of peanut in chocolate, such as 13-42% recovery of peanut[9].

Therefore, this work sought to develop an MS method for the detection and quantification of peanut protein in processed food matrices, specifically cookie and dark chocolate, as their complexity has resulted in inaccurate quantitation of peanut protein by commonly used detection methods. Both selected matrices are considered complex in

nature. Cookies may contain a gluten network, which is rubbery and viscoelastic in dough and after extensive thermal processing, proteins may be structurally or irreversibly changed[10]. Dark chocolate is complex due to its high fat content and phenolic compounds[11]. MS-based methods may be more advantageous for thermally processed matrices compared to immunological assays because the structure of the target protein need not be conserved as MS targets peptides, instead of proteins, which also allows for a more rigorous protein extraction[12].

For the method, it is necessary to detect low levels of peanut protein in a final food product. Thus, one consideration for matrix generation is whether the allergen source material is added prior to (incurred matrices) or following processing of the food matrix (spiked matrices)[13, 14]. The use of incurred matrices allows for the discernment of two primary effects on analyte recovery, the first being the interactions of the allergenic proteins with matrix components (matrix effect) and the effect of processing the material on the proteins targeted by the method (processing effect). Spiked matrices may allow for only limited determination of the effect of the food matrix and does not account for any processing-induced changes of the protein analyte with respect to their interaction with matrix proteins[13, 15]. A wide variety of protein modifications can occur during the processing of a food matrix, including protein denaturation, protein aggregation, as well as chemical modifications such as results of the Maillard reaction, matrix lipid oxidation, or enzymatic modification[16]. These changes may impact the solubility and extractability of allergenic proteins and therefore affect their recovery in detection methods[15]. Spiked matrices also fail to accurately mimic real-world contamination of food products during

production because cross contact may occur before the final product stage, for example by shared manufacturing equipment[17]. Therefore, utilizing incurred matrices, instead of spiked matrices, for the development of allergen detection methods is advantageous as it inherently encapsulates both the effects of a food matrix and its processing.

Other MS-based peanut allergen detection methods have been developed previously for cookie and dark chocolate matrices from only spiked materials or pseudo-incurred materials[18-20]. Because of the nature of the food materials used to develop the method, the final method may not effectively account for the comprehensive matrix effect. MS-methods for incurred cookies and chocolate have been developed, but some have limits of detection and quantitation insufficient compared to the needed method performance determined for this work[21-23].

One of the most critical components of an MS method is the target peptides on which the method is based. In MS-based allergen detection methods, detection of selected target peptides from the allergen source is used to indicate and quantify the presence of the allergenic food in the sample analyzed[24]. There are two primary approaches to target peptide selection for an MS method, which are bioinformatic or *in silico*-based target selection and discovery-based target selection[25]. In the first approach, protein sequences are digested *in silico* with a protease, typically trypsin, to generate a list of peptide sequences to consider as targets. Selected peptides also must be unique to the species of the allergenic source and therefore, should be analyzed using Basic Local Alignment Search Tool (BLAST) to determine their specificity to the intended species[24]. If a target peptide is not unique, it is not fit for a quantitative MS detection method[26]. One limitation

for this selection approach is the availability of protein sequence information, as this strategy may not be feasible unless sequence data is accessible[27]. Secondly and arguably more important, is the limitation that a bioinformatic-based selection approach may not consider the effect of the food matrix or its processing on proteins and respective peptides[25]. The method that overcomes this deficit and considers the food matrix effect is discovery-based target selection. In a discovery-based approach to target selection, the food sample containing the allergenic food is analyzed using discovery MS and detected mass events are searched against a protein sequence database to identify detected peptides[25]. These peptides are then evaluated considering further criteria to select a number of target peptides that show greatest promise as quality targets for an MS method. Limitations of this approach to target selection include reliance on protein sequence availability for peptide identification, the requirement of high-resolution MS instrumentation, and a certain expertise in analysis of discovery MS data[25]. These target peptides should also be analyzed for their specificity to the intended species, as with bioinformatically generated target peptides, and only specific peptides are fit for quantitative MS methods[24, 26].

For the method developed in this work, a discovery-based approach to target selection was utilized, and screening of identified target peptides for sensitivity and robustness was completed. Following the identification of candidate peanut peptides in matrix, an empirical filtration of peptides was completed using targeted MS, specifically parallel reaction monitoring (PRM), to determine the best performing peptides for each matrix to consider as target peptides for a quantitative MS method.

III. MATERIALS AND METHODS

i. Analysis of Cookie Matrix Ingredients

Ingredients for the cookie matrix, including unsalted butter, granulated sugar, table salt, baking soda, and all-purpose wheat flour were obtained from a local market in Lincoln, Nebraska. Prior to the generation of the peanut-incurred cookie matrix, ingredients to be used in the cookie formulation were evaluated for the presence of peanut using a commercially available ELISA kit (Veratox® for Peanut ELISA kit, Product no. 8430 Neogen® Lansing, Michigan). The ingredients analyzed for the presence of peanut were wheat flour, butter, and sugar. Ingredients at low risk for peanut cross-contact, such as salt, baking soda, and dextrose (Spectrum® Chemical MFG Corporation, New Brunswick, New Jersey) were not evaluated. For the wheat flour, butter, and sugar, three samples (5.00 grams each) were taken randomly from ingredient packages. ELISA analysis was conducted according to the protocol provided by the kit manufacturer. Extractions from the samples for each ingredient were evaluated in duplicate wells.

ii. Incurring Strategy and Homogeneity Evaluation for Cookie Matrix

Light roast Old Virginia Byrd Mill peanut flour (PF) (12% fat) was sourced from the Golden Peanut and Tree Nuts Company (Alpharetta, Georgia). The PF was determined to be 52.12% protein according to Dumas analysis. PF was incurred into the cookie matrix prior to processing by incorporation in the sugar to be used subsequently in the cookie formulation. The peanut-incurred sugar was prepared initially at a high concentration and serially diluted to all needed incurred levels using blank sugar. A

KitchenAid® Stand Mixer (4.5 quart) was used to prepare peanut-incurred sugar levels. To create the highest incurred sugar level (36,359.33 ppm PF in sugar), 651.46 g of sugar was added to a mixing bowl, followed by the addition of 24.58 g of PF. To ensure a homogenous mixture of the PF into the sugar, the mixture was thoroughly mixed for five minutes.

To create subsequent levels of peanut-incurred sugar, the previously formulated incurred sugar was serially diluted with blank sugar to achieve the desired concentration. This dilution of was completed with masses according to Table 2.1. Six levels of peanut-incurred sugar were prepared, including 3,636.15, 363.63, 36.36, 7.27, and 3.64 ppm PF in sugar, to prepare six levels of incurred cookie dough.

Table 2.1: Masses Used to Generate Peanut-Incurred Sugar and Subsequent Dilutions

Designed Cookie Matrix Level Matrix (ppm PF)	Designed Cookie Matrix Level (ppm peanut protein)	Actual Cookie Matrix Level (ppm peanut protein)	Concentration in Sugar (ppm PF)	Mass of Previous Incurred Level Added (g)	Blank Sugar Mixed with Previous Level (g)	Total Mass of Incurred Mix (PF + Sugar) (g)	Mass Removed for Formulation (g)
10000	5,000	5,212	36359.33	24.58*	651.46	676.03	550.15
1000	500	521.20	3636.15	66.89	601.98	668.86	550.15
100	50	52.12	363.63	68.72	618.45	687.17	550.15
10	5	5.21	36.36	78.02	702.17	780.19	550.15
2	1	1.04	7.27	180.04	720.18	900.22	550.15
1	0.5	0.52	3.64	300.07	300.07	600.15	550.15

*PF

After mixing, two levels (36,359.33 and 363.63 ppm PF) of the peanut-incurred sugar were transferred to separate baking trays and spread to even thickness. Nine samples (1.00 g each) were taken from each tray. Locations for sampling were equidistant from one another and evenly distributed to provide a holistic view of the

distribution of PF in the sugar. The samples were analyzed using ELISA to ensure homogeneity of the sample. For ELISA analysis, the manufacturer's protocol was followed with minor modifications. The procedure was adjusted for analysis of a 1.00 g sample and sample extractions were diluted using the kit's extraction buffer to ensure a level of peanut protein within the kit's quantification range of 2.5-25 ppm peanut and evaluated in single wells.

iii. Generation of Cookie Matrix

Following determination that the wheat flour, butter, and sugar did not contain detectable levels of peanut and confirmation of the homogeneity of peanut-incurred sugar mixes, the cookie matrix was generated. The method and formulation used for the cookie matrix was based on the American Association of Cereal Chemists (AACC) International method 10-50-0.5, with modifications. All equipment and utensils were washed prior to matrix generation and in between each level produced to prevent cross-contact. The generation of the matrices began with the 0 ppm peanut protein cookie, followed by generation of the 0.52, 1.04, 5.21, 52.10, 521.20, 5,212 ppm peanut protein cookies. This order of increasing peanut protein concentration was selected in order to minimize the potential for allergen cross contact. Ingredient masses used to generate the cookie matrices are summarized in Table 2.2.

Table 2.2: Cookie Formulation for Peanut-Incurred Cookie Matrices

Ingredient	Mass in Formulation Blank Matrix (g)	Mass in Formulation Incurred Matrices (g)
Unsalted Butter	270.84	270.84
Granulated Sugar	550.15	550.15*
Salt	8.89	8.89
Baking Soda	10.58	10.58
Dextrose Solution	139.65	150.00
Distilled Water	67.71	67.71
All-Purpose Flour	952.18	952.18
Total:	2000.00	2010.35

*At appropriate PF concentration

To prepare the cookie matrices, the butter and sugar (blank or incurred at the appropriate PF concentration) were added to a Hobart© Commercial mixer (Model A200, 20 quart) and mixed for two and a half minutes. For all breaks between mixes, any ingredients that had adhered to the sides of the mixing bowl or the beater attachment were scraped with a spatula. Distilled water and dextrose solution were added second, followed by additional mixing for two and a half minutes. Half of the wheat flour, salt, and baking soda mix were added to a KitchenAid mixer and thoroughly mixed for 30 seconds. This mixture was incorporated into the Hobart mixer to be combined with the previously added ingredients. As this mixing continued, the second half of the wheat flour, salt, and baking soda mix was added to the Hobart mixer until mixed for four minutes and fully incorporated.

The dough was removed, and a portion was rolled to an approximate thickness of one quarter inch. Next, cookies were cut using a square cookie cutter with sides that were 4.2 centimeters in length. Cookies were transferred to a baking tray lined with parchment

paper and appropriately spaced to ensure separation after spreading during baking. The cookies were baked for 20 minutes in Reed Revolving Reel Oven at 176.67 °C. Mass determinations of reserved dough and the cookies after baking allowed for a calculation of the percent water lost during baking. Using water loss, experimental peanut protein concentrations were calculated for each cookie level (0, 0.63, 1.24, 6.21, 62.08, and 6,206.93 ppm peanut protein). After weighing, baked cookies were stored at room temperature in an unsealed container for 12 hours to ensure proper drying. Following drying, cookies were stored at 4 °C.

iv. Dark Chocolate Matrix Manufacture

The dark chocolate matrix was manufactured in the pilot plant of a prominent chocolate manufacturer under the supervision of University of Nebraska staff. A common formulation used by the manufacturer for dark chocolate was used for the peanut-incurred dark chocolate, which included sugar, chocolate liquor, cocoa butter, soy lecithin, and vanillin. The materials used for the dark chocolate were well characterized by the manufacturer. The dark chocolate formulated was approximately 45.95% cocoa. The PF used in preparation of the dark chocolate was light roasted, 12% fat PF obtained from the Golden Peanut Company, which was determined to be 55.7% protein by Dumas analysis. The PF was added at the refining step to ensure consistent particle size and prior to the conching step to ensure homogeneity. Therefore, the dark chocolate is appropriately deemed an incurred matrix. Two levels of peanut-incurred dark chocolate were generated (100 and 5,000 ppm peanut protein) and used to obtain desired levels of incurred dark chocolate by mixing and tempering with a 0 ppm peanut protein dark chocolate.

v. Untargeted MS Analysis of Incurred Matrices

a. Grinding

Blank cookie and dark chocolate samples (0 ppm peanut protein) and the highest concentration of peanut-incurred cookie and dark chocolate (6,206.93 ppm peanut protein cookie and 5,000 ppm peanut protein dark chocolate), as well PF, were ground into finer particles prior to analysis by discovery proteomics techniques. To grind the baked cookie samples, approximately 100 g of the cookies were ground in a Cuisinart® Mini-Prep® Plus food processor (24 oz). For cookie dough samples, partitioning into finer particles was completed manually with a spatula. The dark chocolate was ground manually with a razor blade. The PF had a fine particle size and thus, was not ground prior to protein extraction.

b. Extraction

Protein extraction of the incurred matrix samples was completed in triplicate using a chaotropic buffer containing 6M Urea (Bio-Rad Laboratories©), 2M Thiourea (Sigma-Aldrich®), 20 mM DL-1,4-Dithiothreitol (DTT) (Acros Organics, Thermo Scientific™), and 50 mM Tris-hydrochloride (Tris-HCl) (pH 8.8) (Trizma® HCl, Sigma-Aldrich). For all matrix samples, this extraction was completed at a 1:20 w/v ratio. This equated to 0.500 g of matrix extracted in 10 mL buffer. PF samples were extracted at a concentration equivalent to a 5,000 ppm peanut protein matrix. For PF samples, the extraction concentration was selected based on the amount of PF that would theoretically be present in 0.5 g of the 5,000 ppm peanut protein cookie dough. For weighing accuracy, this amount of PF and corresponding buffer was scaled up by a factor of three.

This equated to 0.015 g (15 mg) of PF extracted in 30 mL buffer. This extraction is referred to as a 5,000 ppm peanut protein matrix equivalence.

To begin extractions, sample tubes were vortexed to promote suspension of the solid sample in buffer. Sample tubes were transferred to a shaking water bath (200 rpm) at 60 °C for 10 minutes (Julabo SW22 Water Bath). This was followed by a one minute vortex of each sample. Following this, samples were transferred to a water bath sonicator (Bransonic® S800 Ultrasonic Bath) and sonicated for 10 minutes at room temperature (RT). Next, samples were vortexed briefly to resuspend sample solids in buffer. Samples were returned to the 60 °C shaking water bath and incubated for 10 minutes, followed by a centrifugation at 3,000 x g for 10 minutes at 4 °C for clarification (Centra® MP4R Refrigerated Tabletop Centrifuge). Next, two samples (1 mL each) of each extract supernatant were transferred to 1.5 mL microcentrifuge tubes and further centrifuged at 17,000 x g for 10 minutes at RT (Sorvall Legend Micro 17 Centrifuge). Then, 800 µL of supernatant from each duplicate sample was combined in a 1.5 mL microcentrifuge tube and stored at -20 °C.

Next, an SDS-PAGE gel was completed using NuPAGE™ 4-12% Bis-Tris Gels (1.00 mm x 12 well) (Invitrogen™, Thermo Scientific™) to ensure extraction of protein from samples analyzed. Sample extract (20 µL) was combined with 7.5 µL 4X NuPAGE LDS Buffer and 1.5 µL water and reduced using 1 mL of ß-mercaptoethanol (BME) (Sigma-Aldrich) at 70 °C for 10 minutes. NuPAGE SDS Running Buffer was used in a Mini-Cell Electrophoresis Chamber (Invitrogen, Thermo Scientific). From the 30 µL of prepared sample, 20 µL of each sample was loaded into each well. The gel was run for 35

minutes at a constant voltage of 200V. Alongside samples, Precision Plus Protein™ Dual Xtra Standards were run (Bio-Rad Laboratories). Protein bands were fixed in a 50% methanol and 10% acetic acid solution for 15 minutes (Fisher Chemical™, Thermo Scientific). Gels were stained with Coomassie Brilliant Blue R-250 Staining Solution for 1 hour and destained using Coomassie Brilliant Blue R-250 Destaining Solution for 3 hours (Bio-Rad Laboratories). The gel was rinsed with water and imaged under bright light. Identification of protein bands in extract samples informed the determination of the efficacy of the extraction protocol.

Following confirmation of extraction, protein quantification of sample extracts was completed using the Cytiva 2-D Quant Kit, according to the manufacturer's instructions. Each extraction replicate was analyzed in duplicate. The volume of the extract analyzed varied by sample and protein content to accommodate the kit standard curve. Using the theoretical protein content of each matrix and sample type, an extraction efficiency was determined.

c. PF Extraction Optimization

To address the variability observed with PF extractions prepared at 15 mg PF in 30 mL buffer, improvement of the PF extraction was pursued. To optimize this extraction, extractions were conducted as previously described, but at four different concentrations. The four samples analyzed were 15 mg PF/30 mL buffer, 60 mg PF/30 mL buffer, 250 mg PF/10 mL buffer, and 500 mg PF/10 mL buffer. Triplicate extracts were completed for each of the levels. Extract samples were analyzed in duplicate by 2D-

Quantification, and extraction efficiencies were calculated to determine an optimal PF extraction concentration to minimize variability in weighing and quantification.

d. Digestion

Samples were prepared for tryptic digestion with reduction and alkylation. Methods were based on the manufacturer's recommendation for In-Solution Tryptic Digestion for Pierce™ Trypsin Protease, MS Grade (Thermo Scientific), with minor modifications. In the first untargeted experiment, 21 µL of cookie and PF sample extracts were buffered with 30 µL of 50 mM ammonium bicarbonate (ABC) (Fluka® Analytical, Sigma-Aldrich) and reduced with 3 µL of 100 mM DTT. For the dark chocolate untargeted analysis, 10.5 µL of dark chocolate matrix and PF sample extracts were prepared for digestion with 30 µL of 50 mM ABC and reduced with 3 µL of 100 mM DTT. Reduction was conducted at 95 °C for five minutes. Following reduction, samples were cooled. Next, samples were alkylated with 6 µL of 100 mM iodoacetamide (IAA) (BioUltra, Sigma-Aldrich) and incubated in the dark for 20 minutes. Trypsin protease (2 µL, 100 ng/uL) was added to each sample and incubated for one hour at 37 °C. Next, an additional 2 µL of trypsin was added to samples. Digestion continued overnight, for a period not to exceed 16 hours, at 30 °C. Digestion was halted by transfer of digested samples to a 20 °C freezer. An SDS-PAGE gel was completed in the manner described previously to confirm trypsin digestion.

e. Desalting

Sample digests were desalted to remove non-peptide components from the samples using Pierce™ C18 Spin Columns (Thermo Scientific). The manufacturer's protocol was followed with minor modifications. Formic acid (FA) was used instead of the recommended trifluoroacetic acid (TFA). All solvents used in desalting were Optima™ LC/MS Grade including FA, acetonitrile (ACN), methanol, and water (Fisher Chemical, Thermo Scientific).

f. Lyophilization and Resuspension

Desalted samples were lyophilized using a Savant SpeedVac SPD120 Vacuum Concentrator and a Savant RVT105 Refrigerated Vapor Trap to lyophilize peptide samples. Prior to injection for liquid chromatography and tandem mass spectrometry (LC-MS/MS), cookie matrix samples and the accompanying PF samples (5,000 ppm peanut protein matrix equivalence) were resuspended in 60 µL of a 5% ACN and 0.1% FA buffer (Optima Grade, Thermo Scientific). For the dark chocolate samples, as only 10.5 µL of extract was digested, the resuspension volume was halved with respect to the cookie samples. Thus, dark chocolate samples were resuspended in 30 µL of 5% ACN and 0.1% FA prior to MS analysis. The optimized version of the PF samples (5,000 ppm peanut protein matrix equivalence) was run alongside dark chocolate samples.

g. LC-MS/MS

Peptide separation was completed using a Dionex UltiMate 3000 UHPLC+ system for liquid chromatography and a Hypersil GOLD™ Dim. (mm) 11x1 (Part No 25002-101030) column at 35 °C (Thermo Scientific). Triplicate injections (9 µL) were

analyzed for each sample. The LC method began with a six minute equilibration at 2% ACN (0.060 mL/min flow rate). An ACN gradient from 2% ACN to 40% ACN (0.060 mL/min) over 70 minutes was used to elute peptides. The LC method continued for six minutes at 40% ACN (0.060 mL/min). Next, was a five minute wash at 98% ACN (0.060 mL/min) followed by a six minute wash using 100% methanol (0.150 mL/min). Re-equilibration at 2% ACN was completed for 17 minutes (0.150 mL/min) and then for four minutes at a 0.060 mL/min flow rate. All solvents, including ACN, FA, methanol, and water were of Optima LC/MS Grade (Fisher Chemical, Thermo Scientific).

A Thermo Scientific™ Q Exactive™ Plus hybrid quadrupole-Orbitrap™ mass spectrometer was used for this analysis and was set in data dependent acquisition (DDA) mode. The full scan MS method was run in positive ion mode at 70,000 resolution with a scan range of 400 to 1,400 m/z. Settings for MS1 scans included the automatic gain control (AGC) target set at 3e6 and the maximum injection time (IT) at 100 milliseconds (ms). The top 20 precursor ions were selected for fragmentation. Precursors with unassigned charges, a charge of +1 or greater than 5+ were excluded. For MS2 scans, a scan range of 200 to 2,000 m/z was used, and analysis was completed at a resolution of 70,000. The AGC target was set at 1e5, and the maximum IT was set at 240 ms. The normalized collision energy (NCE) used was 27. Dynamic exclusion for 20 seconds (s) was also used.

h. Peptide Identification and Semi-Quantitation

Data analysis, including peptide identification and semi-quantitation of identified peptides, for untargeted MS runs was conducted using PEAKS® Studio 8.5 Software (Bioinformatics Solutions). To accommodate the PEAKS project workflow, identification began with a *de novo* search. The *de novo* search was completed using a parent mass error tolerance of 5.0 ppm and a fragment mass error tolerance of 0.05 Daltons (Da). A fixed modification of carbamidomethylation (+57.02 Da) was included due to the alkylation of reduced sulfide bonds by IAA. Variable modifications included in the search were the oxidation of methionine (+15.99 Da) and hydroxyprolination (+15.99 Da). Data from the *de novo* search was not utilized but completed to comply with the software workflow required prior to PEAKS database searches.

Next, PEAKS database searches were used to identify peptides detected in the discovery MS analysis of the incurred matrices. Databases searches for peanut peptides utilized a genome-derived peanut protein sequence database, as used by Marsh, et al.(2020)[28]. This protein sequence database was focused on proteins of the peanut seed as opposed to a comprehensive peanut plant proteome. Nomenclature for protein isoforms, as defined by Marsh et al. (2020), were used for this work[28]. Protein sequence databases for the wheat proteome (*Triticum aestivum*) and cacao (*Theobroma cacao*) were downloaded from UniProt Proteomes. For the cookie samples, the MS spectra were analyzed against the peanut protein database and the *Triticum aestivum* proteome separately. In the same way for the dark chocolate samples, the spectra were analyzed against the peanut protein database and the *Theobroma cacao* proteome separately.

PEAKS database searches were conducted with a 5.0 ppm parent mass error tolerance and a 0.05 Da fragment mass error tolerance. Carbamidomethylation (+57.02 Da) was included as a fixed modification, as well as oxidation of methionine (+15.99 Da) and hydroxyprolination (+15.99 Da) as variable modifications. Enzyme settings were used for trypsin, and the maximum number of missed cleavages was set at zero, and no non-specific cleavages were included. Three maximum variable post-translational modifications (PTM) were allowed per peptide. A contaminant database was also included in these searches. A comparative analysis between the peanut protein database search and the matrix proteome search was completed within PEAKS in order to exclude peptides which were detected in both the peanut and the respective matrix proteome.

Following identification of peptides, semi-quantitation of detected peanut peptides was completed using Label Free Quantification (LFQ) in PEAKS. LFQ settings included a 5.0 ppm mass error tolerance and a 6.0 minute retention time shift tolerance. A false discovery rate (FDR) threshold of 1% was used. LFQ data, which was normalized to the total ion current (TIC) for identified peptides, was exported to Microsoft® Excel® (Microsoft 365) and the "protein-peptides" export file was used for further data analysis.

The average peak area (MS1) for each peptide in each sample were determined by averaging the three injection replicates and the three extraction replicates for each sample. This value was noted as the abundance for each peptide. Next, peptide peak areas were used to determine the relative allergen abundance in the incurred cookie, incurred dark chocolate, and PF samples. To complete this relative quantitation of allergenic proteins using the LFQ data, the top three peptides from each protein detected were

averaged to deduce an overall peak area value per peptide. Peanut proteins were only relatively quantified in this way if there three peptides were detected from the protein in the untargeted analysis. Isoforms of peanut proteins were considered in this analysis, as some detected peptides are present in one, multiple, or all isoforms of a protein. Graphs and figures were created GraphPad Prism, Version 9.3.0 (463).

vi. Selection of Candidate Target Peptides in Incurred Matrices

From all identified and quantified peanut peptides in LFQ, a smaller list was generated of peptides that demonstrated promise as quality targets. series of three exclusion steps were conducted, followed by application of two selection criteria to determine candidate target peptides.

First, any peptide found to be in common between the peanut PEAKS database search and the matrix proteome PEAKS database search through the comparative analysis was excluded. Next, any peptide that had a detection in more than one injection in the blank matrix was removed from the potential peptide list for specificity purposes. Any peptide which contained variable PTMs of hydroxyprolination or oxidation of methionine were eliminated.

Peptides that were not removed by the three exclusion steps were then subjected to two predetermined selection criteria, designed to select the best performing target peptides in the analyzed matrix. The first selection criterion was abundance and was applied through the selection of only the top 60% most abundant peptides in the sample set based on the MS1 peak areas of peptides. The second selection criterion evaluated the detection of the peptide in matrix against its detection in PF as a measure of robustness or

matrix performance. For this, the selection criterion was established to select only peptides with a recovery ratio of 0.1 or greater in the matrix compared to PF alone. The ratio was calculated by dividing the average peak area of the peptide in matrix by the average peak area of the peptide in PF. Only peptides that met both selection criteria were deemed candidate target peptides.

vii. Large-Scale Sample Preparation for Targeted MS

Sample preparation that was used to prepare samples for targeted MS was similar to that of the sample preparation for untargeted MS, and any deviations from the original protocol were for the purpose of achieving a greater amount of protein in samples prior to analysis by MS. Extraction was completed at the same 1:20 w/v ratio described for the untargeted MS sample preparation, except the PF extraction used was the optimized concentration for its extraction efficiency and reduced variability (60 mg PF/30 mL buffer). In the large-scale digestion, 105 µL of sample extracts were buffered with 150 µL of 50 mM ABC and reduced with 15 µL of 100 mM DTT. For alkylation, a volume of 30 µL of 100 mM IAA was used. Digestion was initiated using 10 µL of trypsin with a one hour incubation at 37 °C. Next, an additional 10 µL of trypsin was added for digestion. The total digestion volume was 320 µL.

For desalting, a larger capacity column was used (Strata™-X 33 um Polymeric Reversed Phase (10 mg/1 mL)(Phenomenex®), and the manufacturer's protocol was followed with minor modifications. Samples were lyophilized as mentioned previously and were resuspended in 300 µL of 5% ACN/0.1% FA. For filtration experiments, samples were diluted to various concentrations using resuspension buffer (5% ACN/0.1%

FA) and injected (15 µL) for LC-MS/MS analysis. For these samples, LC was completed using a larger capacity column, a Hypersil GOLD aQ Dim. (mm) 20 x 2.1 (Part No 80000-506) at 35 °C. Chromatographic adjustments were made for the column. The chromatography used for the subsequent filtration experiments begins with a six minute equilibration at 2% ACN (0.300 mL/min). Next, a 19-minute ACN gradient from 2% to 40% ACN (0.300 mL/min) was used for peptide elution. This was followed by a five minute wash using 98% ACN (0.300 mL/min). Re-equilibration at 2% ACN (0.300 mL/min) was completed for five minutes.

The large-scale samples were used for targeted MS analysis (PRM) to filter candidate target peptides down to a list of the most sensitive and robust targets for each matrix. Candidate peptide precursor m/z values were added to a scheduled PRM inclusion list. The MS was run in positive mode at a resolution of 35,000. The AGC target was set at 1e6, and the maximum IT was set at 500 ms. A loop count of 1 was used for compatibility with an inclusion list. An isolation window of 0.8 m/z was used for precursors and no isolation offset was utilized. The NCE for this method was 27. Analysis of targeted MS data was completed using Skyline Software from the University of Washington, MacCoss Lab[29].

viii. Targeted MS Filtration of Candidate Target Peptides in Cookie

Based on the number of candidate target peptides selected after untargeted MS analysis, it was necessary to filter these targets down to a reasonable number for inclusion in a targeted MS method. To accomplish this filtration, iterative rounds of PRM were completed to experimentally determine the best performing target peptides for the

analyzed matrix using the large-scale samples. Overall, six rounds of PRM filtration were completed for determination of the most sensitive and robust target peptides for the incurred cookie matrix. For PRM filtration analyses, samples are referred to by their peanut concentration in ppm PF. A summary of the methods used for each of the six rounds of iterative PRM for the cookie matrix are shown in Table 2.3.

Table 2.3: Method Description for PRM Filtration Rounds for the Cookie Matrix

Round of PRM	**Description of Method**	**Data Analysis Approach**
Round 1	Samples: PF Levels: 10,000 ppm PF Injections: 2 µL, single Inclusion list: all candidate target peptides in two batches All candidate target peptides, as selected based on untargeted MS analysis, were added to one of two inclusion lists and each was analyzed against the PF sample.	For precursors that had multiple charge states in the inclusion, the most abundant charge state was selected, and the least was removed, if could be determined. The two inclusion lists were combined into one and run again with the PF sample with a scheduled inclusion list. A scheduling window of 2 minutes was created based off peptide retention time (RT) from the initial run.
Round 2	Samples: Incurred cookie dough and incurred baked cookie Levels evaluated: 10,000 5,000, 500, 100, 50, and 10 ppm PF Injections: 15 µL, duplicate Inclusion list: remaining precursors after Round 1 removal of least abundant charge states for some target peptides. Remaining peptides included in scheduled inclusion list and evaluated against various concentrations of incurred samples.	The lowest concentration of PF that had at least three product ions detected was recorded for each sample type, cookie dough and baked cookie. Target peptides were eliminated from the method inclusion list if the peptide was not detected, if the lowest detected concentration level was greater than 5,000 ppm PF, or if visual inspection of the chromatogram indicated inappropriate or incomplete peak shape.
Round 3	Samples evaluated: Incurred baked cookie and PF Levels evaluated: 10,000, 5,000, 2,500, 1,000, 500, 100, 50, and 10 ppm PF Injections: 15 µL, duplicate Inclusion list: remaining precursors after Round 1 and Round 2 eliminations. Peptides included in scheduled inclusion list and evaluated against various concentrations of incurred samples.	The sum of the peak area of the top three product ions was calculated, and dilution curves were created for each peptide and matrix. The sensitivity of potential target peptides was evaluated and peptides which were not detected below 1,000 ppm PF in either sample type were eliminated. The linearity of the dilution curves was considered and peptides with non-linear curves were eliminated.

Round 4	Samples evaluated: Incurred baked cookie and PF Levels evaluated: 1,000, 750, 500, 250, 100, 50, 10, and 1 ppm PF Injections: 15 µL, duplicate Inclusion list: remaining targets after Round 1-3 eliminations. Peptides included in scheduled inclusion list and evaluated against various concentrations of incurred samples.	Using the top three product ions and sum of peak area as described for Round 3, dilution curves were created, and their linearity was evaluated. Peptides with nonlinear curves were eliminated. The lowest concentration of detection was considered and peptides which did not have detections at or below 500 ppm PF were removed.
Round 5	Samples: Incurred baked cookie and PF Levels evaluated: 500, 100, 50, 10, and 1 ppm PF Injections: 15 µL, duplicate Inclusion list: remaining targets after Round 1-4 eliminations. Peptides included in scheduled inclusion list and evaluated against various concentrations of incurred samples.	Using the top three product ions and sum of peak area as described for previous rounds, dilution curves were created, and their linearity was evaluated. Peptides with nonlinear curves were eliminated. The lowest concentration of detection was considered and peptides which did not have detections at or below 50 ppm PF in both sample types were removed.
Round 6	Samples: Incurred baked cookie, blank cookie dough, blank baked cookie, and PF Levels evaluated: 100, 50, 25, 10, and 1 ppm PF Injections: 15 µL, duplicate Inclusion list: remaining targets after Round 1-5 eliminations. Peptides included in scheduled inclusion list and evaluated against various concentrations of incurred samples. Additionally blank cookie matrix samples analyzed to evaluate sensitivity of remaining peptides and product ions.	Using the top three product ions and sum of peak area as described for previous rounds, dilution curves were created, and their linearity was evaluated. Peptides with nonlinear curves were eliminated. The lowest concentration of detection was considered. Relative performance in sensitivity was compared across remaining peptides in both sample types to eliminate any peptides which appeared to be underperforming.

ix. Targeted MS Filtration of Candidate Target Peptides in Dark Chocolate

The number of candidate target peptides selected based on the two outlined selection criteria for the dark chocolate matrix required further filtration of targets. An empirical approach to the reduction of candidate target peptides to a reasonable number of robust and sensitive target peptides was completed using iterative rounds of PRM analysis of dark chocolate samples. Overall, five rounds of PRM were completed in order

to determine the final list of sensitive and robust target peptides for the dark chocolate matrix. A summary of the methods used for each of the five rounds of iterative PRM for the dark chocolate matrix are shown in Table 2.4.

Table 2.4: Method Description for PRM Filtration Rounds for the Dark Chocolate Matrix

Round of PRM	**Description of Method**	**Data Analysis Approach**
Round 1	Samples: PF Levels: 10,000 ppm PF Injections: 15 µL, single Inclusion list: all candidate target peptides in four batches All candidate target peptides, as selected based on untargeted MS analysis, were added to one of four inclusion lists and each was analyzed against the PF sample.	For precursors that had multiple charge states in the inclusion, the most abundant charge state was selected, and the least was removed, if could be determined. A scheduling window of 4 minutes was created based off peptide RT from this run to be used in subsequent analyses.
Round 2	Samples: Incurred dark chocolate and PF Levels: 10,000, 5,000, and 1,000 ppm PF Injections: 15 µL, duplicate Inclusion list: remaining target peptides after Round 1, one inclusion list. Peptides included in scheduled inclusion list and evaluated against various concentrations of incurred samples.	For peptides that still had two charge states included, the least abundant was removed. The sensitivity of detection of analyzed peptides was considered. Peptides which did not have three product ions detected at 5,000 ppm PF or lower were removed from the method.
Round 3	Samples: Incurred dark chocolate and PF Levels: 2,500, 1,000, 500, 100, 50, and 10 ppm PF Injections: 15 µL, duplicate Inclusion list: remaining target peptides after Round 1-2, two inclusion lists. Peptides included in two scheduled inclusion lists, and each was evaluated against various concentrations of incurred samples.	The sum of the peak area of the top three product ions for each peptide was determined and dilution curves were created. The linearity of the peptide's response in accordance with the concentration of peanut. Peptides with nonlinear curves were eliminated. The sensitivity of each peptide was also evaluated, and peptides not detected below 1,000 ppm PF were removed.

Round 4	Samples: Incurred dark chocolate and PF Levels: 500, 250, 100, 50, 10, and 1 ppm PF Injections: 15 µL, duplicate Inclusion list: remaining target peptides after Round 1-3, two inclusion lists. Peptides included in two scheduled inclusion lists, and each was evaluated against various concentrations of incurred samples.	Dilution curves were created for each peptide, as described for Round 3. Any peptides with nonlinear dilution curves were removed. The sensitivity parameters allowed for the elimination of any peptides not detected at levels below 250 ppm PF in the dark chocolate or below 500 ppm PF in the PF.
Round 5	Samples: Blank dark chocolate, incurred dark chocolate, and PF Levels: Injections: 15 µL, duplicate Inclusion list: remaining target peptides after Round 1-4, one inclusion list. Peptides included in scheduled inclusion list and evaluated against various concentrations of incurred samples. Additionally blank dark chocolate matrix samples analyzed to evaluate sensitivity of remaining peptides and product ions.	Dilution curves were created for each peptide, as described and peptides with nonlinear calibration curves were removed from the method. Peptides without a detection at a concentration below 100 ppm PF in PF or dark chocolate were eliminated from the list of target peptides. .

IV. RESULTS AND DISCUSSION

i. Analysis of Cookie Ingredients

The purpose of this evaluation was to verify ingredients used in the cookie matrix did not contain detectable traces of peanut. All samples analyzed by ELISA were below the limit of quantification (LOQ = 2.5 ppm peanut) for peanut (n=6).

ii. Cookie Matrix: Incurring Strategy and Homogeneity Evaluation

The strategy of incurring PF into the sugar prior to the generation of the cookie matrix was selected in order to promote homogeneity of the PF in the final matrix and increase the accuracy of PF amounts at lower levels by eliminating the need to weigh small masses of PF. Sugar was selected as the vehicle to be incurred with the PF primarily due to its particle size and its ability to equitably mix with the PF.

Samples from two peanut-incurred sugar levels (36,359.33 and 363.63 ppm PF) were determined to be sufficiently homogeneous based on their analysis by ELISA. Based on the average measured peanut concentration, the coefficient of variation (%CV) was determined for each level as a measure of homogeneity, shown in Table 2.5. The %CV values for both incurred sugar levels were less than 20%, thus these levels were determined to be sufficiently homogenous. Furthermore, because the two evaluated peanut-incurred sugar levels were deemed homogenous, it was assumed that all peanut-incurred sugar mixes were amply homogenous for use in the generation of the cookie matrix.

Table 2.5: ELISA Results for Homogeneity Analysis of Peanut-Incurred Sugar

	36,359.33 ppm PF in sugar	**363.63 ppm PF in sugar**
Theoretical Concentration of Peanut in Sugar (ppm peanut)	72,718.66	727.26
Average Measured Result ± Standard Deviation (ppm peanut)	26,017.78 ± 3468	382.29 ± 21
Coefficient of Variation (%)	13.33	5.73

(N=9).

iii. Cookie Matrix Generation

Generation of the PF-incurred cookie matrices was completed according to formulations shown in Table 2.1. Water loss calculations were completed for each level of the cookie matrix (Table 2.6). The average water loss for the batches of cookies was 16.6%. The %CV for the water loss measure was 3.84%.

The actual concentration of peanut protein in the cookies after baking was calculated with consideration for the protein content of the peanut flour, as well as for

water loss and its effect on the total mass of the cookie batches. The actual concentrations of peanut protein in the cookies after baking are shown in Table 2.6.

Table 2.6: Adjusted Peanut Protein Concentrations Considering Water Loss

Designed Level (ppm peanut protein)	Concentration of Cookie Dough (ppm peanut protein)	Water Loss (%)	Concentration of Baked Cookie (ppm peanut protein)
0	0	16.25	0
0.5	0.52	17.98	0.63
1	1.04	16.56	1.24
5	5.19	16.33	6.21
50	51.86	16.05	62.08
5,000	5,185.96	16.45	6,206.93

The designed levels of peanut protein concentration were selected for this work according to the needed method performance for this detection method. This was determined using risk management principles. For determination of the lowest concentration of peanut protein per mass of food material (mg/kg) that should be detected by the method, there are two values which must be considered. The first is the relevant reference dose for peanut which, for the purposes of this study, is 0.2 mg peanut protein[30]. This is the amount of peanut in one eating occasion that would elicit a reaction for the most sensitive 1% of peanut-allergic individuals if consumed. This is the Voluntary Incidental Trace Allergen Labeling (VITAL) reference dose for peanut[30]. The second factor in determining risk of an allergic reaction is the typical amount of food matrix consumed by individuals. However, to maximize safety, an overestimate of the serving size much greater than the average consumption was used. A 100 g serving size for the cookie was used, though the median food intake for cookies in the US is only 36.0 g[31]. For an individual to receive a total of 0.2 mg of peanut protein while consuming 100

g of cookie, the concentration of peanut protein would equate to 2 ppm peanut protein in the cookie.

iv. Dark Chocolate Matrix Manufacture

The dark chocolate matrix was manufactured at various levels of incurred peanut and the levels used for this work were 0, 2, 20, 100, 5,000 ppm peanut protein. In the US, the median intake for dark chocolate in one eating occasion is 31.2 g, but a serving size of 50 g was used to calculate the needed method performance for this project to maximize safety for allergic consumers[31]. For an individual to consume the reference dose for peanut (0.2 mg peanut protein) through the consumption of 50 g of dark chocolate, the concentration of peanut protein would have to be 4 ppm peanut protein in matrix[30].

v. Untargeted MS Analysis of Incurred Matrices

a. Extraction Confirmation by SDS-PAGE

SDS-PAGE gels qualitatively confirmed protein extraction from samples to be analyzed in untargeted MS experiments. Triplicate extracts were run, including PF (5,000 ppm peanut protein matrix equivalence), blank cookie dough (0 ppm peanut protein), blank baked cookie (0 ppm peanut protein), incurred cookie dough (5,185.96 ppm peanut protein), and incurred baked cookie (6,206.93 ppm peanut protein), which are shown in Figure 2.1.

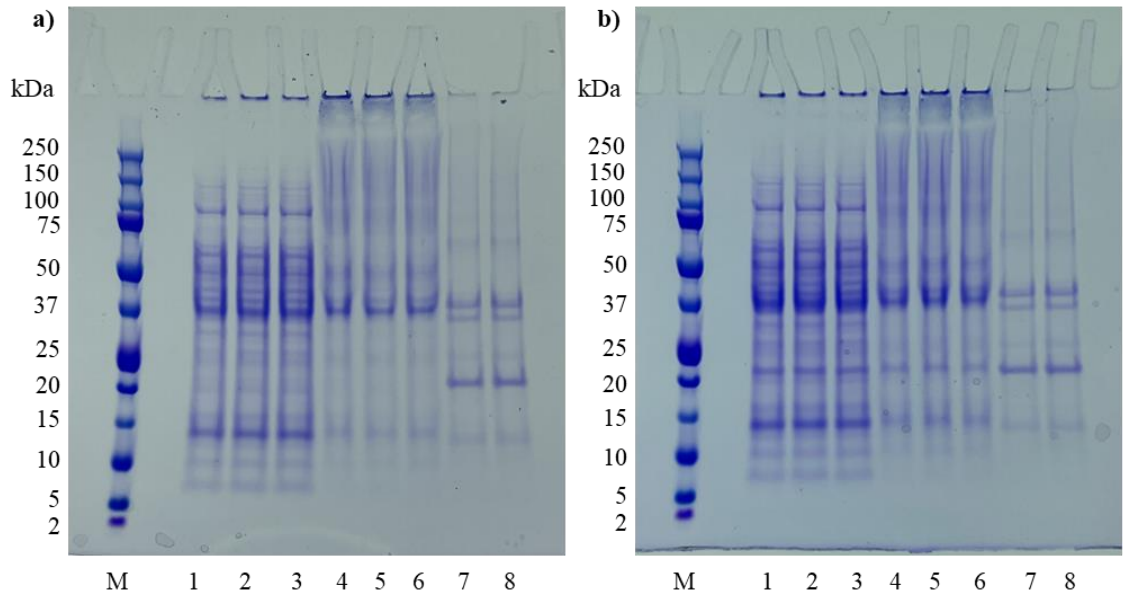

Figure 2.1: SDS-PAGE for Confirmation of Protein Extraction in the Cookie Matrix. **A)** Blank cookie dough and baked cookie samples. **B)** Peanut-incurred cookie dough and baked cookie samples. M: Molecular weight markers; lanes 1-3: replicate extracts of **a)** 0 or **b)** 5,185.96 ppm peanut protein in cookie dough; lanes 4-6: replicate extracts of **a)** 0 or **b)** 6,206.93 ppm peanut protein in baked cookies; lanes 7-8: replicate extracts of PF (5,000 ppm peanut protein matrix equivalence).

Gels for all extracted samples indicated successful protein extraction. Congruent protein banding patterns between triplicate extracts suggested similar extraction results across replicates. With the blank cookie dough and blank baked cookie samples, wheat protein was the primary protein source extracted and a different protein profile was observed for wheat proteins prior to versus after baking. This finding is consistent with the idea that thermal processing may impact the general extractability of proteins due to changes in structure and aggregation induced by heat during baking[32]. The PF-incurred cookie dough and PF-incurred baked cookie mirrored this result.

All PF extract samples have identical banding patterns and characteristic protein bands from peanut seeds can be observed on the gels. The protein band at approximately 65 kDa is likely the monomeric form of Ara h 1, which has an expected molecular weight

(MW) of 64 kDa when in its monomer form[33]. Prior to denaturation, Ara h 1 would be a tetramer with an estimated MW near 180 kDa[33]. The typical Ara h 3 acidic subunit protein bands can be observed near 37 kDa[34]. This protein band doublet represents different isoforms of the Ara h 3 acidic subunit[35]. The band near 20 kDa can be attributed the basic subunit of the Ara h 3 protein[34]. At 15 kDa, a band that may be Ara h 6 can be observed[34].

The protein bands observed for the incurred cookie dough and baked cookie samples indicated the presence of peanut in the extract material. This can be observed by the 20 kDa basic subunit of Ara h 3, which is noticeably present in the incurred samples and noticeably absent from the blank samples. This provided confidence in the extraction of peanut protein from the incurred cookie samples.

Blank dark chocolate (0 ppm peanut protein), incurred dark chocolate (5,000 ppm peanut protein), and PF (5,000 ppm peanut protein matrix equivalence) triplicate extracts are shown in Figure 2.2. The blank dark chocolate extract samples indicated little protein in the observable range for the gel. There is a faint protein band near 21 kDa, which has been attributed to a subunit of an albumin protein in cacao[36]. The extract samples of the 5,000 ppm peanut protein dark chocolate and the PF extract were nearly identical in their banding patterns which indicated probable extraction of peanut proteins from the incurred dark chocolate material.

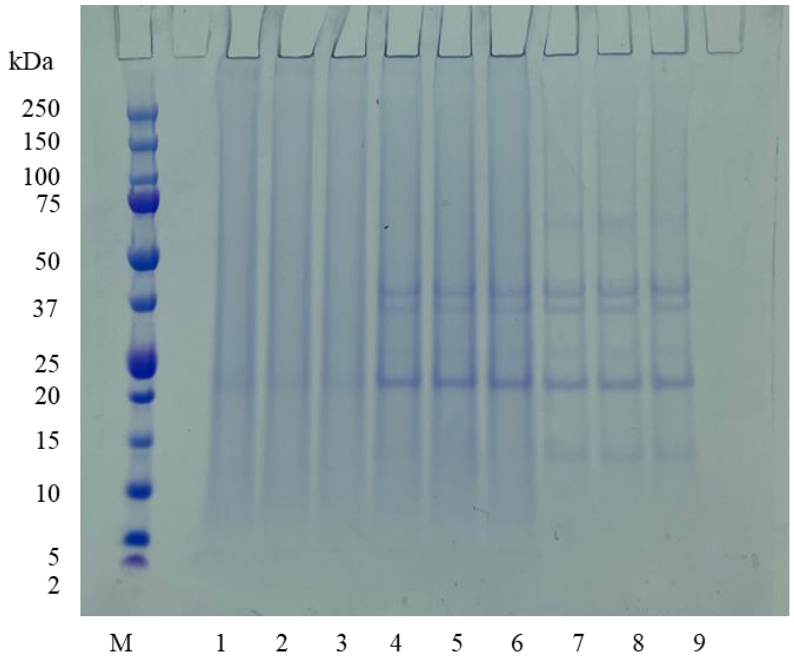

Figure 2.2: SDS-PAGE for Confirmation of Protein Extraction in the Dark Chocolate Matrix.
Blank and peanut-incurred dak chocolate samples. M: Molecular weight markers; lanes 1-3: replicate extracts of 0 ppm peanut protein in dark chocolate; lanes 4-6: replicate extracts of 5,000 ppm peanut protein in dark chocolate; lanes 7-0: replicate extracts of PF (5,000 ppm peanut protein matrix equivalence).

b. Extraction: PF Extraction Optimization

An optimization of the PF extraction was completed in order to evaluate the extraction efficiency and variability of four different extraction concentrations. An overestimation of protein by 2D-Quantification for the PF extraction (15 mg PF/30 mL buffer) which equated to an extraction efficiency of 119.00% prompted this optimization analysis.

The average extraction efficiency was calculated for each of the four extraction types: 15 mg/ 30 mL (136.45%), 60 mg/30 mL (63.96%), 250 mg/10 mL (66.22%), and 500 mg/10 mL (64.34%). The 15 mg PF/30 mL buffer extraction was again found to have

overestimated protein with a 136.45% extraction efficiency. In evaluating the variation between extracts for these sample types, the standard deviation (SD) of the extraction efficiency was 20.78% (15 mg PF/30 mL buffer), 2.77% (60 mg PF/30 mL buffer), 4.17% (250 mg PF/10 mL buffer), and 2.80% (500 mg/10 mL buffer). As the extraction increased from 15 mg PF/30 mL buffer to 60 mg PF/30 mL buffer, the variability decreased. A further increase of the extraction concentration, as with 250 mg PF/10 mL buffer to 500 mg PF/10 mL did not appear to decrease the variability any less than that observed with the 60 mg PF/30 mL buffer samples. The larger sample size for PF may have overcome issues with the heterogeneity of the flour sample or resulted in an improved accuracy in weighing of the material.

The untargeted MS experiment for the cookie matrix was completed prior to this optimization and therefore has a PF extraction of 15 mg PF/30 mL buffer, whereas the dark chocolate untargeted MS experiment was completed following this optimization and utilized the 60 mg PF/30 mL buffer extraction concentration with a proportionately scaled up resuspension volume to account for the final concentration prior to injection for MS.

c. Extraction: 2D-Quantification

Quantification of the soluble protein in sample extracts was completed to determine the protein concentration of extracts to evaluate extraction efficiency and to inform trypsin digestion. Extraction efficiencies were determined from the theoretical protein content of the sample type (Table 2.7).

Table 2.7: Quantification of Soluble Protein in Cookie, Dark Chocolate, and PF Extracts

	Average Measured Protein Concentration (ug protein/uL)	**Theoretical Protein Concentration (ug protein/uL)**	**Extraction Efficiency (%)**
Cookie Dough (5,185.96 ppm peanut protein)	0.868	2.617	33.18
Baked Cookie (6,206.93 ppm peanut protein)	0.665	3.132	21.25
Dark Chocolate (5,000 ppm peanut protein)	0.544	2.55	21.34
PF (15 mg/ 30 mL buffer)*	0.296	0.249	119.00
PF (60 mg/ 30 mL buffer)	0.762	1.00	76.16

(*N=6, *N=8).*

The results of the protein quantification confirmed some degree of protein extraction for all matrices and samples evaluated. A higher extraction efficiency was observed for the incurred cookie dough (33.18%) when compared to the incurred baked cookie (21.25%). This result indicated proteins may have endured structural changes or aggregation during the baking process which impacted their ability to be extracted. Others have shown a general decrease in protein extractability in wheat matrices after baking[32, 37]. Both incurred matrices had similar extraction efficiencies, at approximately only 20% of the theoretical protein in the matrices, which suggested an effect of either matrix components or processing of the matrix on the extractability of proteins. It also indicated the poor solubility of gluten proteins in aqueous conditions.

d. Peptide Identification

Results from the data analysis of the untargeted MS runs against the peanut database, as genereated for the PEAKS Summary Report, are shown in Table 2.8.

Table 2.8: Untargeted MS Results, PEAKS Summary Report for Cookie and Dark Chocolate Analyses Against Peanut Protein Database

	Cookie Matrix	**Dark Chocolate Matrix**
# of MS Scans	296,235	231,201
# of MS/MS Scans	300,614	109,479
PSMs	2,898	2,453
Peptide Sequences	115	217
Proteins	27	26
FDR (PSM)	1.0%	1.4%
FDR (Peptide Sequences)	10.4%	10.6%
FDR (Protein)	17.4%	18.2%
de novo Only Spectra	25,932	11,090

PSM: peptide spectrum match
FDR: false discovery rate

e. Quantification of Peptides

LFQ was completed for the cookie and dark chocolate datasets separately as a semi-quantitative analysis of peptides within each dataset. In the cookie dataset, the most apparent difference in relative allergen abundances was between the PF samples (no matrix) and the cookie dough and baked cookie matrices (Figure 2.3). The matrix samples had a decrease in relative peanut protein detected by the instrument compared to the peanut protein detected in the no matrix PF samples. This indicated an effect of the matrix on the detection of peanut protein using MS methodologies. There are a number of effects that could contribute to this decrease in recovery of peanut protein. One explanation is the change in protein structure or aggregation of protein in the matrix and the thermal processing of the matrix. These changes in protein structure may impact the

ability of the buffer to effectively extract these proteins from the matrix samples.

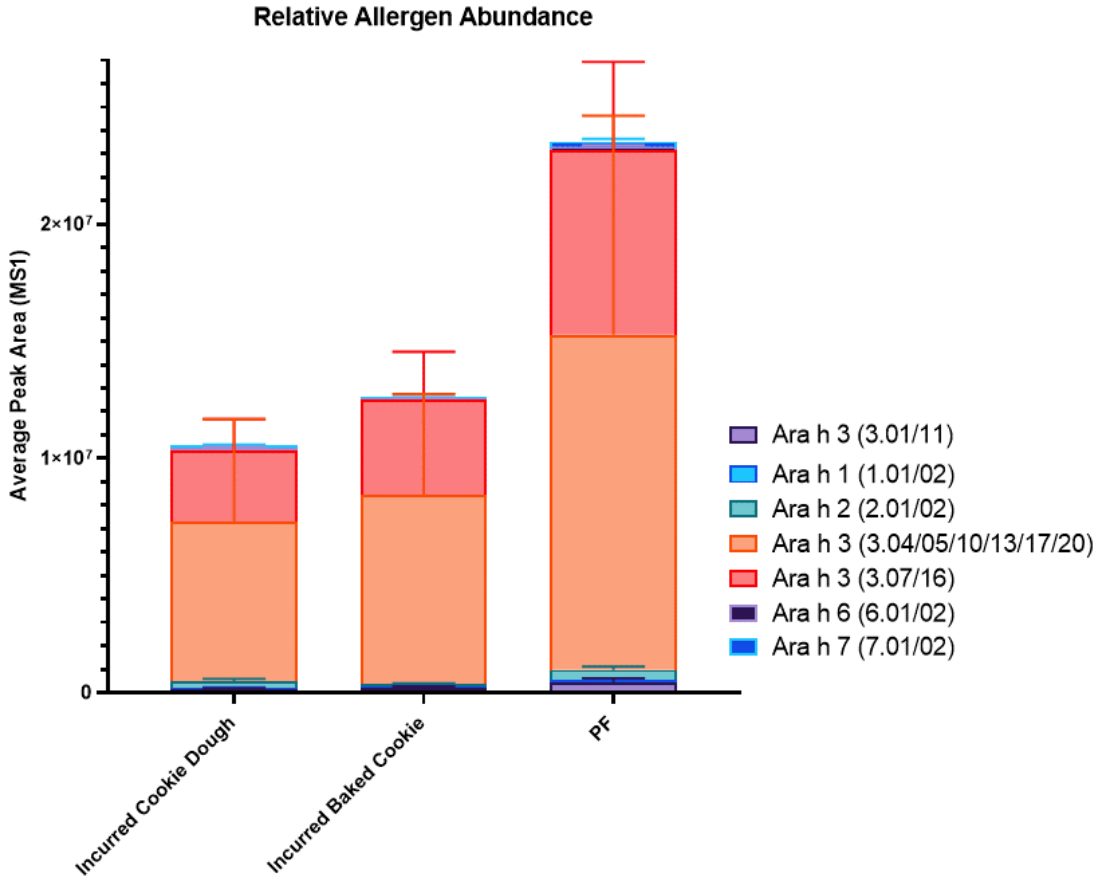

Figure 2.3: Relative Abundance of Major Peanut Allergens in the Incurred Cookie Matrix as Determined by LFQ
Quantification of peanut proteins based on LFQ results for cookie dough and baked cookie (5,185.96 and 6,206.03 ppm peanut protein), as well as PF (5,000 ppm peanut protein matrix equivalence). Average relative allergen abundances determined using the peak areas of the top three most abundant peptides from each protein. *N= 9 ± SEM.*

Next, the overall abundance of proteins in the baked cookie appeared to be greater than that in the cookie dough. It is important to note that the peanut protein that is contributing greatly to the relative allergen abundance graph is the multiple isoform groups of Ara h 3, primarily Ara h 3.04/05/10/13/17/20 and Ara h 3.07/16. This observed effect may be an effect of the matrix or its processing but may also be an effect of the

varying peanut concentrations between the dough (5,185.96 ppm peanut protein) and the baked cookie (6,206.93 ppm peanut protein) due to water loss. Additionally, the observed effect in the LFQ may be due to both the concentration change, as well as the matrix and processing effects. With respect to the processing effects, one factor that may be contributing to this observed effect is the change in protein structure after baking reduced the extraction of other non-Ara h 3 proteins and thus, promoted the preferential extraction of Ara h 3 isoforms. Peptides from Ara h 1, 2, 6, and 7 were also detected and the quantification of these proteins was completed. However, their relative abundance is much less in comparison to Ara h 3. This was an expected result, as Ara h 3 makes up over 70% of the protein in a peanut seed[38]. Additionally, it can be observed that the Ara h 2 quantity decreased in its detection between the cookie dough and baked cookie. Because Ara h 2 is known to be heat stable, the decrease suggested chemical modifications during baking or a change in its extractability after baking, highlighting the possibility of its change in structure or possible aggregation after thermal processing[39].

For the dark chocolate matrix, a similar trend between non-matrix PF samples and the incurred matrix material can be observed as was noted for the cookie matrix (Figure 2.4). The dark chocolate matrix also had a decreased relative quantity of proteins compared to the PF. Again, this suggested an effect of the matrix on the detection of peanut proteins by MS. An additional similarity between the dark chocolate and the cookie matrix is the dominance of Ara h 3 and its isoform groups in the relative allergen quantitation. The majority of peanut protein detected in each the dark chocolate, cookie, and PF samples originated from Ara h 3 isoforms. Ara h 1 appears to differ in its

abundance between the dark chocolate samples and the PF samples. Ara h 1 recorded a greater abundance in the dark chocolate material, compared to the PF alone, which is unexpected due to the heat lability of Ara h 1 and the moderate thermal processing of dark chocolate production[40]. However, this could have been an artifact of the relative quantification approach or a result of the decrease of Ara h 3 in the dark chocolate, allowing for greater Ara h 1 detection by the instrument.

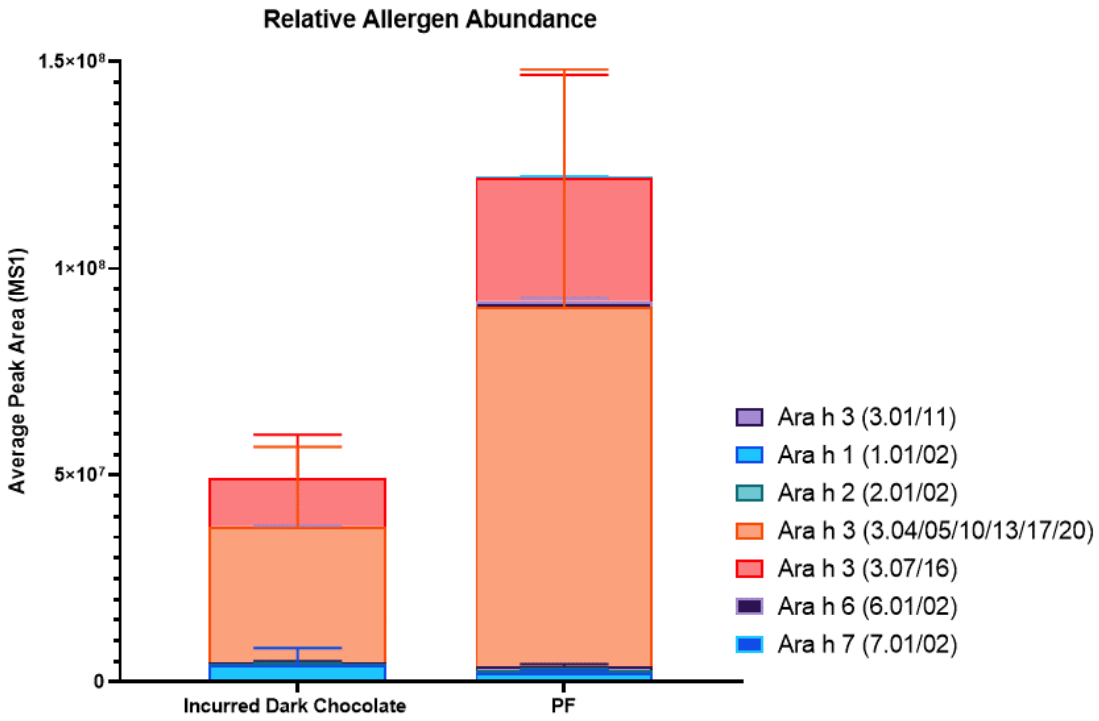

Figure 2.4: Relative Abundance of Major Peanut Allergens in the Incurred Dark Chocolate Matrix as Determined by LFQ

Quantification of peanut proteins based on LFQ results for cookie dough and baked cookie (5,000 ppm peanut protein), as well as PF (5,000 ppm peanut protein matrix equivalence). Average relative allergen abundances determined using the peak areas of the top three most abundant peptides from each protein. *N*= 9 ± *SEM*.

vi. Selection of Candidate Target Peptides in Incurred Cookie

The goal of exclusion and selection steps was to identify a subset of the peanut peptides identified in untargeted MS which indicated the potential to be quality targets in a targeted MS method. A summary of peptide exclusion for peptides in the cookie matrix is shown in Table 2.9.

Table 2.9: Candidate Target Peptide Tracking Through Selection Criteria: Cookie Matrix

Elimination Criteria:	**# of Target Peptides Eliminated**	**# of Target Peptides Remaining**
Initial LFQ Data		88
Detections in Blank Matrix	13	75
Variable PTMs	7	68
10% Recovery in Dough vs. PF and Top 60% Overall Abundance (Dough)	28	40
10% Recovery in Dough vs. PF and Top 60% Overall Abundance (Baked)	32	36
Qualification in Dough and Baked Matrices	7 (dough) 3 (baked)	33
Other Elimination	3	30
Peptides Deemed “Candidate Target Peptides”		30 peptides 32 targets

LFQ: label-free quantification
PTM: post-translational modification

The removal of any peptides that had positive detections in more than one replicate of the blank cookie dough or baked cookie samples (0 ppm peanut protein) was completed because the mass events may have been wheat peptides mis-detected as peanut peptides or peanut peptides that possess too similar of mass-to-charge ratios (m/z) to wheat peptides to have high specificity to peanut in matrix. Peptides with the variable modifications outlined (hydroxyprolination and oxidation of methionine) were removed for the purposes of sensitivity and precision. If a peptide contains a variable modification,

a certain proportion of its abundance is modified while the inverse proportion does not contain the modification. This cuts the overall abundance of that target peptide and creates, in theory, a less sensitive target. If the modification occurs inconsistently, the quantitative precision will also be impacted.

Next, peptides were subjected to the two selection criteria outlined to determine candidate target peptides (recovery ratio > 0.1 and top 60% abundance), examples shown in Figure 2.5. This figure displays peptides from Ara h 2 and Ara h 3, though all peptides which persisted through exclusion steps were subjected to the selection criteria.

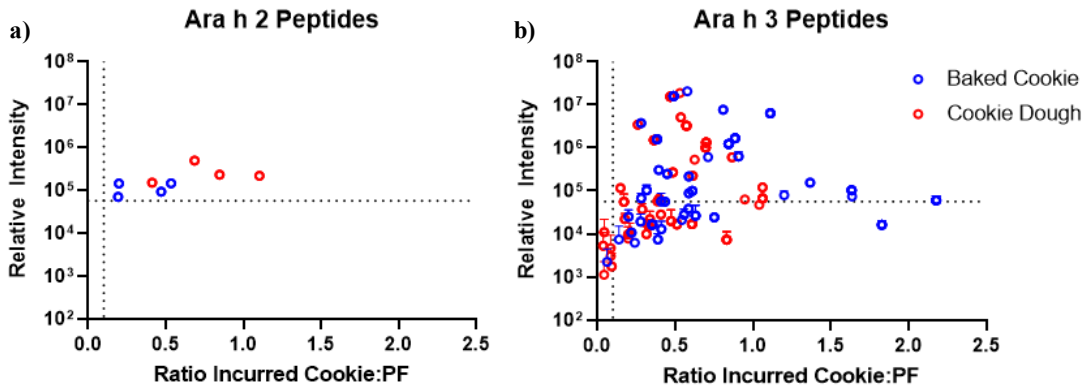

Figure 2.5: Selection Criteria for Abundance and Recovery in Matrix Applied to Peanut Peptides Identified in the Cookie Matrix
Abundance (top 60%) and ratio (>0.1) selection criteria applied to peptides from **a)** Ara h 2 or **b)** Ara h 3 for both the baked cookie and cookie dough incurred matrices (5,185.96 and 6,206.93 ppm peanut protein). Peptides meeting both criteria fell in the upper right hand quadrant created by the dotted lines representing selection criteria. *N= 9 ± SEM.*

Peptides from Ara h 2 (a 2S albumin) in the incurred cookie dough appeared to have greater abundances and were less affected by the matrix than the baked cookie, as indicated by the higher recovery ratios observed. This suggested Ara h 2 extraction from the baked cookie is decreased due to the thermal processing of the matrix. For peptides

form Ara h 3, a cupin, it appeared that the relative abundances and ratios of peptides were slightly greater in the baked cookie than the same measures for the cookie dough. This may be that due to the structural and aggregation changes of proteins induced by thermal processing, thus cupins are preferentially extracted and/or digested compared to other proteins after processing, which caused the greater abundance observed.

Of the 32 targets that were deemed candidate target peptides, 21 of the candidates originate from Ara h 3. This is an expected result, as Ara h 3 is the most abundant protein in a peanut seed[38]. Three candidate targets were selected from Ara h 1, the next most abundant protein in peanut[38]. From Ara h 2, four candidate targets were selected and from Ara h 6, only one target peptide was selected. Additionally, three candidate targets were selected that originate from Ara h 7, a very minor peanut protein with respect to abundance at approximately 0.04 -0.015% of peanut protein[41]. There were more factors than simply abundance that affect the abundance and recovery of these candidate target peptides in the incurred matrices, as selection does not solely follow abundance trends. This suggests an effect of the matrix or of matrix processing in the accessibility and extractability of peanut proteins.

vii. Selection of Candidate Target Peptides in Incurred Dark Chocolate

The strategy for the selection of candidate target peptides in the incurred dark chocolate mirrored that of the cookie matrix and is summarized in Table 2.10.

Table 2.10: Candidate Target Peptide Tracking Through Selection Criteria: Dark Chocolate Matrix

Elimination Criteria:	**# of unique peptides eliminated**	**# of unique peptides remaining**
Initial LFQ Data		145
Detections in Blank Matrix	16	129
Variable PTMs	20	109
10% Recovery in Dough vs. PF and Top 60% Overall Abundance	50	59
Other Elimination	0	59
Peptides Deemed "Candidate Target Peptides"		59 peptides 67 targets

LFQ: label-free quantification
PTM: post-translational modification

Two examples of selection criteria applied to peptides from the untargeted MS dataset for the dark chocolate are shown in Figure 2.6. Though all peptides were subjected to selection criteria, the figure displays peptides from Ara h 2 and Ara h 3 as examples.

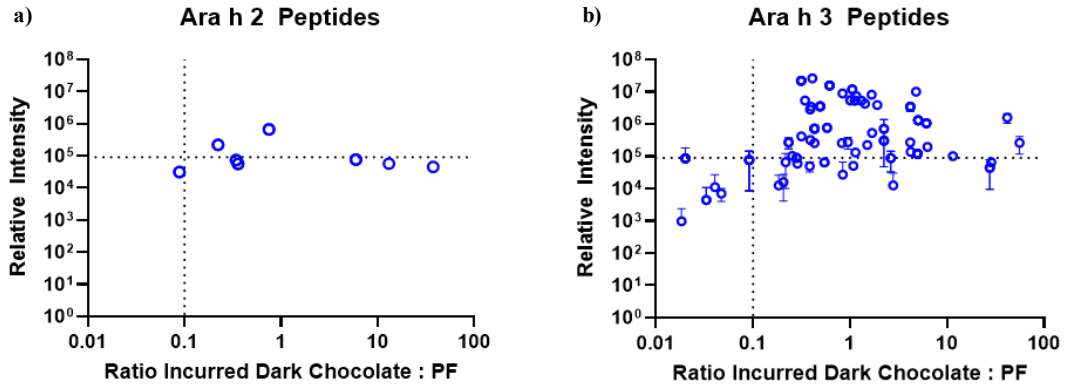

Figure 2.6: Selection Criteria for Abundance and Recovery in Matrix Applied to Peanut Peptides identified in the Dark Chocolate Matrix.
Abundance (top 60%) and ratio (>0.1) selection criteria applied to peptides from **a)** Ara h 2 or **b)** Ara h 3 for the incurred dark chocolate matrix (5,000 ppm peanut protein). Peptides meeting both criteria fell in the upper right hand quadrant created by the dotted lines representing selection criteria. *N= 9 ± SEM.*

Of the 67 candidate targets, a similar proportion of the targets are from Ara h 3 as were selected in the incurred cookie matrix. For the incurred dark chocolate, 49 of the 67 candidates were selected from Ara h 3 (for cookie, 21 of the 32 candidates were from Ara h 3). The large number of targets selected from Ara h 3 was presumably an effect of the sheer abundance of this protein in peanut. The next greatest number of candidates, 12, were from Ara h 1. Ara h 2 and Ara h 6 each contributed three candidate targets in this selection step. While three candidate targets were selected from Ara h 7 for the cookie matrix, zero targets from this protein qualified as candidate target peptides in the dark chocolate matrix. Therefore, there may be some effect of the dark chocolate matrix on the accessibility and extractability of Ara h 7 in the matrix.

viii. Targeted MS Filtration of Candidate Target Peptides in Cookie

a. Round 1

In the first run of Round 1 for filtration of candidate target peptides by targeted MS, the number of target peptides included in the PRM method was 32, all selected candidate target peptides. After analysis of the 5,000 ppm peanut protein sample of PF, the selection of the most abundant charge state for two peptide sequences was completed, thus removing two targets. Additionally, seven target peptides were not detected in these PRM rounds. These target peptides were not eliminated, but a broad scheduling window of either five or six minutes was used in the subsequent PRM run. For all other target peptides, the retention time as observed in the first iteration of Round 1 was used to create a two minute scheduling window for the second iteration of Round 1. In the second run of Round 1, there were 29 target peptides detected of the 30 included. The peptide

that was not detected was eliminated because the lack of detection at such a high concentration of PF indicated that this target peptide would not be a sensitive target peptide in the final method.

b. Round 2

Round 2 of PRM filtration was completed with 29 target peptides in the method's inclusion list. Filtration criteria for this round of PRM required that target peptides have at least three product ions, manually determined in Skyline, detected at a concentration lower than 5,000 ppm PF and chromatograms with appropriate peak shape. This caused the elimination of five target peptides inclusion list.

c. Round 3

In Round 3, 24 target peptides were analyzed. Four peptides were eliminated due to non-linear dilution curves. An example of a non-linear dilution curve compared to an ideal linear dilution curve is shown in Figure 2.7. A disproportionate change in the sum of the peak area with respect to the concentration of peanut or PF in a sample indicated that these peptides would be poor quality targets if included in a quantitative MS method. A quality target for a quantitative method has a linear response to the amount of peanut present in the analyzed sample. Therefore, peptides with non-linear dilution curves were not considered for inclusion in the method due to their lack of quantitative robustness. Additionally, six peptides were removed due to their lack of sensitivity in detection in the

analyzed samples. In total, Round 3 of PRM filtration resulted in the elimination of 10 target peptides.

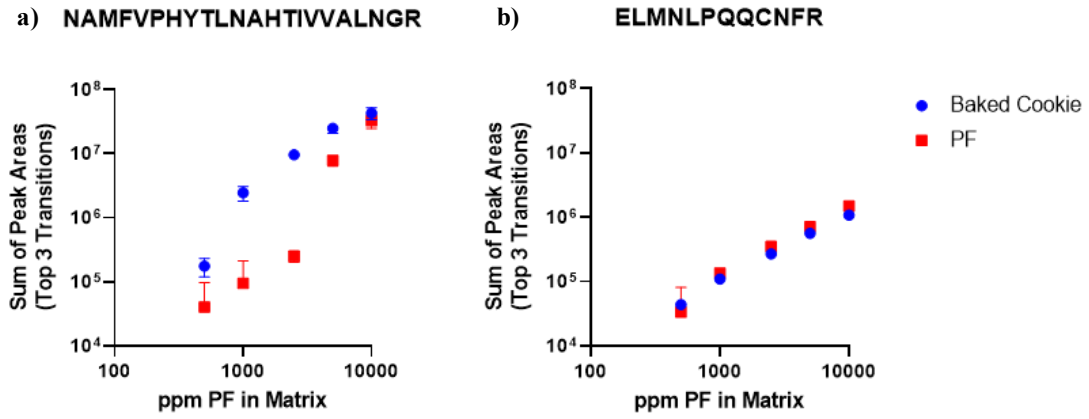

Figure 2.7: Representative Examples of Nonlinear and Linear Dilution Curves from Round 3 of PRM Filtration in the Cookie Matrix.
Dilution curves for **a)** NAMFVPHYTLNAHTIVVALNGR, a peptide from Ara h 3 (3.7/16) and **b)** ELMNLPQQCNFR, a peptide from Ara h 6 (6.1/2) constructed from Round 3 of PRM filtration of targets for the cookie matrix. Analyzed concentrations include 10,000, 5,000, 2,500, 1,000, 500, 100, 50, and 10 ppm PF in matrix. *N=2, ± SD.*

d. Round 4

Of the 15 target peptides analyzed in this round of PRM filtration, five were eliminated following data analysis. Four peptides had dilution curves with non-linear responses to the change in peanut concentration of the sample and lacked sensitivity. One peptide had a linear dilution curve but did not have ample sensitivity for inclusion in the quantitative method.

e. Round 5

In this round of PRM filtration, there were 10 target peptides in the inclusion list. Two peptides were removed due to their lack of sensitivity in both the PF and baked cookie sample types. One peptide (WLGLSAEYGNLYR) was eliminated from the

method due the ratio of its peak area in the baked cookie compared to the PF. The difference between the sum of the peak area in the baked cookie and in the PF was so large that this peptide would not be a quality peptide in the final method considering calibration to PF. For a representative peptide in Round 5 of PRM filtration, the ratio of peak area in the baked cookie to PF was between 1.0 and 2.0. Whereas the ratios for WLGLSAEYGNLYR ranged from 3.4-17.4. The large discrepancy between the response in the baked cookie and the response in the PF indicated that calibration to PF for this peptide would lead to inaccurate quantification in the final method. As a result, this peptide was eliminated from the list of potential target peptides.

f. Round 6

Seven target peptides were analyzed in Round 6 of PRM filtration. Additionally, the blank cookie dough and baked cookie (0 ppm peanut protein) were evaluated using the method to verify that the peptides in the method were specific to peanut and confirm no issue of cross reactivity or misdetection of the method's peptides in a cookie matrix. Results indicated that the remaining peptides were specific to peanut as they did not have any detections that would meet final method detection criteria for the forthcoming quantitative MS method in the blank matrices.

Second, the peanut-containing samples (PF and baked cookie) were analyzed for each of the seven target peptides. The sensitivity of these peptides was evaluated, and it was determined that one peptide was less sensitive than the other six target peptides. The lowest concentration of detection for ADFYNPAAGR was 25 ppm PF in the baked cookie sample. The other six peptides were detected at 10 ppm PF in the baked cookie

and therefore were more sensitive. Thus, the ADFYNPAAGR peptide was removed from the list. The result was six target peptides that demonstrated sensitivity and robustness in the cookie matrix. Dilution curves for all seven peptides evaluated in Round 6 of PRM filtration are shown in Figure 2.8.

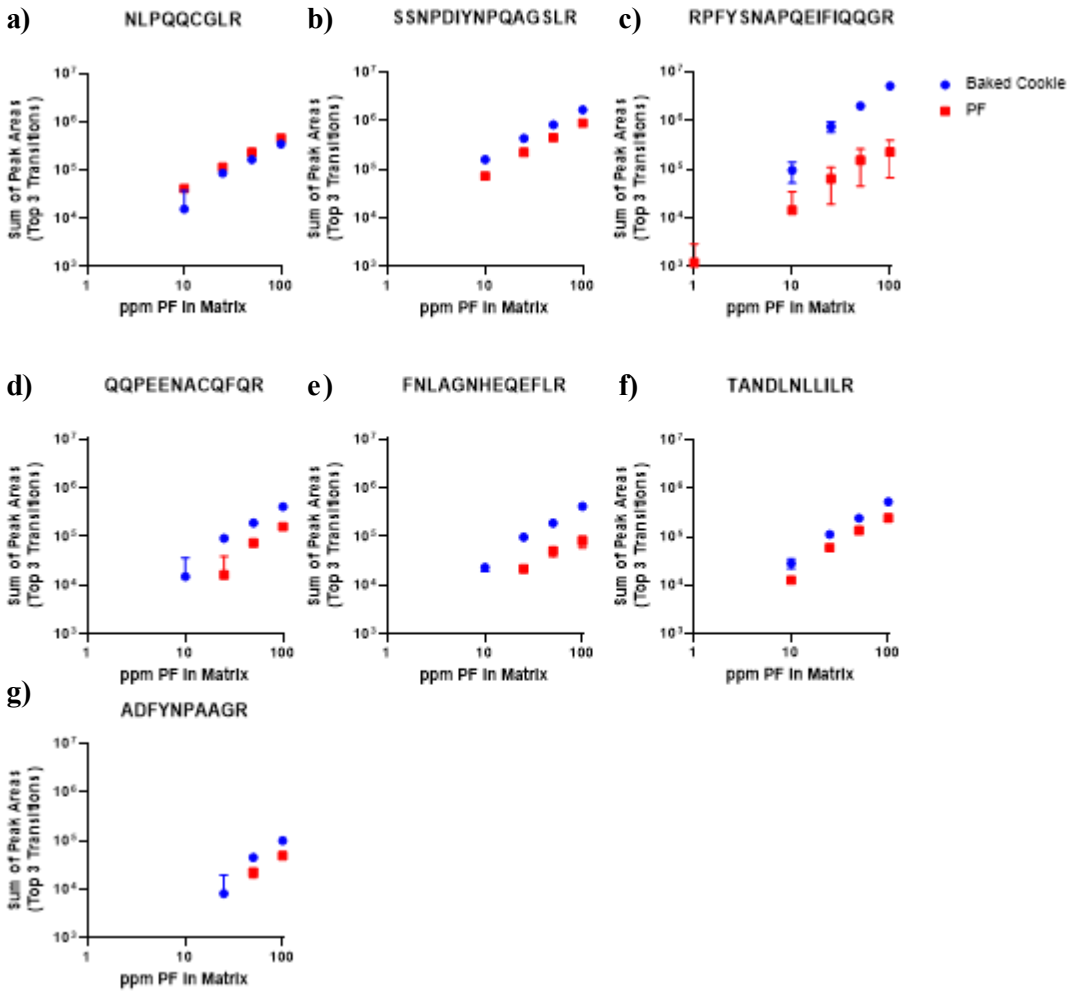

Figure 2.8: Dilution Curves for the Seven Peptides Analyzed in Round 6 of PRM Filtration in the Cookie Matrix.
Dilution curves for **a)** NLPQQCGLR (Ara h 2.1/2) **b)** SSNPDIYNPQAGSLR (Ara h 3.7/16) **c)** RPFYSNAPQEIFIQQGR (Ara h 3.4/5/10/13/17/20) **d)** QQPEENACQFQR (Ara h 3.4/5/10/13/17/20)**e)** FNLAGNHEQEFLR (Ara h 3.4/5/10/13/17/20) **f)** TANDLNLLILR (Ara h 3.4/5/10) **g)** ADFYNPAAGR (Ara h 3.1/11) from Round 6 of PRM filtration. Analyzed concentrations include 100, 50, 25, 10, and 1 ppm PF in matrix. *N=2, ± SD.*

The dilution curves, shown in Figure 2.7, from Round 6 of PRM filtration presented a unique increase in the sum of the peak area for the cookie matrix sample compared to the PF sample. This observed trend was only true for peptides from the Ara h 3 protein in peanut. The peptide from Ara h 2 (NLPQQCGLR) did not show this trend. This indicated that this effect may be a result of the behavior of the protein prior to or during the digestion of the protein into peptides. Perhaps the extraction of certain proteins is decreased in the cookie matrix, which preferentially extracts the proteins of Ara h 3. This may explain why there was an observed increase in the peak are of all peptides from Ara h 3 in the cookie matrix compared to the PF alone. Additionally, dilution curves indicated that six of the seven peptides perform both sensitively and linearly in the PF and baked cookie matrix.

g. Filtration Results

After six rounds of iterative PRM experiments, six target peptides demonstrated ample sensitivity and robustness in the PF samples and in the baked cookie matrix. Table 2.11 shows the narrowing of the candidate target peptide list over the course of PRM filtration. Peptides that persisted through all rounds of PRM filtration were deemed final target peptides.

Of the six final target peptides, only one is from a protein that is not Ara h 3. One peptide, NLPQQCGLR is from Ara h 2 (Ara h 2.1/2). Five peptides are from isoforms of the Ara h 3 protein: SSNPDIYNPQAGSLR (Ara h 3.7/16), RPFYSNAPQEIFIQQGR (Ara h 3.4/5/10/13/17/20), QQPEENACQFQR (Ara h 3.4/5/10/13/17/20), FNLAGNHEQEFLR (Ara h 3.4/5/10/13/17/20), and TANDLNLLILR (Ara h 3.4/5/10).

It was expected to obtain final target peptides that were from Ara h 3, due to the abundance of this protein in peanut. However, the experimental approach to filtration by PRM confirmed, empirically, the efficacy of these final target peptides in the cookie matrix.

Table 2.11: Lowest Concentration of Detection and Elimination of Target Peptides by PRM Round for the Cookie Matrix

Peptide Sequence	Round 1 (PF)	Round 2 (Cookie)	Round 3 (Cookie)	Round 4 (Cookie)	Round 5 (Cookie)	Round 6 (Cookie)
C(+57.02)QSQLER	5000	X	X	X	X	X
YQQQQGSRPHYR	5000	5000	500	250	X	X
AHVQVVDSNGNR	5000	X	X	X	X	X
AHVQVVDSDGNR	5000	X	X	X	X	X
QFQNLQNHR	10000	5000	1000	750	X	X
QGGEENEC(+57.02)QFQR	5000	5000	1000	X	X	X
ANLRPC(+57.02)EEHIR	X	X	X	X	X	X
QQPEENAC(+57.02)QFQR	1000	500	100	50	50	10
NLPQQC(+57.02)GLR	5000	5000	500	250	10	10
WGPAEPR	5000	5000	2500	X	X	X
ANLRPC(+57.02)EQHLMQK	10000	5000	1000	500	X	X
NLPQNC(+57.02)GFR	5000	5000	1000	X	X	X
ADFYNPAAGR	5000	5000	500	500	50	25
C(+57.02)C(+57.02)NELNEFENNQR	5000	5000	500	500	100	X
SSNPDIYNPQAGSLR	500	10	10	10	10	10
FYLAGNPEEEHPETQQQQPQTR	5000	5000	500	750	X	X
FNLAGNHEQEFLR	100	500	50	50	10	10
ELMNLPQQC(+57.02)NFR	5000	5000	500	750	X	X
RPFYSNAPQEIFIQQGR	100	100	10	50	1	10
WLGLSAEYGNLYR	100	500	10	50	50	X
TVNELDLPILNR	5000	5000	500	250	100	X
NAMFVPHYTLNAHTIVVALNGR	5000	5000	500	X	X	X
GIPADVLINAFGLR [+2]	5000	5000	2500	X	X	X

GIPADVLINAFGLR [+3]	X	X	X	X	X	X
TANDLNLLILR	100	500	50	100	10	10
C(+57.02)MC(+57.02)QALQQILQNQSFR	5000	5000	500	X	X	X
GYFGLIFPGC(+57.02)PSTYEEPAQQGR [+2]	X	X	X	X	X	X
GYFGLIFPGC(+57.02)PSTYEEPAQQGR [+3]	5000	5000	500	X	X	X
IPSGFISYILNR	100	5000	1000	X	X	X
TDSRPSIANLAGENSFIDNLPEEVVANS YGLPR	100	10000	X	X	X	X
SVNELDLPILGWLGLSAQHGTLYR	100	5000	10	X	X	X
QGHLLAIPAGVPYWSFNYGNEPIVAITL LDTSNLDNQLDPSPR	5000	X	X	X	X	X

X = peptide not detected or eliminated from method

ix. Targeted MS Filtration of Candidate Target Peptides in Dark Chocolate

a. Round 1

All 67 candidate target peptides included in the targeted MS method for dark chocolate were detected in the 10,000 ppm PF sample analyzed for Round 1. In the inclusion lists used for this round, there were eight peptides which had more than one charge state. The most abundant charge state for six of the eight peptides were discerned. This resulted in the removal of six potential target peptides from the list. For all peptides remaining in the method, a four minute scheduling window was created and added to the method based on the retention time observed in this round. The scheduling of the peptides was used in subsequent rounds of PRM and refined to a two minute window in subsequent work.

b. Round 2

Round 2 of PRM filtration assessed 61 peptides in PF and the dark chocolate matrix. In the inclusion list used for this round, there were two peptides that had two different charge states analyzed. The least abundant charge states for each of these two peptides were removed. Six potential target peptides were eliminated for lack of detection at the high peanut concentration level. After Round 2, there remained 53 potential target peptides.

c. Round 3

Batching of target peptides into two inclusion lists was used for Round 3 for the purpose of reducing the possibility of co-eluting targets. In Round 3 of PRM, 21 target peptides were eliminated from the list either due to poor sensitivity or nonlinear calibration curves, which left 32 target peptides for consideration.

d. Round 4

Round 4 prompted the elimination of 17 total target peptides from the method. Based on dilution curves, three peptides were eliminated from the method due to their nonlinear response to the amount of peanut protein present in the sample. An example of a peptide with a nonlinear dilution curve compared to a peptide with a linear dilution curve is shown in Figure 2.9.

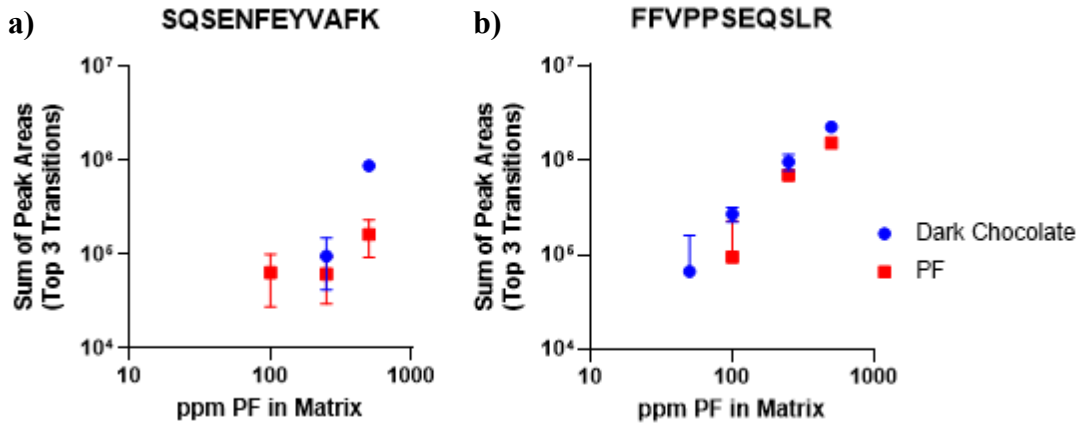

Figure 2.9: Representative Examples of Nonlinear and Linear Dilution Curves from Round 4 of PRM Filtration in the Dark Chocolate Matrix.
Dilution curves for **a)** SQSENFEYVAFK, a peptide from Ara h 3 (3.10/13/17/18/20) and **b)** FFVPPSEQSLR, a peptide from Ara h 3 (3.17/20) constructed from Round 4 of PRM filtration of targets for the dark chocolate matrix. Analyzed concentrations included 500, 250, 100, 50, 10, and 1 ppm PF in matrix. *N=2*, ± *SD*.

The dilution curve for SQSENFEYVAFK suggests a nonlinear relationship between the concentration of peanut in the PF sample and the detection of the peptide by MS, therefore this target was of poor quality. Second, 14 potential target peptides were removed from the method based on their lowest detected concentration in this round of filtration and indication of a lack of ample sensitivity.

e. Round 5

15 potential target peptides were analyzed in Round 5. Evaluation of the 0 ppm peanut protein dark chocolate by the method indicated appropriate specificity of the peptides to peanut. From analysis of the incurred dark chocolate and the PF samples, eight total peptides were eliminated, one for lack of a linear dilution curve. For this peptide (DQSSYLQGFSR), a nonlinear response at lower concentrations of PF was observed. This effect indicated this target would be of poor quality in the final method, as

the decrease in peanut protein does not produce a proportionate decrease in the sum of the peak area for the peptide. Additionally, seven peptides were eliminated because they had poor sensitivity in the PF and dark chocolate. The result was seven target peptides deemed to be the most robust and sensitive in the dark chocolate matrix per the iterative PRM approach used to filter candidate target peptides (Figure 2.10).

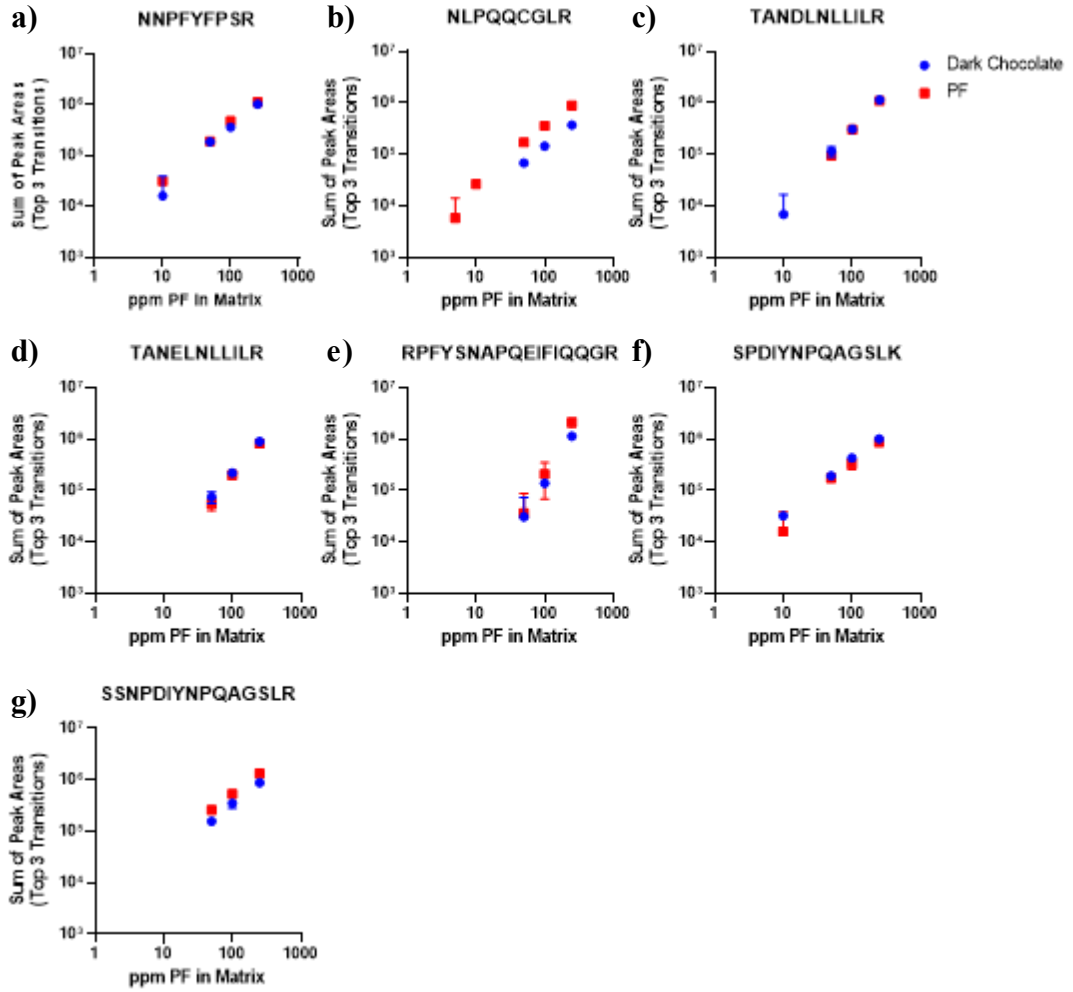

Figure 2.10: Dilution Curves for the Seven Peptides Analyzed in Round 5 of PRM Filtration in the Dark Chocolate Matrix.
Dilution curves for **a)** NNPFYFPSR (Ara h 1.1/2) **b)** NLPQQCGLR (Ara h 2.1/2) **c)** TANDLNLLILR (Ara h 3.4/5/10) **d)** TANELNLLILR (Ara h 3.13/17/20) **e)** RPFYSNAPQEIFIQQGR (Ara h 3.4/5/10/13/17/20) **f)** SPDIYNPQAGSLK (Ara h 3.4/5/10/13/17/20) and **g)** SSNPDIYNPQAGSLR (Ara h 3.7/16) from Round 5 of PRM filtration. Analyzed concentrations include , 250, 100, 50, 10, 5, and 1 ppm PF in matrix. *N=2, ± SD.*

For most of the peptides, the dilution curves showed a similar response between the dark chocolate matrix and the PF samples. However, for some peptides, NLPQQCGLR, SSNPDIYNPQAGSLR, and to some extent RPFYSNAPQEIFIQQGR, the dark chocolate samples have reduced peak area compared to the PF samples. For NLPQQCGLR (Ara h 2.1/2), there is greater detection in the PF sample than the dark chocolate. This may be due to the effect of the dark chocolate matrix in that the extractability of Ara h 2 proteins is impaired by the matrix, suggesting an influence of the matrix affect, not necessarily thermal processing as Ara h 2 is relatively heat stable[39]. SSNPDIYNPQAGSLR, from the variable isoforms of Ara h 3 (Ara h 3.7/16) shows a similar trend, though to a lesser extent than the NLPQQCGLR peptide.

f. Filtration Results

After five rounds of iterative PRM filtration, seven target peptides were experimentally determined to be the most robust and sensitive target peptides of those identified in untargeted MS and selected as candidate target peptides. Table 2.12 shows the narrowing of the candidate target peptide list over the course of filtration. Peptides which persisted through all rounds of PRM filtration were deemed final target peptides.

Table 2.12: Lowest Concentration of Detection and Elimination of Target Peptides by PRM Round for the Dark Chocolate Matrix

Peptide (m/z)	Round 1 (PF)	Round 2 (Chocolate)	Round 3 (Chocolate)	Round 4 (Chocolate)	Round 5 (Chocolate)
AGFLTALNTPNLPVLQYVQLGVDR	10000	5000	X	X	X
AGQEEENEGGNIFSGFTPEFLAQAFQVDDR	10000	5000	2500	X	X
AGQEEENEGGNIFSGFTPEFLEQAFQVDDR	10000	5000	X	X	X
AGQEQENEGGNIFSGFTPEFLAQAFQVDDR	10000	5000	2500	X	X
AGQEQENEGGNIFSGFTSEFLAQAFQVDDR	10000	5000	X	X	X
AHVQVVDSDGNR	10000	X	X	X	X
AHVQVVDSNGNR	10000	1000	1000	X	X
AQSENYEYLAFK	10000	1000	500	250	X
C(+57.02)C(+57.02)NELNEFENNQR [+2]	10000	1000	500	250	X
C(+57.02)C(+57.02)NELNEFENNQR [+3]	10000	1000	X	X	X
C(+57.02)DLDVSGGR	10000	1000	1000	X	X
DQSSYLQGFSR	10000	1000	50	50	50
EGALMLPHFNSK	10000	1000	500	X	X
EGEQEWGTPGSEVR	10000	5000	500	X	X
EGEQEWGTPGSHVR	10000	1000	100	250	X
FFVPPFQQSPR	10000	1000	100	50	50
FFVPPSEQSLR	10000	1000	100	50	100
FFVPPSQQSLR	10000	1000	50	50	50
FFVPPSQQSPR	10000	1000	50	100	100
FHLAGNQEQEFLR	10000	1000	50	100	250
FNLAGNHEQEFLR	10000	1000	50	50	100
GENESDEQGAIVTVR	10000	1000	500	500	X
GENESEEEGAIVTVR [+2]	10000	1000	500	X	X
GENESEEEGAIVTVR [+3]	10000	X	X	X	X
GIPADVLINAFGLR [+2]	10000	5000	1000	X	X
GIPADVLINAFGLR [+3]	10000	X	X	X	X

GTGNLELVAVR	10000	1000	50	100	100
IESEGGYIETWNPNNQEFQC(+57.02)AGVALSR	10000	5000	2500	X	X
ILNPDEEDESSR	10000	1000	500	250	X
IMGEQEQYDSYDIR	10000	5000	500	X	X
IPSGFISYILNR	10000	1000	500	X	X
IQVVNSQGNAVFNGVLR [+2]	10000	X	X	X	X
IQVVNSQGNAVFNGVLR [+3]	10000	5000	1000	X	X
ISMPVNTPGQFEDFFPASSR	10000	5000	1000	X	X
ISSANSLTFPILR	10000	1000	500	250	X
IVQIEAKPNTLVLPK	10000	1000	500	X	X
LNAQRPDNR	10000	X	X	X	X
NAMFVPHYTLNAHTIVVALNGR [+3]	10000	X	X	X	X
NAMFVPHYTLNAHTIVVALNGR [+4]	10000	5000	2500	X	X
NLPQQC(+57.02)GLR	10000	1000	50	50	50
NNPFYFPSR	1000	1000	10	50	10
QIVQNLR	1000	5000	500	X	X
QMVQQFK	1000	1000	X	X	X
RPFYSNAPQEIFIQQGR	1000	1000	500	50	50
SFNLDEGHALR	1000	1000	50	250	X
SPDIYNPQAGSLK	1000	1000	10	10	10
SQSDNFEYVAFK	1000	1000	100	100	X
SQSENFEYVAFK	1000	1000	100	250	X
SSNPDIYNPQAGSLR	1000	1000	50	100	50
SVNELDLPILGWLGLSAQHGTLYR [+3]	1000	1000	500	X	X
SVNELDLPILGWLGLSAQHGTLYR [+4]	1000	X	X	X	X
TANDLNLLILR	1000	1000	10	10	10
TANELNLLILR	1000	1000	50	50	50
TDSRPSIANLAGENSFIDNLPEEVVANSYGLPR [+3]	1000	5000	2500	X	X
TDSRPSIANLAGENSFIDNLPEEVVANSYGLPR [+4]	1000	5000	X	X	X

TDSRPSIANLAGENSIIDNLPEEVVANSYGLPR [+3]	1000	5000	2500	X	X
TDSRPSIANLAGENSIIDNLPEEVVANSYGLPR [+4]	1000	X	X	X	X
TDSRPSIANLAGENSIIDNLPEEVVANSYR	1000	5000	2500	X	X
TDSRPSIANLAGENSVIDNLPEEVVANSYGLPR	1000	5000	2500	X	X
TVNELDLPILNR	1000	1000	500	500	X
VFDEELQEGHVLVVPQNFAVAGK	1000	X	X	X	X
VLLEENAGGEQEER	1000	5000	500	250	X
VYDEELQEGHVLVVPQNFAVAAK	1000	1000	1000	X	X
VYDEELQEGHVLVVPQNFAVAGK	1000	5000	500	X	X
WFQLSAEHVLLYR	1000	5000	1000	X	X
WGPAEPR	1000	1000	100	250	X
WLGLSAEYGNLYR	1000	1000	100	250	X

X = Peptide not detected or eliminated from method.

There were seven final target peptides determined for the dark chocolate matrix. Five of the seven peptides are from isoforms of the Ara h 3 protein: TANDLNLLILR (3.4/5/10), TANELNLLILR (3.13/17/20), RPFYSNAPQEIFIQQGR (3.4/5/10/13/17/20), SPDIYNPQAGSLK (3.4/5/10/13/17/20), and SSNPDIYNPQAGSLR (3.7/16). There was one final target peptide from each, Ara h 1 and Ara h 2. These final target peptides were NNPFYFPSR from Ara h 1 (1.1/2) and NLPQQCGLR Ara h 2 (2.1/2). It was expected to observe a large number of target peptides from the most abundant protein in peanut, Ara h 3. Additionally, the peptides not from Ara h 3 were from the second and third most abundant protein in peanut (Ara h 1 and Ara h 2). However, abundance was likely not the only factor contributing to the efficacy of target peptides since final peptides originated from multiple proteins and many peptides from Ara h 3 were filtered out during the iterative PRM approach. Secondly, the complex matrix and possible matrix interactions

appeared to have affected peptide recovery in analyzing the dark chocolate material compared to PF, as the lowest detected concentration for a peptide varied across sample types which indicated some effect of matrix components or matrix processing.

x. Comparison of Filtered Targets for Cookie and Dark Chocolate

The final peptides determined after PRM filtration in the cookie matrix (six) were compared to the final peptides determined for the dark chocolate matrix (seven). For the two matrices, there were four peptides which were determined to be sensitive and robust in both the cookie and the dark chocolate (NLPQQCGLR, SSNPDIYNPQAGSL, RPFSNAPQEIFIQQGR, and TANDLNLLILR). This result indicated potential extensibility of the subsequent MS method as the target selection approach was applied to two compositionally different matrices and four peptides were determined as quality targets in both. There were two final peptides that were unique to the cookie matrix (both from Ara h 3) and three peptides that were unique to the dark chocolate matrix (one from Ara h 1 and two from Ara h 3). Though selected through a specific matrix filtration approach, all final target peptides were combined into one for the subsequent MS method. The result was a list of nine unique peanut target peptides. The final nine target peptides and the lowest detected peanut concentration for each sample type in the final round of PRM filtration are shown in Table 2.13.

Table 2.13: Lowest Detected Concentration for Filtered Peptides Matrix in ppm PF

			Cookie		Dark Chocolate	
Peptide		**Protein and Isoforms**	**PF**	**Cookie Matrix**	**PF**	**Dark Chocolate Matrix**
Cookie and Dark Chocolate	NLPQQCGLR	Ara h 2.1/2	10	10	5	50
	SSNPDIYNPQAGSLR	Ara h 3.7/16	10	10	50	50
	RPFYSNAPQEIFIQQGR	Ara h 3.4/5/10 /13/17/20	1	10	50	50
	TANDLNLLILR	Ara h 3.4/5/10	10	10	50	10
Unique to Cookie	QQPEENACQFQR	Ara h 3.4/5/10 /13/17/20	25	10		
	FNLAGNHEQEFLR	Ara h 3.4/5/10 /13/17/20	25	10		
Unique to Dark Chocolate	NNPFYFPSR	Ara h 1.1/2			10	10
	TANELNLLILR	Ara h 3.13/17 /20			50	50
	SPDIYNPQAGSLK	Ara h 3.4/5/10 /13/17/20			10	10

The final nine target peptides that resulted from this work's target selection and filtration approach originate from three different peanut proteins (Ara h 1, Ara h 2, and Ara h 3). Ara h 1 and Ara h 2 are each targeted by one target peptide. The remaining seven target peptides target Ara h 3 and its many isoforms. The variable isoforms of Ara h 3 (Ara h 3.7/13/16) are covered by one target peptide included in the final list[35]. For all six peptides selected for the cookie matrix, sensitivity in the matrix was promising with detections at 10 ppm PF in post-digestion dilutions of the incurred cookie. In the dark chocolate, the three of the seven final target peptides indicated comparable sensitivity at 10 ppm PF, while the other four have recorded lowest levels of concentration at 50 ppm

PF in matrix. As the final target peptides indicated robustness in the matrix and moderate sensitivity in their detection, it appeared that the target selection and filtration was preliminarily successful.

The majority of these final target peptides have been frequently included in other published MS peanut detection methods. Many of these target peptides have been selected for inclusion in MS methods from both *in silico* trypsin digestion approaches and discovery-based target selection approaches. NNPFYFPSR (Ara h 1.1/2) has been identified as a target peptide in several methods that targeted incurred or spiked matrices[12, 28, 42-45]. Other peptides that are commonly used to target Ara h 1 are DLAFPGSGEQVEK and IFLAGDKDNVIDQIEK, though these peptides were not selected as candidate target peptides after untargeted MS for this method[44, 46-50]. NLPQQCGLR (Ara h 2.1/2) is a commonly used target peptide for the 2S albumin, Ara h 2, in incurred and complex matrices[18, 19, 21, 43, 47, 51, 52].

With respect to Ara h 3 peptides, there are three peptides determined as final target peptides for this method that are commonly targeted in other MS peanuts. These three peptides RPFYSNAPQEIFIQQGR (Ara h 3.4/5/10/13/17/20), FNLAGNHEQEFLR (Ara h 3.4/5/10/13/17/20), and SPDIYNPQAGSLK (Ara h 3.4/5/10/13/17/20) are frequently used in peanut protein detection methods[12, 18, 20, 21, 42, 43, 48, 51-59]. Two peptides with similar sequences that originate from different isoforms of Ara h 3 (TANDLNLLILR and TANELNLLILR) have been included in some targeted MS peanut detection methods. TANDLNLLILR (Ara h 3.4/5/10) has been referenced in peanut detection methods in various matrices[55, 60, 61]. TANELNLLILR (Ara h 3.13/17/20) has

been included in some methods used to analyze incurred or spiked chocolate and other spiked matrices like jam and mayonnaise[18, 61].

The QQPEENACQFQR (Ara h 3.4/5/10/13/17/20) peptide was found less commonly in the literature but was included in a method that investigated incurred chocolate desserts and chocolate bars[47]. Last, SSNPDIYNPQAGSLR (Ara h 3.7/16) is a peptide not often included in quantitative MS peanut detection methods. Though not generally used quantitatively for targeted peanut MS methods, probably due to its variability across cultivars, it has been detected and reported by untargeted MS[35, 61, 62]. One study reports using it for quantitation in an untargeted MS analysis[35]. This peptide was not found using Allergen Peptide Browser, a resource that reports target peptides included in published SRM/MRM methods[26].

V. SUMMARY

PF-incurred cookie and dark chocolate matrices were generated for analysis by proteomic techniques for the development of an MS-based quantitative peanut protein detection method. Peanut-incurred cookie matrices were generated at 0, 0.63, 1.24, 6.21, 62.08, and 6,206.93 ppm peanut protein after water loss calculations and protein content of the PF was considered. The peanut-incurred dark chocolate matrix was manufactured in the pilot plant of a prominent chocolate manufacturer at levels including 0, 2, 20, 100, 5,000 ppm peanut protein in dark chocolate.

Untargeted proteomics techniques were used to analyze the incurred materials for the selection of candidate peanut peptides for a targeted MS method. Selection criteria allowed for the determination of 32 candidate target peptides in the cookie matrix and 67

in the dark chocolate matrix. Iterative rounds of PRM were used to empirically filter the candidate target peptides down to a number of robust and sensitive target peptides in each matrix. This resulted in six final target peptides for the cookie and seven in the dark chocolate. Combination of the final target peptides list indicated nine unique peanut peptides which have all demonstrated sensitivity and robustness in the complex, processed matrices analyzed. Many of these identified peptides have been utilized in other published MS-based peanut detection methods.

VI. REFERENCES

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CHAPTER III: QUANTITATIVE MS METHOD DEVELOPMENT, OPTIMIZATION, AND EVALUATION OF PEANUT-INCURRED COOKIE AND DARK CHOCOLATE MATRICES

I. ABSTRACT

Peanut allergy, an IgE-mediated hypersensitivity to peanut protein, is estimated to effect approximately 2% of individuals in the United States (US)[1, 2]. Peanut-allergic individuals can react to as little as 0.2 mg of peanut protein, and the elicited reaction is associated with particularly severe reactions[3, 4]. To protect allergic consumers, US mandates plain-language labeling of food products to which commonly allergenic foods, including peanut, have been intentionally added[5]. Thus, for the food industry, the ability to detect peanut protein in food products is imperative to comply with labeling requirements and to monitor the unintentional presence of peanuts in food products. Often, this detection is based on immunochemical means, though these methods have demonstrated poor recovery and quantification of peanut in processed and complex food matrices, such as cookie and dark chocolate[6, 7]. Mass spectrometry (MS) has been used to overcome the deficits of immunoassays for the detection of peanut in these matrices, but these methods struggle with incurred matrices or sufficiently sensitive detection to protect peanut-allergic individuals[8-10].

In previous work, nine target peptides were identified as sensitive and robust in their detection in peanut-incurred cookie and dark chocolate matrices using targeted MS. A quantitative strategy was developed using stable isotope labeled (SIL) target peptides and an external calibration with peanut flour (PF) materials to report sample

concentration in ppm peanut protein. Method sensitivity was improved through a number of optimizations including extraction, calibrant preparation, large-scale digestion, chromatography, and instrument parameters. To protect the most sensitive peanut-allergic individuals, the method sought to detect peanut at concentrations less than 2 ppm peanut protein in the cookie and 4 ppm peanut protein in the dark chocolate[4, 11]. After optimization, the method was successful in detecting incurred matrices at 1.24 ppm peanut protein in cookie and 2 ppm peanut protein in dark chocolate. Recovery of peanut protein was generally high for the low concentration of peanut in cookie matrix (1.24 ppm peanut protein, 270.62 - 456.81%), but reasonable for the higher incurred level (6.21 ppm peanut protein, 42.43 - 117.81% recovery). For the dark chocolate incurred matrices, recoveries were generally acceptable for the 2 ppm peanut protein level (124.08 -2 88.02% recovery) and the 20 ppm peanut protein level (44.40 - 103.07% recovery). The method, however, reported high variability in the value for the quantification of peanut protein. In this work, a quantitative LC-MS/MS method for peanut was developed using nine target peptides to achieve a method performance which is both highly sensitive and generally robust in its detection and quantification of peanut in cookie and dark chocolate matrices.

II. INTRODUCTION

Food allergy has been increasing in prevalence over recent decades and has become a prominent food safety concern for many stakeholders[12, 13]. The prevalence of specifically IgE-mediated peanut allergy has also increased over recent decades and is considered to affect approximately 2% of individuals in the US[1, 2, 14]. To protect allergic consumers, the US has regulated the labeling of the presence of commonly allergenic foods, known as the "Big Eight," which includes peanut[5, 15]. In order to comply with regulation and to protect consumers, the food industry utilizes a number of food allergen detection methods to detect the unintended presence of food allergens in food products, to inform labeling decisions, and to advise risk management. Enzyme-linked immunosorbent assays (ELISA) are the most commonly used detection method but have demonstrated deficiencies in recovery of target proteins when analyzing processed food matrices[16]. Further, undeclared peanut in commercially available cookie and dark products has been observed and the inaccuracy of ELISA detection methods in quantifying the contamination of peanut has been demonstrated[6, 7, 16]. This is likely the result of matrix components or processing effects on the target analyte. Mass spectrometry (MS) is emerging as an alternative or orthogonal detection method as it detects mass-to-charge (m/z) values of peptides to indicate the presence of an allergenic food source and therefore does not need to preserve the conformation of the protein as with ELISAs[17]. MS methods for peanut detection in cookie and dark chocolate matrices have been developed, but a greater sensitivity for detection and quantification is needed to protect allergic consumers[8-10].

A vital component of a quantitative MS method is the peptides targeted by the method. In the previous chapter, nine target peptides that were empirically determined to be robust and sensitive for MS detection of peanut in incurred food matrices were identified. These nine target peptides originated from three major peanut allergens including, Ara h 1, Ara h 2, and Ara h 3. Target peptides were determined using untargeted MS analysis of incurred matrices, followed by selection of candidate target peptides that were then iteratively filtered to a number of robust and sensitive peptides using targeted MS.

After identification of these nine target peptides, the quantitative aspects of the method can be established. Further, optimization of the quantitative method can be pursued in order to increase the method sensitivity to achieve the desired method performance. Lastly, the developed and optimized MS method can be evaluated against the incurred cookie and incurred dark chocolate matrices, as described in Chapter 2, containing various concentrations of peanut.

The development of a quantitative MS method relies on the quantification strategy, as it must consider how the peak areas of a target peptides are translated into concentrations of the analyte in the tested sample. There are several quantification strategies utilized by MS methods for allergen quantification. One approach achieves absolute quantification using a known amount of stable isotope labeled (SIL) peptides in the sample and then utilizes the ratio of the signal for the analyte peptide to the SIL peptide's signal (light-to-heavy ratio) to determine a molar amount of the target peptide[18]. SIL peptides act as an internal standard. This quantification can be very specific and

sensitive, however often requires optimization of many steps and is not applicable when the allergenic source is unknown[18]. An external calibration strategy is another approach to quantification that can be utilized for MS methods and even for other detection methods[19]. In this approach, standard samples containing the analyte or the analyte itself can be analyzed at various concentrations to create a calibration standard curve[19]. Using the calibration curve, interpolation of the analyte signal can be used to determine analyte concentration[20].

For the method presented in this work, the approach to quantification is based on both internal standards using SIL peptides and a calibration to the allergen source material, peanut flour (PF). The strategy of using both standards and SIL peptides has been used for other MS methods such as that by Planque et.al[20]. SIL peptides for each of the nine target peptides were added to samples (both matrix samples and PF calibrant samples) at known concentrations and the light-to-heavy ratio was determined. Using the light-to-heavy ratio and a calibration curve formed from the PF calibrant samples, a concentration of peanut contamination in a test sample was determined.

The desired method performance for the targeted MS method that this work sought to develop was established using risk assessment principles. Ideally, the MS method would be able to detect and quantify the presence of peanut protein at 2 ppm peanut protein in cookie and 4 ppm peanut protein in dark chocolate. This needed method performance is based on the Voluntary Incidental Trace Allergen Labeling (VITAL) reference dose for peanut, 0.2 mg peanut protein, which is the amount of peanut that would elicit a reaction in the most sensitive 1% of peanut-allergic individuals if

consumed in one eating occasion[4]. This determination utilizes an overestimation of the median intake amount of a cookie in the US at one eating occasion which is 36.0 g, by using a 100 g intake of cookie amount to maximize food safety[11]. For an individual to receive 0.2 mg of peanut protein in 100 g of cookie, the concentration of peanut protein would have to be 2 ppm peanut protein. The same calculation can be completed for dark chocolate, of which the median intake is 31.2 g[11]. Using an overestimation of the intake amount of 50 g dark chocolate, the concentration of peanut protein equated to 4 ppm peanut protein in order for an individual to consume the reference dose of peanut protein in one eating occasion. Thus, to protect the majority (99%) of peanut-allergic consumers, the needed method performance is detection and quantification of peanut protein at or below the 2 and 4 ppm peanut protein concentrations in cookie and dark chocolate, respectively.

To achieve the desired method sensitivity, several optimization strategies were evaluated for their impact on the concentration of peanut which the method could detect. A number of optimization approaches were employed, including chromatographical refinements, instrument parameter improvements, sample preparation additions, increased peptide concentrations, and optimized extraction concentrations.

III. MATERIALS AND METHODS

A targeted MS method for the detection and quantification of peanut protein in processed food matrices was developed based on nine target peptides that were determined to be sensitive and robust in Chapter 2. Sample preparation for MS analysis mirrored that used in the experiments used in Chapter 2 to select and filter candidate

target peptides. All materials and solvents used were identical to those listed in Chapter 2, unless otherwise noted. A summary of the materials and protocols used to prepare samples for MS is provided in Table 3.1.

Table 3.1: Overview of Methods Used for Sample Preparation for MS Analysis

Sample Preparation	Procedural Description	Procedure Details
Grinding	Cookie matrices were ground using a food processor prior to extraction. Dark chocolate matrices were ground manually using a spatula. PF materials were fine in particle size and were not ground.	Approximately 100 g of each matrix ground and stored at -20 °C.
Extraction	Extractions at 1:20 w/v were completed using a 6M Urea, 2M Thiourea, 20 mM DL-dithiothreitol (DTT), and 50 mM Tris-hydrochloride (Tris-HCl) buffer. Extraction utilized 60 °C shaking water bath incubation, sonication, and centrifugation.	0.500 g of matrix/ 10 mL buffer 0.060 g of PF/ 30 mL buffer
Digestion	Protein digestion was completed using trypsin protease and ABC for buffering. DTT was used to reduce disulfide bonds at 95 °C for five minutes. Alkylation was completed using IAA and a 20-minute dark incubation. Trypsin digestion was completed for one hour at 37 °C, followed by additional trypsin and overnight incubation at 30 °C.	105 µL of sample extracts digested with 150 µL of 50 mM ABC, 15 µL of 100 mM DTT, alkylated with 30 µL IAA, and digested with 20 µL of 100 ng/uL trypsin
Desalting	Desalting was completed using large capacity columns (Strata™-X 33 um Polymeric Reversed Phase (10 mg/1 mL).	320 µL of samples desalted
Lyophilization and Resuspension	Desalted samples were lyophilized in a vacuum concentrator. Resuspension of peptides was completed using 5% ACN/0.1% FA and SIL peptides. SIL peptides were included at a concentration to accomplish a 100 fmol load.	100 µL resuspension volume for matrices 400 µL resuspension volume for PF
LC	Peptide separation was completed using a Dionex UltiMate 3000 UHPLC+ system for liquid chromatography and a Hypersil GOLD™ Dim. (mm) 20x2.1 (Part No 25002-101030) column at 35 °C (Thermo Scientific). Method shown in Figure 3.1.	15 µL injection volume
MS/MS	Thermo Scientific™ Q Exactive™ Plus hybrid quadrapole-Orbitrap™ mass spectrometer was used with instrument parameters described previously, set for PRM.	15 µL of sample analyzed with 100 fmol SIL peptide

The chromatography used for this targeted method was developed and optimized for the nine target peptides selected for the method, shown in Figure 3.1.

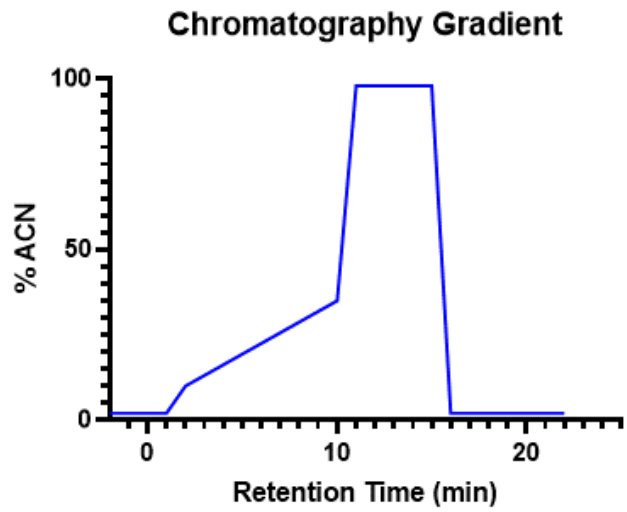

Figure 3.1: Chromatography Gradient Used for Peptide Separation Prior to Targeted MS
The %ACN gradient used to elute target peptides before analysis by MS/MS. The chromatography method was developed after determination of the nine target peptides that were included in the method. The elution gradient occurs between 10 - 35% ACN. Further optimization of this chromatography occurred in later method optimization stages.

The targeted MS method developed was a total of 22 minutes in duration, including LC separation. This method had a parallel reaction monitoring (PRM) run time from 1 to 10 minutes in the method, with the remainder of the gradient used for column washing and re-equilibration. The instrument was set in positive ion mode and utilized a resolution of 35,000, an automatic gain control (AGC) target of 1e6, and a maximum injection time of 500 ms. A loop count of 1 was utilized to accommodate using an inclusion list for PRM. The inclusion list contained m/z values for the nine target peptides and the nine stable isotope labeled (SIL) target peptides. A scan window within the inclusion list was used to schedule the PRM scan and the window was 2 minutes wide. An isolation window of 0.8 m/z was used and no isolation no offset was utilized. A

normalized collision energy (NCE) of 27 was used. Peptide details used for the inclusion list for this targeted MS method are shown in Table 3.2. This method for targeted MS was used for the duration of method optimization experiments, except when method parameters or sample preparation procedures were changed for improvement of the method, as noted for individual experiments.

Table 3.2: Light and Heavy Peptide Inclusion Lists for PRM

Peptide	Target Type	Mass (m/z)	Charge (z)	Start (min)	End (min)
NLPQQ[C]GL[**R**]	Heavy	548.2838	2	3.30	5.30
NLPQQ[C]GLR	Light	543.2797	2	3.30	5.30
SSNPDIYNPQAGSL[**R**]	Heavy	814.8988	2	4.40	6.40
SSNPDIYNPQAGSLR	Light	809.8946	2	4.40	6.40
RPFYSNAPQEIFIQQG[**R**]	Heavy	687.6895	3	6.00	8.00
RPFYSNAPQEIFIQQGR	Light	684.3534	3	6.00	8.00
TANDLNLLIL[**R**]	Heavy	633.3762	2	7.90	9.90
TANDLNLLILR	Light	628.3721	2	6.00	8.00
QQPEENA[**C**]QFQ[**R**]	Heavy	772.8429	2	2.90	4.90
QQPEENA[C]QFQR	Light	767.8388	2	2.90	4.90
FNLAGNHEQEFL[**R**]	Heavy	528.9313	3	5.50	7.50
FNLAGNHEQEFLR	Light	525.5952	3	5.50	7.50
NNPFYFPS[**R**]	Heavy	576.2790	2	6.10	8.10
NNPFYFPSR	Light	571.2749	2	6.10	8.10
TANELNLLIL[**R**]	Heavy	640.3840	2	8.10	10.10
TANELNLLILR	Light	635.3799	2	8.10	10.10
SPDIYNPQAGSL[**K**]	Heavy	699.3612	2	4.40	6.40
SPDIYNPQAGSLK	Light	695.3541	2	4.40	6.40

Spectra obtained from PRM experiments were imported into Skyline Software from the University of Washington, MacCoss Lab for data analysis[21]. The sum of the peak area for the top three product ions for each peptide was taken manually, until product ions were permanently established for the quantitative method. The sum of the peak areas was used to determine the light-to-heavy ratio, which was used as the primary quantitative value for samples until the final quantification strategy was adopted in the quantitative method evaluation. The final quantification strategy utilized calibration techniques within Skyline software[22].

i. Method Development: Stable Isotope Labeled Peptides

HeavyPeptide™ AQUA Basic stable isotope labeled (SIL) versions of the nine target peptides were obtained from Thermo Scientific™. The SIL target peptide sequences and molecular weights (MW) provided by the manufacturer are shown in Table 3.3. The three SIL peptides (QQPEENA[**C**]QFQ[**R**], FNLAGNHEQEFL[**R**], and TANELNLLIL[**R**]), which had not previously been utilized in the group's methods, were evaluated for any light peptide contamination using direct infusion MS.

Table 3.3: Calculated and Experimental MW of SIL Peptides as Provided by Manufacturer

Peptide	Protein	Isoforms	Calculated MW (Da)	Experimental MW (Da)
NLPQQ[**C**]GL[**R**]	Ara h 2	2.1/2	1094.55	1095.17
SSNPDIYNPQAGSL[**R**]	Ara h 3	3.7/16	1627.78	1628.65
RPFYSNAPQEIFIQQG[**R**]	Ara h 3	3.4/5/10/13/17/20	2060.05	2061.21
TANDLNLLIL[**R**]	Ara h 3	3.4/5/10	1264.74	1265.4
QQPEENA[**C**]QFQ[**R**]	Ara h 3	3.4/5/10/13/17/20	1544.65	1545.5
FNLAGNHEQEFL[**R**]	Ara h 3	3.4/5/10/13/17/20	1584.73	1585.38
NNPFYFPS[**R**]	Ara h 1	1.1/2	1150.54	1151.17
TANELNLLIL[**R**]	Ara h 3	3.13/17/20	1279.52	1279.43
SPDIYNPQAGSL[**K**]	Ara h 3	3.4/5/10/13/17/20	1396.71	1397.46

Lyophilized SIL peptides were resuspended using 50% ACN to achieve a concentration of 10 pmol SIL peptide/uL. An equimolar mix of the nine SIL peptides was created to obtain a solution of 500 fmol of each SIL peptide/ µL in 50% ACN. The equimolar mix of SIL peptides was stored at -20 °C prior to use. Prior to analysis by MS, the equimolar mix was diluted to an appropriate SIL concentration in 5% ACN and 0.1% FA that would yield the desired molar amount for each SIL peptide per LC-MS/MS injection of 15 µL.

The optimal molar amount of SIL peptide for each injection was determined empirically. Six loading amounts including, 50, 100, 150, 200, 250, and 500 fmol of each SIL peptide in a 15 µL injection were evaluated for their signal quality and variation across injections. One loading amount was selected for continued use throughout the method development and method optimization stages.

ii. Method Optimization of Instrument Parameters: Automatic Gain Control (AGC) and Injection Time (IT)

Initial evaluation of SIL peptides by the method indicated issues with variability, as observed through large %CVs of peak areas, between injections and samples. In attempt to mitigate the large variation of SIL peptide responses observed, two instrument parameters were optimized. The parameters optimized were the AGC target value and the maximum IT value. The original AGC target was set at 1e6, and the maximum IT was set at 500 ms. An AGC target of 5e5 and an IT of 50 ms were tested in these analyses. Five injections (15 µL) from an equimolar mix at 6.667 fmol SIL/ µL in 5% ACN/0.1 % FA were analyzed (100 fmol on column) for each combination of these two instrument parameters (AGC = 1e6 with IT = 500 ms, AGC = 1e6 with IT = 50 ms, AGC = 5e5 with IT = 500 ms, and AGC = 5e5 with IT = 50 ms). The sum of the peak area for the top three product ions was evaluated for each injection and the average peak area, standard deviation, and %CV were determined for each combination of instrument parameters. The variability between injection replicates was considered through a weighted ranking system to select the least variable combination of instrument parameters to use for further optimization of the method.

iii. Method Evaluation: Stability of SIL Equimolar Mix

The stability of the equimolar mix of SIL peptides over time was evaluated. An equimolar mix that had been stored at -20 °C for two weeks was compared to an equimolar mix which was prepared on the same day of analysis by LC-MS/MS. Both equimolar mixes were prepared according to identical protocols to achieve an SIL

concentration of 500 fmol/uL in 50% ACN and then were further diluted to various SIL concentrations to yield 1, 10, 25, and 50 fmol SIL on column with a 15 µL injection in 5% ACN/0.1% FA. Triplicate injections of each equimolar mix at each SIL concentration were evaluated by LC-MS/MS. The method used to analyze these sample sets utilized an AGC target of 1e6 and an IT of 50 ms, as previously optimized. For each peptide, the sum of the peak area for the top three product ions was compared across the two equimolar mixes, the stored equimolar mix and the freshly prepared equimolar mix, to determine if there were any effects of the storage over time on the signal intensity or signal variability of the peptides. The %CV was also evaluated across injection replicates.

iv. Preliminary Evaluation of Method on Incurred Matrices

To investigate the efficacy of the targeted MS method in its detection of peanut protein in processed food matrices, the method was used to analyze the peanut-incurred cookie and peanut-incurred dark chocolate matrices, as described in Chapter 2. The PF used was light roast (12% fat) from the Golden Peanut and Tree Nuts Company. Incurred cookie, dark chocolate, and PF samples were prepared for MS analysis according to the protocol outlined previously, with two extraction replicates (Table 3. 1). Following resuspension, samples were diluted to various PF concentrations using a solution of 5% ACN/0.1% FA with 6.667 SIL peptide/uL to yield 100 fmol SIL peptide on column with a 15 µL injection. The concentrations analyzed included 0.5, 1, 5, 10, 20, 50, 100, 500, and 1,000 ppm PF. These samples were evaluated in triplicate against the targeted MS method to gauge the sensitivity of the method through detection limits of target peptides. Detection criteria, at this stage in method development, required that three of three pre-

determined product ions were detected in the sample and appropriate peak shape as determined using visual assessment of chromatograms. The lowest concentration of PF detected was compared across sample types to discern the sensitivity of the method at this stage of optimization.

v. Optimization of Instrument Parameters: Injection Time (IT)

To address deficits in method sensitivity, the IT was reconsidered with respect to suggested method parameters for the MS instrument utilized. For the resolution used for this targeted MS method (35,000), the recommended IT is 110 ms, though previous optimization attempts for IT informed the decision of setting the IT at 50 ms[28]. The same samples as were evaluated in the preliminary evaluation of the targeted method in incurred matrices with an IT of 50 ms were analyzed for this experiment, but with an IT of 110 ms. Matrix and PF samples were diluted to various concentrations of PF, including 0.5, 1, 5, 10, 20, 50, 100, 500, and 1,000 ppm PF prior to injection (15 µL). Samples were resuspended with 5% ACN/ 0.1% FA and 6.667 fmol SIL peptide/uL to yield 100 fmol of SIL peptide for each injection. The light-to-heavy ratio for each sample was compared between analyses that utilized the 50 ms and 110 ms for IT. The IT which yielded the greatest signal response, based on the light-to-heavy ratios, for most peptides and samples was selected for continued use throughout.

vi. Method Optimization: Addition of Background Protein

After observation of high variability in both the light and the heavy target peptides, further method optimization of the sample preparation protocol was pursued. The principle tested in this analysis was the inclusion of additional non-target protein into

the dilutions of samples prior to analysis by MS to reduce non-specific binding of the analyte to reduce the hypothesized loss of peptide to plasticware.

Single extracts from the incurred cookie and incurred dark chocolate matrix samples, triplicate extracts of the respective blank matrices, duplicate extracts of PF, and duplicate extracts of instant non-fat dry milk (NFDM) (Nestle® Carnation) were completed. Sample preparation for MS analysis was completed as previously described, except for the generation of the dilution series after peptide resuspension. After samples were resuspended with the appropriate volume of 5% ACN/0.1% FA with 6.667 fmol SIL peptide/ µL, the dilutions were created using resuspended blank matrix samples (for the cookie and the dark chocolate) or with resuspended NFDM samples (for the PF). Samples were diluted with the background protein samples to levels including 1, 10, 100, and 1,000 ppm PF prior to analysis by MS. To control this experiment, samples were also diluted to the respective PF concentrations using the resuspension buffer (5% ACN/ 0.1% FA with 6.667 fmol SIL peptide/ µL). 15 µL of the sample was injected in triplicate for LC-MS/MS. After data analysis using Skyline to determine the sum of the peak area for the top three product ions, dilution curves were constructed to compare the sensitivity of the samples using the background matrix dilution and samples which were resuspended with only buffer. The lowest detected concentration for each sample and resuspension type was recorded and the %CV was evaluated for each peptide, resuspension type, and concentration of PF. The resuspension strategy which provided the least amount of variability, as measured by %CV, and greatest sensitivity was selected for the method.

vii. Method Optimization: Chromatographic Refinements

Optimization of the chromatography gradient used for LC prior to MS/MS was completed in order to achieve greater separation of target peptides prior to MS analysis and to improve peak characteristics, such as points (scans) across the peak. Two chromatography gradients were designed and evaluated against the chromatography that had been used for all previous quantitative method development and optimization included in this chapter. The three tested chromatography gradients each had varying slopes for the peptide elution gradient, which ranged from 1.09% ACN/min to 3.13% ACN/min. Reducing the slope of the elution gradient, in principle, was thought to increase the separation by time of peptides with similar chemical characteristics. Chromatography gradients, with respect to % ACN, are shown in Figure 3.2.

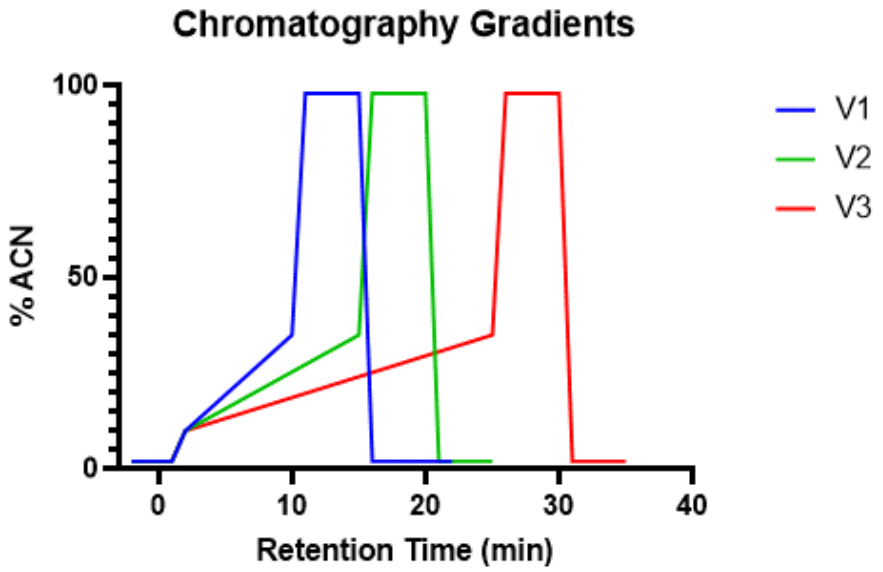

Figure 3.2: Three Chromatography Gradients as Evaluated for Method Optimization
The three versions (V) of chromatography that were analyzed against two concentrations of PF to determine any effect on peak area responses or points across the peak. The respective ACN gradients for the methods are 3.13% ACN/min (V1), 1.92% ACN/ min (V2), and 1.08% ACN/min (V3).

Testing of the chromatography gradients was completed on PF samples diluted to 100 and 1,000 ppm PF in solutions containing 5% ACN/ 0.1% FA with 6.667 SIL peptide/ µL. The sum of the peak area for the top three product ions for each peptide in each chromatography method was compared. Points across chromatogram peaks were recorded and an optimal chromatography method was selected for subsequent LC-MS/MS analyses. At this point in method optimization, the top three product ions that would be used to determine the sum of the peak area were permanently established for each peptide. The product ions selected are shown in Table 3.4. Furthermore, a spectral library created from an untargeted MS analysis of PF (Chapter 2) was added to Skyline. The spectral library allowed comparison of collected spectra to reference spectra to increase detection confidence through library dotp values.

Table 3.4: Target Peptides, Product Ions Selected for Quantification, and Scheduling Window for the Final Method

Peptide (Abbreviation)	Mass (m/z)	Charge (z)	Product Ions for Quantification	Start (min)	End (min)
NLPQQ[C]GLR (NLP)	543.2797	2	P [y7] - 858.4250+ Q [y5] - 633.3137+ P [y7] - 429.7162++	3.60	5.60
SSNPDIYNPQAGSLR (SSN)	809.8946	2	Y [y9] - 1005.5112+ N [y8] - 842.4479+ P [y7] - 728.4050+	6.40	8.40
RPFYSNAPQEIFIQQGR (RPF)	684.3534	3	F [y6] - 748.4100+ I [y5] - 601.3416+ A [b7] - 836.4050+	11.20	13.20
TANDLNLLILR (TAND)	628.3721	2	N [y9] - 1083.6521+ N [y6] - 741.4981+ A [b2] - 173.0921+	15.70	17.70
QQPEENA[C]QFQR (QQP)	767.8388	2	P [y10] - 1278.5532+ P [y10] - 639.7802++ Q [b2] - 257.1244+	3.00	5.00
FNLAGNHEQEFLR (FNL)	525.5952	3	A [y10] - 600.7914++ H [y7] - 479.7407 N [b2] - 262.1186+	9.50	11.50
NNPFYFPSR (NNP)	571.2749	2	F [y6] - 816.4039+ Y [y5] - 669.3355+ F [y4] - 506.2722+	10.40	12.40
TANELNLLILR (TANE)	635.3799	2	N [y9] - 1097.6677+ L [y7] - 854.5822+ N [y6] - 741.4981+	16.30	18.30
SPDIYNPQAGSLK (SPD)	695.3541	2	Y [y9] - 977.5051+ P [y7] - 700.3988+ D [b3] - 300.1190+	6.40	8.40

viii. Method Optimization: Improving Sample Preparation

Further optimization of sample preparation techniques was employed in order to increase method sensitivity and decrease variation in peptide quantification by the method. There were three additions to sample preparation which were evaluated against the control sample preparation (REG). A hexane defatting (HD) of the sample materials prior to extraction, an acetone precipitation (AP) of extracts, and a filter-aided sample preparation (FASP) alongside protein digestion were completed. For HD, samples (0.100

g PF, 0.800 g cookie, and 0.800 g dark chocolate) were defatted with 1.4 mL of hexane (HPLC Grade, Fisher Chemical) three successive times. Following evaporation of hexane, samples were weighed to determine the percent fat lost during HD. The corresponding amount of HD sample was extracted with a mass adjustment to account for the effect of fat loss on mass. The protocol for AP was based off that used by Chen et. al, 2015, with modifications[23]. AP was completed through a two-hour precipitation of extracts using 49.95% acetone/ 49.95% ethanol/ 0.1% FA at -20 °C. Following protein precipitation, samples were washed using 100% acetone and 75% ethanol. Samples were dried and subsequently resuspended to the extract volume for digestion. FASP protocol utilized Amicon® Ultra 0.5 mL centrifugal filters (Millipore Sigma) and a streamlined protocol, adapted from a method for proteomic use outlined by Wisniewski and utilized by Ramachandran, et al[24, 25]. For FASP, reduction and alkylation for digestion was completed as normal and the sample was transferred to the spin filter for 15 min centrifugation at 14,0000 x g at RT. 1 mL of 1M urea and 50 mM ABC were added to the filter, followed by 20 µL of 100 ng/uL trypsin. Spin filters where then covered with parafilm to reduce evaporation loss and samples were digested overnight at 37 °C. The control sample preparation (REG) was completed as described previously for this method. Two extraction replicates of each sample type (cookie, dark chocolate, and PF) and of each procedural type (HD, AP, FASP, and REG) were completed. Following resuspension using 5% ACN/ 0.1% FA with 6.667 fmol SIL peptide/ µL, samples were diluted using the previously optimized dilution with NFDM digest samples, to various

concentrations of PF, including 1, 10, 50, and 100 ppm PF. Samples were injected (15 µL) in duplicate.

ix. Method Optimization: Addressing Method Sensitivity

The primary goal of the final method was the detection and quantification of low levels of peanut contamination in cookie and dark chocolate matrices. Based on observed lowest concentrations of peanut detected for several peptides in both the matrices and the PF calibrant, even after selection of AP of extracts prior to digestion, further optimization of sample preparation parameters was required. One approach to increase the sensitivity of the method in detecting target peptides at low concentrations is to increase the final peptide concentration prior to injection for LC-MS/MS. In addition to the volume changes made in Chapter 2, a large-scale sample preparation protocol was created with adjustments, primarily at the digestion stage, to increase the peptide amount loaded on column. Table 3.5 shows the volume adjustments made to increase the final concentration of peptide in MS samples to, in theory, increase method sensitivity. Additionally, an extraction of 1.00 g matrix in 20 mL buffer was used (1:20 w/v) as compared to the previous 0.500 g matrix in 10 mL buffer (1:20 w/v). The scale up factor for the increased large-scale sample preparation protocol was designed to achieve approximately 9.4 times the peptide concentration prior to injection. One additional adjustment was made for the purpose of optimization with respect to samples prepared using AP. In the previous analysis of samples using AP, the precipitated pellet was dried and then resuspended using water to the original extract volume. To ensure proper buffering of protein, the resuspension of the precipitated pellet was completed using the mix of 50 mM ABC and

100 mM DTT in 50 mM ABC to begin reduction, with volume adjustment for the original extract volume.

Table 3.5: Scaled Up Sample Preparation Procedures Compared to the Previous Protocol

Sample Preparation Step	**Previous Protocol (uL)**	**Scaled Up Protocol (uL)**
Sample Volume Precipitated	105	250
Acetone Precipitated Pellet Resuspended in Water	105	0
Digestion Mix:		
50 mM ABC	150	1000
100 mM DTT	15	75
Water	0	0
50 mM IAA	30	150
Trypsin (100 ng/uL)	20	70
Total Digestion Volume	320	1295
Volume Desalted	320	1280
Resuspension Volume	PF: 400 Matrix: 100	PF: 200 Matrix: 50

Using the increased large-scale sample protocol, duplicate extractions of the matrix and PF samples were completed and precipitated using acetone, as previously described. The samples proceeded through sample preparation according to Table 3.5. Resuspension and dilutions to various PF concentrations (0.5, 1, 10, and 50 ppm PF) were completed using resuspended NFDM digests in 5% ACN/ 0.1% FA with 6.667 fmol SIL peptide/ µL. Samples were injected (15 µL) in duplicate. Dilution curves of the light-to-heavy ratio was constructed and the lowest concentrations for detection were evaluated for each peptide. The expected fold change for the sum of the peak area of the top three product ions, 9.4x, was compared to the observed fold change. Assessments for method sensitivity using the increased large-scale sample preparation protocol were completed.

i. Method Optimization: Calibrant Extraction

The method sought to be quantitative using a calibration to PF material and thus, further optimization of PF samples was investigated to minimize variability observed in the peak area response of calibrant samples. To optimize the calibrant extraction, various extraction concentrations were evaluated using the targeted MS method. Resuspension volumes proportional to the sample PF concentration were used to normalize the final peptide concentration prior to injection for MS.

The method's PF extraction of 0.060 g PF in 30 mL buffer was included in the analysis, as well as 0.120 g PF/30 mL buffer, 0.150 g PF/30 mL buffer, and 0.300 g PF/30 mL buffer. Two extraction replicates were completed for each extraction concentration. Following resuspension, dilutions of the PF samples were created to various concentrations of PF using NFDM digests in 5% ACN/ 0.1% FA with 6.667 fmol SIL peptide/ µL, as previously demonstrated to reduce variability. Each sample was analyzed with duplicate injects.

x. Quantitative Method Evaluation

To demonstrate the efficacy of the quantitative method developed in this work, peanut-incurred cookie and peanut-incurred dark chocolate matrices were evaluated using all optimized method protocols, instrument parameters, and final quantification strategy. Triplicate extracts were completed for the cookies incurred with 2 ppm PF (final concentration after baking of 2.38 ppm PF) and 10 ppm PF (final concentration of 11.91 ppm PF), and dark chocolate incurred with 4 or 40 ppm PF across two days. One set of triplicate extracts of PF (0.150 g PF/ 30 mL buffer) was completed with each set of

matrix samples, including cookie and dark chocolate. One day contained extraction for the cookie matrix and PF and the second day included extractions for the dark chocolate matrix and the PF. This entire experiment was repeated to obtain additional day-to-day measures of the PF calibrant and to obtain initial day-to-day measures for the incurred matrix samples. Resuspension was completed using 5% ACN/ 0.1% FA with 6.667 fmol SIL peptide/ µL for the matrix samples. Dilutions of resuspended PF was completed using NFDM digest samples in 5% ACN/ 0.1% FA with 6.667 fmol SIL peptide/ µL. Triplicate injects (15 µL) of matrix samples and duplicate injects (15 µL) of PF calibrant samples were evaluated by the instrument to yield 100 fmol of SIL peptide on column for each sample. The optimized LC-MS/MS method was used for this analysis. The sequences, product ions, and scheduling windows for target peptides based on optimized chromatography is shown in Table 3.4.

For the quantitative MS method, it was necessary to formalize criteria that a signal obtained from this method must meet in order to be objectively determined as a detection. One inherent detection criterion is that the transition signals must be within the scheduled retention time window. Only detections with library dotp values ≥ 0.80 were considered. A 5.0 ppm mass error tolerance for the average mass error of measured product ions was also used as a detection criterion. Additionally, the peptide peak found ratio (PFR) must be equal to 1, indicating that all three product ions were observed for each peptide. Only signals which met all formalized detection criteria were used for quantification.

The quantification strategy developed for this method was based on the peak area of the light peptide divided by the peak area of the SIL peptide, which is referred to as the light-to-heavy ratio. The peak area was determined using the top three product ions for each peptide as noted in Table 3.4. Further, the light-to-heavy ratios were calibrated to the PF calibrant to obtain a ppm PF concentration for the sample. This calibration to the PF occurred within Skyline. PF samples were denoted as standards in Skyline and their respective PF concentration was recorded in the software. The multi-point calibration curve for the PF was constructed from PF samples with concentrations of 0.1, 0.5, 1, 5, 10, 50, and 100 ppm PF. Following the interpolation of the matrix samples to the calibration curve, Skyline reported a value for PF concentration in ppm PF. Using the protein content of the PF used as the calibrant material (52.1% protein as determined by Dumas analysis), the ppm PF value was converted to a ppm peanut protein value.

For the method, there are three reported values in ppm peanut protein for the four samples analyzed. The first reported value is the average ppm peanut protein value for a peptide. This is the average of all quantified values for each injection completed, this is denoted as the peptide average. Next, for each of the four matrix samples analyzed the maximum of all nine peptide averages is reported (termed the maximum peptide average). Lastly, to provide a conservative value for risk assessment and food safety

purposes, the maximum extract average is also reported. An example of the reported values for the quantitative method is shown in Figure 3.3 using a model peptide.

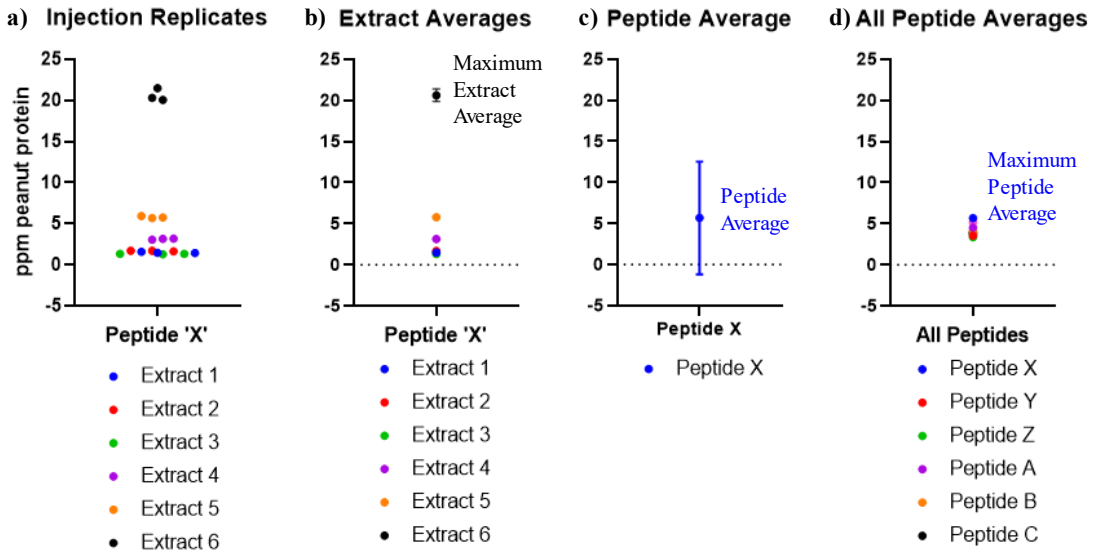

Figure 3.3: Summary of Reported Values for the Quantitative Method
There are three reported values as a result of the quantification strategy and method. **A)** Each quantified value for each injection is plotted (*N=18*), **b)** extract averages calculated from three injection replicates, as shown in **a)**, with the maximum extract average of all peptides reported *(N=3, Mean ± SD)*, **c)** the average quantified value for a peptide, a reported value *(N=18, Mean ± SD)*, and **d)** of all peptide averages the maximum quantified value is reported as the maximum peptide average (*N=18, Mean ± SD)* for each peptide average value as shown in **c)**.

A variation analysis was also completed to quantify the observed variability. For this analysis, a number of components with possible contribution to variation were considered, primarily method variation, extraction variation, and injection variation. In this experiment, there are a total of 18 data points for each matrix and level analyzed. These values were obtained in experiments across two different days, nine data points for day one and nine data points for day two. The quantified value, the ppm peanut protein values, were used as the data points for this variation analysis.

An overall method variation measure was calculated using the %CV of all reported data points for a sample, including all values from both days (*N=18*). A within-

day method variation was also calculated for each day and was represented by the %CV (*N=9*). Day-to-day variation of the method was determined using the %CV of the within day overall averages (*N=2*). Extraction variation within each day was calculated using the average reported values for each extract replicate and determination of the %CV between these values (*N=3*). Day-to-day extraction variation was calculated using the average reported values for each extract across both of the experiments and is displayed as %CV (*N=6*). Injection variation was calculated by the %CV of values that were injection replicates of the same sample (*N=3*) and a range of the injection variation values were recorded to show the maximum and minimum %CV per sample.

IV. RESULTS AND DISCUSSION

For results and discussion, a three- or four-letter abbreviation was used to refer to target peptides. Table 3.4 shows the abbreviation used for each peptide sequence.

i. Method Development: Stable Isotope Labeled Peptides

The optimal molar amount of SIL peptide for each injection was determined through analysis of six loading amounts including, 50, 100, 150, 200, 250, and 500 fmol of each SIL peptide. The purpose of determining the optimal loading amount for SIL peptides was to establish a molar amount for SIL peptides that is both robust in its detection and minimally variable, as SIL peptides play a critical role in the quantitative aspect of the method. From the top three product ions for each peptide, the sum of the peak area was determined. The %CV of the sum of the peak areas for injection replicates was determined (Figure 3.4).

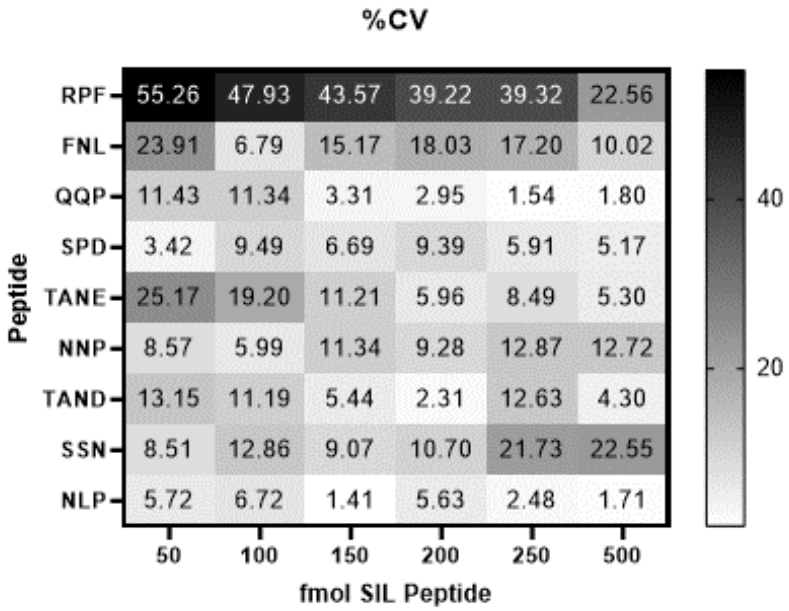

Figure 3.4: The %CV Between Injection Replicates for SIL Peptides at Various Loading Amounts.
A heatmap displaying the %CV of the sum of the peak area between injections calculated for each peptide at five SIL loading amounts including 50, 100, 150, 200, 250, and 500 fmol. *N=3.*

For a majority of the SIL peptides analyzed (except for NLP and SPD) the %CV between injection triplicates was much higher than anticipated for a standard material. The %CV results did not indicate the expected trend, in which increasing the fmol of SIL peptide analyzed directly decreased the variability between injections. Therefore, selection of the highest loading amount was not necessary. RPF and FNL peptides had particularly high %CVs, which suggested some chemical characteristics of the peptides may be contributing to the variability in the detection of the peptide by the instrument. One factor that may be contributing to the variable nature of the response of these peptides is hydrophobicity. These peptides elute later in the ACN gradient, indicating a greater hydrophobicity relative to other peptides in the method. Hydrophobicity of peptides was thought to contribute to nonspecific binding and loss of analyte peptide.

Grand average of hydropathicity indices (GRAVY) values for these two peptides were found to be -0.929 (RPF) and -0.815 (FNL) using ProtParam (Expasy). GRAVY values represent the hydrophobicity of a peptide using hydropathy values of amino acids in the peptide[26]. Negative GRAVY values indicate hydrophilicity, while positive values predict hydrophobicity[26]. While the GRAVY scores for these two peptides are negative (indicating some hydrophilicity), they are more hydrophobic than some peptides included in the method, for example QQP = -1.958. Two other variable peptides, TAND and TANE, appear to have some hydrophobicity as well with GRAVY scores of 0.527 each. However, hydrophobicity, as indicated by GRAVY scores, does not follow identically the trend observed or fully explain the variability observed in this experiment.

The 100 fmol loading amount was used for subsequent analyses, except when noted, as it demonstrated sufficient detection, comparable variability compared to other loading amounts, and was predicted to be an appropriate level of SIL peptide for most samples that would be analyzed for the final method. As the 100 fmol loading amount had similar variability to other loading amounts, as demonstrated by the %CV, it still indicated an issue in the variability of the response of most SIL peptides.

ii. Method Optimization of Instrument Parameters: Automatic Gain Control (AGC) and Injection Time (IT)

As previously observed, the variability of the response of SIL peptides by the method proved to be an issue as the variability is too large for a robust quantitative method. In order to reduce the variability observed, two instrument parameters were investigated (AGC and IT). AGC is an instrument parameter that regulates the number of

ions present in the Orbitrap to reduce space charge effects[27]. The IT parameter sets a maximum amount of time that ions can accumulate in the ion trap before being sent the mass analyzer. These are not independent parameters, as mass events depend on which parameter (AGC or IT) is met first[27]. Since these are not independent parameters, multiple combinations of these settings were investigated. Optimizing these parameters was predicted to increase the points (scans) across peptide peaks which could, in theory, decrease the variability in detection. The %CV for the sum of the peak area of the top three product ions was determined for each peptide and method parameter combination (Figure 3.5).

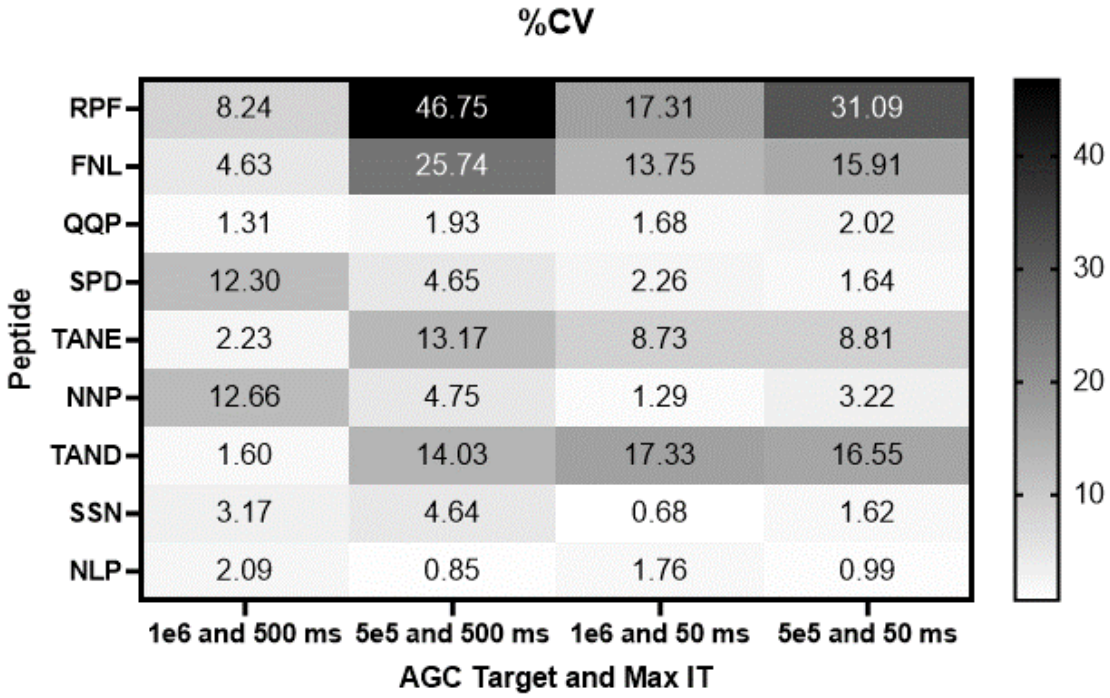

Figure 3.5: The %CV Between Injection Replicates for SIL Peptides Using Various Instrument Parameters.
A heatmap displaying the %CV of the sum of the peak area between injections calculated for each peptide at each SIL loading amount in fmol for different instrument parameters. AGC targets evaluated include 1e6 and 5e5. Maximum IT values evaluated included 50 and 500 ms. *N=5.*

As the goal of this analysis was to determine which instrument settings resulted in peptide responses with the least variability, the %CV was the primary result considered for the selection of an optimal AGC target and IT. In observation of %CVs for peptides and instrument parameters, there is no clear parameter setting combination which results in the lowest variability for all peptides. There are some peptides (QQP, SSN, and NLP) which seem to have low %CV regardless of the instrument parameter used. In general, it seems the IT of 500 ms generally caused a greater %CV for most peptides, though it is important to note that the two parameters tested are not independent. Overall, the effect of the AGC target and IT varied by peptide. To objectively discern the least variable method, a weighted point system was used to determine which parameter combination was the least variable. The instrument parameters selected, as determined to be the least variable for the greatest number of peptides, included the AGC target of 1e6 and a maximum IT of 50 ms. The second least variable combination of settings was 1e6 for AGC and 500 ms for IT. These settings (AGC = 1e6 and IT = 50 ms) would be used in subsequent MS analyses using this targeted method, unless otherwise noted. Further optimization of IT occurred later in method optimization steps.

iii. Method Evaluation: Stability of SIL Equimolar Mix

An important consideration for the use of SIL peptides through an equimolar mix is the stability of the SIL peptides over time. If an equimolar mix of SIL peptides is stored and used in numerous quantitative analyses over time, it is important to determine the effect of storage on the response expected from the sample. The sum of the peak area for the top three product ions for the SIL peptide for each equimolar mix type (prepared

same day and stored for two weeks) was calculated and is shown for three representative peptides in Figure 3.6.

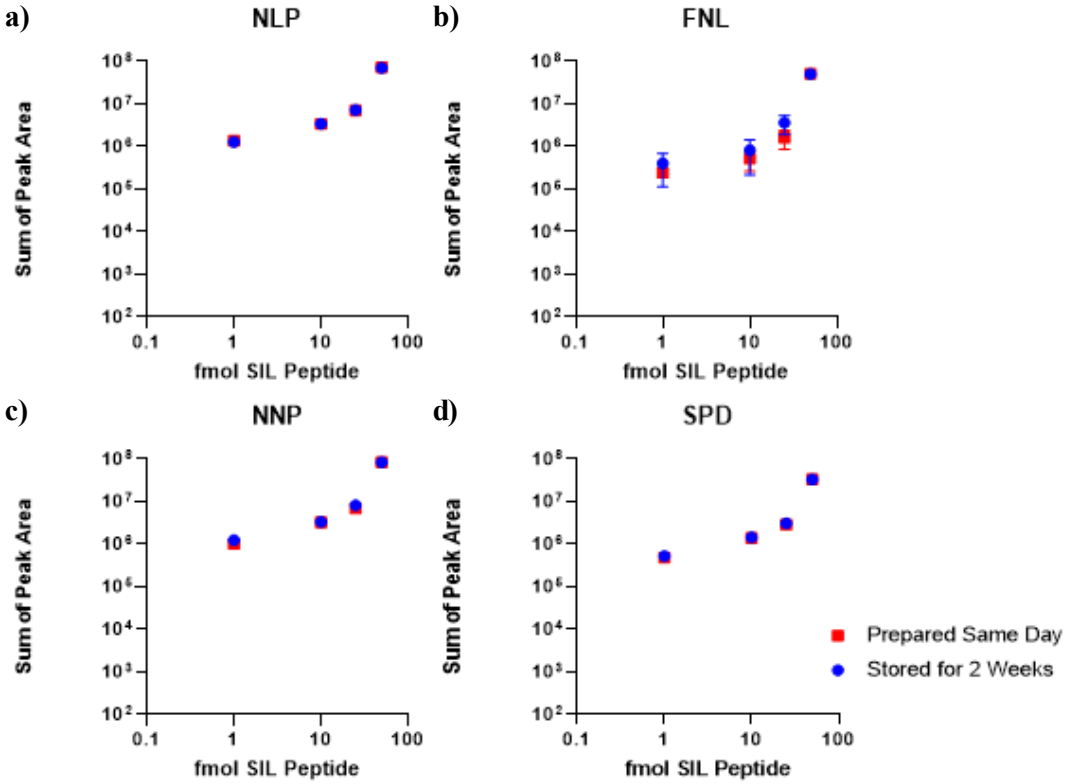

Figure 3.6: Peak Area Responses at Various SIL Peptide Loading Amounts for Stored and New Equimolar Mixes.
The average sum of the peak area for the top three product ions for equimolar mixes prepared on the same day of analysis or prepared and stored for two weeks prior to analysis at -20 ºC. Dilution curves are shown for three peptides, **a)** NLP (Ara h 2), **b)** FNL (Ara h 3), **c)** NNP (Ara h 1), and **d)** SPD (Ara h 3). Product ions used for peak area measures included NLP (y7 - 868.4333+, y6 - 771.3806+, y5 - 643.3220+), FNL(y6 - 831.4235+, y11 - 662.3376++, y10 - 605.7956++), NNP (y7 - 923.4649+, y6 - 826.4122+, y5 - 679.3438+) and SPD (y9 - 985.5193+, y8 - 822.4559+, y7 - 708.4130+). *N=3, Mean ± SD.*

Dilution curves for SIL peptide equimolar mixes indicated highly comparable peak area responses for both the equimolar mix was prepared on the same day of analysis and for the equimolar mix prepared and stored at -20 ºC for two weeks. For one peptide shown, FNL, there appears to be a greater variability as apparent in the graphed SD. This is a result which has been observed in previous experiments indicating high variability

with late-eluting, partially hydrophobic target peptides. Overall, this result showed relative stability of the equimolar mix at the storage concentration (500 fmol SIL peptide/ µL in 50% ACN). Further, the similar responses in peak area between new and stored equimolar mixes provided support for the continued use of a stock equimolar mix of SIL peptides throughout method development and for the SIL peptide dilution strategy used to achieve the desired SIL loading amount (100 fmol). In the dilution curves, an effect is observed in which decreasing analyte concentrations did not produce the expected linear decrease in reported analyte signal, but instead an increase compared to the expected point. This is observed primarily at the 1 fmol SIL peptide point on the dilution curve. Though not satisfactory, the level of 1 fmol is only 1% of the SIL peptide loading amount selected for the quantitative method, and thus, is not considered an issue for the quantitative aspect of the method.

iv. Preliminary Evaluation of Method on Incurred Matrices

A desired component of the targeted MS method is the ability to detect peanut protein at low concentrations in food matrices, namely cookie and dark chocolate. Therefore, analysis of post-digestion dilutions of the incurred matrices to discern the current sensitivity of the method and its approximate detection limits for each of the nine target peptides was completed. A representative dilution curve is shown in Figure 3.7 for the TAND peptide.

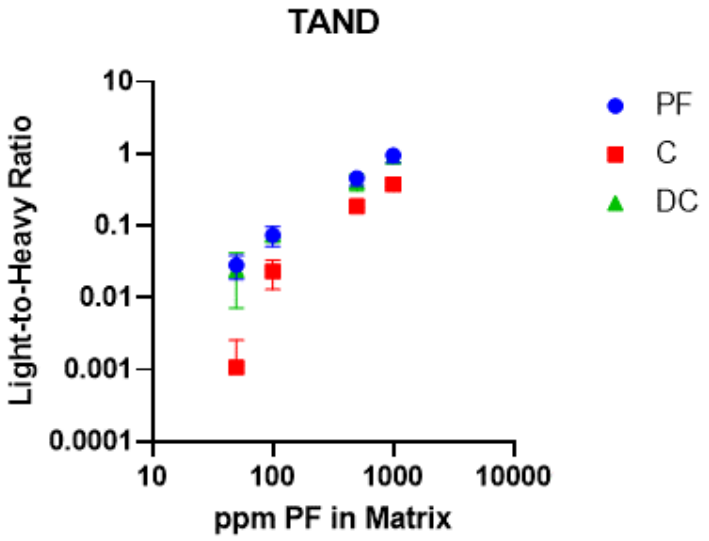

Figure 3.7: A Representative Dilution Curve from the Preliminary Evaluation of the Method on Incurred Matrix Samples.
The light-to-heavy ratio for the TAND peptide (Ara h 3) in all three sample types, PF, cookie, and dark chocolate. Analyzed concentrations included 0.5, 1, 5, 10, 20, 50, 100, 500, and 1,000 ppm PF. Product ions used for the sum of the peak area for TAND include (y9 - 1083.6521+, y7 - 854.5822+, y6 - 741.4981+). *N= 6, Mean ± SD.*

The dilution curve for the TAND peptide (Ara h 3) indicated one general trend observed in the preliminary evaluation of the method on incurred matrices. The dilution curve displayed comparable light-to-heavy ratio values for the PF and dark chocolate samples, but a decrease in the light-to-heavy ratios for the cookie samples. This effect was apparent at each of the concentrations of PF and appeared to intensify at lower concentrations. The same trend was observed in the Ara h 1 peptide (NNP) and two Ara h 3 peptides (TANE and SPD). This suggested the cookie matrix obtained lower recoveries of certain proteins or peptides compared to the other food matrix (dark chocolate) or no matrix (PF). This could be attributed to the effect of the matrix components or an effect of the processing of matrix components causing a reduction in extractability, digestibility, or other factors for certain proteins. Three peptides (SSN, FNL, RPF) recorded very similar light-to-heavy ratios across the three sample types, while the NLP peptide

indicated similar matrix responses (cookie and dark chocolate) but a greater recovery in the no matrix samples (PF). This suggested that the recovery of NLP peptide, or likely the Ara h 2 protein, is impacted by interactions of the matrix components or its processing.

The dilution curve in Figure 3.7 also displayed the lowest detected concentration of PF in the three sample types to be 50 ppm PF, which was not considered sufficiently sensitive for the needed method performance. This lowest detected concentration is one of the most common limits for the other target peptides as well. Of analyzed concentrations, including 0.5, 1, 5, 10, 20, 50, 100, 500, and 1,000 ppm PF, the lowest detected PF concentration for each peptide and sample type is shown in Table 3.6.

Table 3.6: Lowest Concentrations of Detection by Peptide and Matrix (ppm PF in matrix) from the Preliminary Evaluation of the Method on Incurred Matrices

Target Peptide	PF	Cookie	Dark Chocolate
NLPQQ[C]GLR	50	100	100
SSNPDIYNPQAGSLR	50	50	50
TANDLNLLILR	50	50	50
NNPFYFPSR	50	50	50
TANELNLLILR	50	100	50
SPDIYNPQAGSLK	100	100	50
QQPEENA[C]QFQR	500	500	1000
FNLAGNHEQEFLR	50	50	100
RPFYSNAPQEIFIQQGR	20	50	50

The lowest detected concentrations for the nine target peptides indicated a further need for method optimization to improve the sensitivity of the method. One peptide, QQP (Ara h 3), indicated a particular lack of sensitivity compared to the other eight peptides. In this evaluation, the RPF peptide (Ara h 3) was the most sensitive. All peptides

recorded lower or identical levels of detection in the PF (no matrix) compared to the matrices (cookie and dark chocolate). This suggested, again, the effect of matrix components or the processing of the matrix on proteins and resulting peptides targeted by the method. Additionally, large %CVs were observed in these samples, particularly for the SIL peptide. This issue was later addressed in method optimization through the addition of background protein to resuspended and diluted MS samples.

Generally, most peptides reported lowest concentrations of detection at 50 or 100 ppm PF. This necessitated further method optimization to improve sensitivity because these concentrations were not low enough to achieve the needed method performance for this detection method. The desired method performance for this method was in the low ppm PF range for the cookie (4 ppm PF or 2 ppm peanut protein) and dark chocolate samples (8 ppm PF or 4 ppm peanut protein) based on calculated risk assessment principles using the VITAL reference dose for peanut and US consumption data[4, 11].

v. Optimization of Instrument Parameters: Injection Time (IT)

Further optimization of the IT setting was pursued based on the continued observation of high variability in target peptide responses across injection replicates. Additionally, for the resolution utilized in this method (35,000), the recommended IT is 110 ms[28]. Though previous work suggested an optimal IT of 50 ms compared to 500 ms, this investigation displayed superior peak area response in samples analyzed with an IT of 110 ms compared to an IT of 50 ms (Figure 3.8).

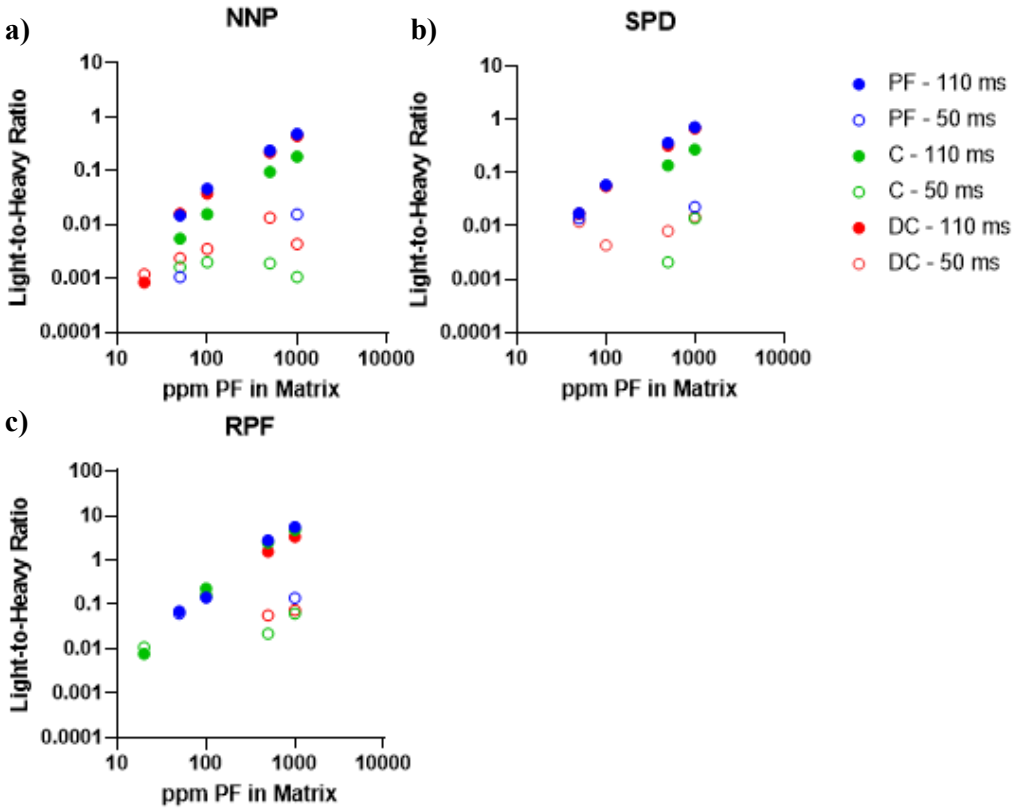

Figure 3.8: Dilution Curves of PF, Cookie, and Dark Chocolate Samples Analyzed with an IT 110 ms vs 50 ms.
The mean light-to-heavy ratio for three representative target peptides **a)** NNP (Ara h 1), **b)** SPD (Ara h 3), and **c)** RPF (Ara h 3). Product ions used for determination of the light-to-heavy ratio include NNP (y6 - 816.4039+, y5 - 669.3355+, y4 - 506.2722+), SPD (b2 - 185.0921+ , b3 - 300.1190+, b4 - 413.2031+), and RPF (y6 - 748.4100+, y5 - 601.3416+, b6 - 765.3678+). *N=6. Mean.*

The effect of IT (110 ms or 50 ms) for NNP, SPD, and RPF peptides is representative of the effect observed for a majority of the nine target peptides. The results indicated an increase in the light-to-heavy ratio for samples using the longer IT time for all sample types, including cookie, dark chocolate, and PF samples. The IT time of 110 ms likely allowed a greater number of ions to accumulate in the ion trap prior to being sent to the mass analyzed compared to the IT time of 50 ms. This obvious effect of the IT time indicated that IT may be providing greater control of the flow of ions than the AGC

target which would suggest that the AGC target may not have been met prior to the IT setting for most previously analyzed samples. The increase in the light-to-heavy ratio for the 110 ms IT informed its use as an instrument parameter setting for subsequent analyses using the method because it improved the detection of target peptides compared to a 50 ms IT, as was previously used.

vi. Method Optimization: Addition of Background Protein

One observation from the preliminary evaluation of the method in analyzing incurred matrices that prompted this method optimization procedure was the variability of the SIL peptide and its relationship to sample (light) protein concentration. This trend was observed for several of the SIL peptides but was considered to have a predominant effect on five of the nine target peptides (TAND, NNP, TANE, FNL, and RPF). Two examples of peptides which displayed the observed trend (FNL and RPF), alongside two peptides that did not have the same effect (SPD and QQP) are shown in Figure 3.9.

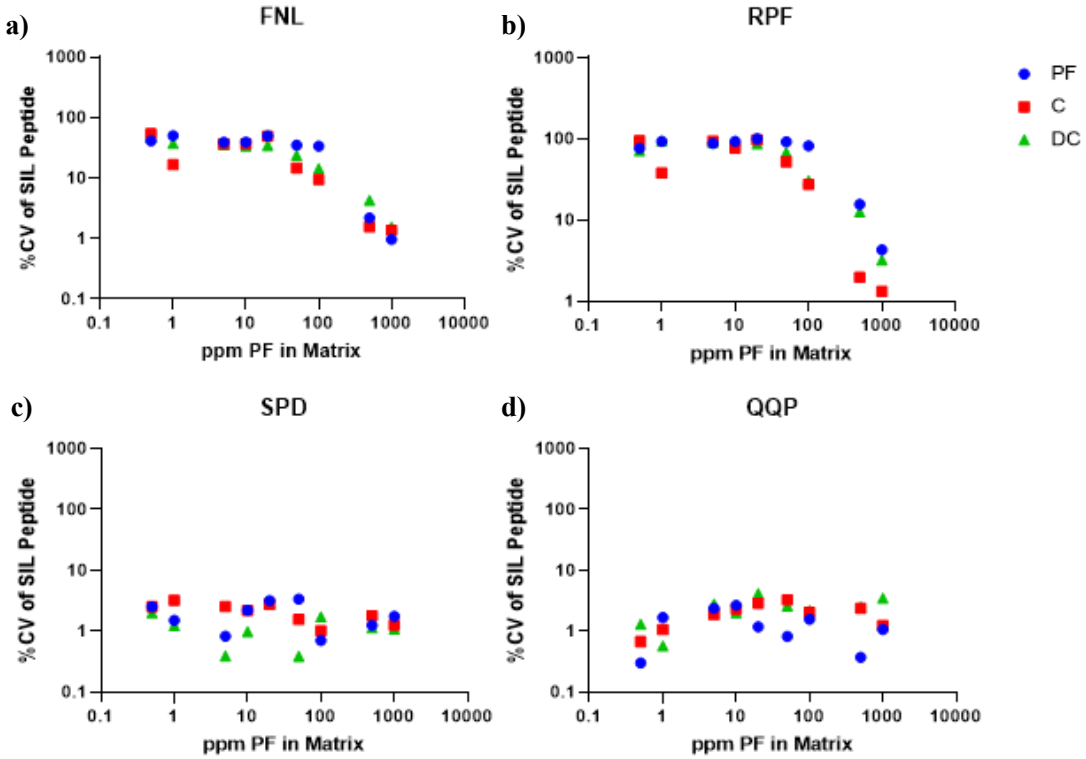

Figure 3.9: The %CV of SIL Peptides with Respect to the Light Peptide Concentration of Samples.
The %CV for the respective SIL peptide, **a)** FNL, **b)** RPF, **c)** SPD, and **d)** QQP, plotted against the concentration of the light sample through ppm PF in matrix. *N=6.*

In Figure 3.9, it can be observed that there were two main trends in the effect of the light sample concentration on the %CV of the SIL peptide. The expected trend, as seen in the plots for SPD and QQP, is no correlation between the light peptide concentration the variation calculated for the SIL peptide. In a simple linear regression of the data points in SPD and QQP plots, the slopes were considered not to be significantly different from zero. In principle, this should occur when an SIL peptide is used as an internal standard because its response should not be affected by different concentrations of the target analyte. The reverse, observed with the FNL and RPF peptides, was an apparent trend of the effect of the light concentration of the sample analyzed on the %CV

of the SIL peptide, specifically that as the light concentration decreases, the variability of the response of the SIL peptide increases. This trend was also observed for other late-eluting, partially hydrophobic peptides in the method. A simple linear regression for the data sets for FNL and RPF indicated many slopes that were significantly different than zero. The slopes for the PF (-0.04844) and dark chocolate (-0.03808) were significantly different from zero for the FNL peptide, however the slope for the cookie (-0.03805) was not significantly different from zero. For the RPF peptide, all slopes (PF = -0.9757, cookie = -0.08422, and dark chocolate = -0.08880) were considered significantly different from zero. This observed trend is not ideal for a quantitative method, as the quantification of the target peptides is dependent on the response of the SIL peptide. It would not be a robust method if the internal standards were impacted by the concentration of the target analyte. Thus, further optimization was pursued in order to address this deficit in the robustness of the method.

To combat the problematic effect of the light concentration on the variation of the SIL peptide response, an optimization strategy of diluting samples with background protein was employed. This approach was selected because it was hypothesized that increasing the concentration of background peptides in the sample would allow for a decrease in the variation of the SIL peptide, as had been previously observed. It is important to note that the background peptide in the samples did not contain analyte or peanut peptides. For PF, NFDM digests were used as background peptide to dilute samples for MS. For the cookie and dark chocolate, digests of the blank matrices (0 ppm PF) were used as background material to create the dilution series for MS.

The results indicated that the background matrix dilutions did accomplish a moderate decrease in the variability of the SIL peptide. This was observed especially for partially hydrophobic peptides, like FNL (Figure 3.10).

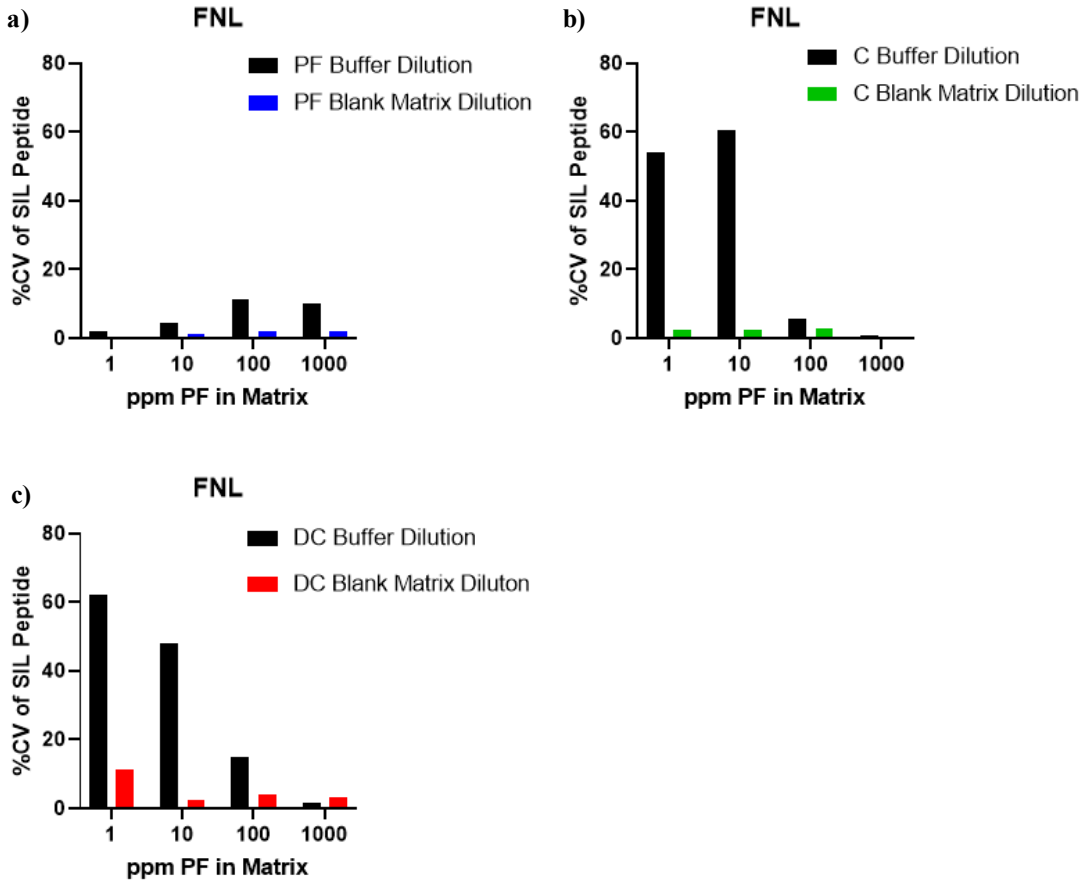

Figure 3.10: The %CV of SIL Peptides for Samples Diluted with Buffer or With Background Protein.
The %CV for the FNL peptide for **a)** PF, **b)** cookie, and **c)** dark chocolate samples diluted with buffer (5% ACN/0.1% FA/ 6.667 fmol SIL peptide) or with blank matrix dilutions (NFDM, blank cookie, or blank dark chocolate resuspended in 5% ACN/0.1% FA/ 6.667 fmol SIL peptide). *N=3.*

The background protein dilution strategy appeared to reduce the %CV of the SIL peptides in general, as well as address the trend of increasing %CV with decreasing light peptide concentration. The strategy also increased the sensitivity of the method for some

light peptides, especially those that are partially hydrophobic, such as FNL,TANE, and RPF (Table 3.7).

Table 3.7: Lowest Levels of Detection for All Sample Types Using Buffer Dilution (BD) or Background Protein Dilutions (BPD) Prior to MS Analysis

Matrix Type	**PF**		**C**		**DC**	
Dilution Type	**BD**	**BPD**	**BD**	**BPD**	**BD**	**BPD**
FNLAGNHEQEFLR	100	100	10	10	100	10
TANELNLLILR	100	10	100	10	100	10
RPFYSNAPQEIFIQQGR	100	10	1	1	10	1

This increase in detection and thus, sensitivity, for some peptides suggested that the background protein dilution benefited the stability of the analyte peptides, not just the SIL peptides. This may be due to the partially hydrophobic nature of the light peptides being lost due to nonspecific binding to plasticware during sample preparation following digestion or in MS vials prior to injection for LC-MS/MS. The background protein dilution strategy was adopted for the remainder of the method optimization analyses. However, to simplify the sample preparation and to reduce the number of samples necessary to create enough digest material for the dilution series, the background protein dilutions were modified to utilize NFDM digests for all sample types (PF, cookie, and dark chocolate). Furthermore, it is important to note that the background protein dilution was only necessary when creating dilutions of incurred materials, which only occurred when diluting high concentrations of incurred materials (10,000 ppm PF cookie or dark chocolate) to create lower concentrations for analysis or for the PF calibrant. When low levels of incurred matrices were evaluated for the quantitative method evaluation (2 and 10 ppm PF cookie and 4 and 40 ppm PF dark chocolate) no background protein dilution

was necessary as these samples were not diluted prior to analysis for MS, simply resuspended.

vii. Method Optimization: Chromatographic Refinements

Optimization of the chromatography used for this method was pursued because further separation of target peptides was hypothesized to increase points across the peak and peak width, thus increasing the sensitivity of the method. An increase in sensitivity was desired for method optimization based on the previous lowest concentrations of PF that were observed to be in the range of 1-100 ppm PF. These levels are not sufficient for the method and thus chromatographic refinements were analyzed. In addition to the previously used chromatography gradients for this work (Version 1 = V1), two additional chromatography gradients were evaluated (Version 2 = V2 and Version 3 = V3) (Figure 3.2).

After analyzing each of the three chromatography gradients against PF samples diluted to 100 and 1,000 ppm PF, it was clear that the peak area responses of all target peptides were not generally different between chromatography gradients. However, the points across the peak were notably different between V1, V2, and V3 of the chromatography. As hypothesized, decreasing the slope of the ACN gradient increased peptide separation and thus, increased points across the peak. This was directly observed with respect to V3, the shallowest of ACN gradients, as it recorded the greatest number of points across the peak for a majority of the light peptides. While this chromatography method (V3) extended the method duration to 35 minutes, it was decided that the possible increase in sensitivity that the shallow gradient and increased points across the peak

would cause at lower levels of peanut contamination outweighed the increase in method time. As a result, the V3 chromatography was selected and used in all subsequent analyses using this LC-MS/MS method.

viii. Method Optimization: Improving Sample Preparation

Further method optimization through different sample preparation additions was explored in order to improve the sensitivity of the method to reach the desired method performance. Hexane defatting (HD) was analyzed because it was hypothesized that the fat in the samples, particularly the PF and dark chocolate, was impacting some component of the sample preparation. HD has been used in other MS methods for the detection of peanut[29, 30]. Acetone precipitation (AP) has been demonstrated to improve recovery of peptides by MS and was therefore tested in this analysis[31, 32]. Filter-aided sample preparation (FASP) is another sample preparation addition which has been commonly employed in the field of food allergen proteomics[30, 33]. The effect of FASP, AP, HD, and REG sample preparation protocols on light-to-heavy ratios and variability of target peptides was analyzed. Three representative peptides and the dilution curves for each sample type are shown in Figure 3.11.

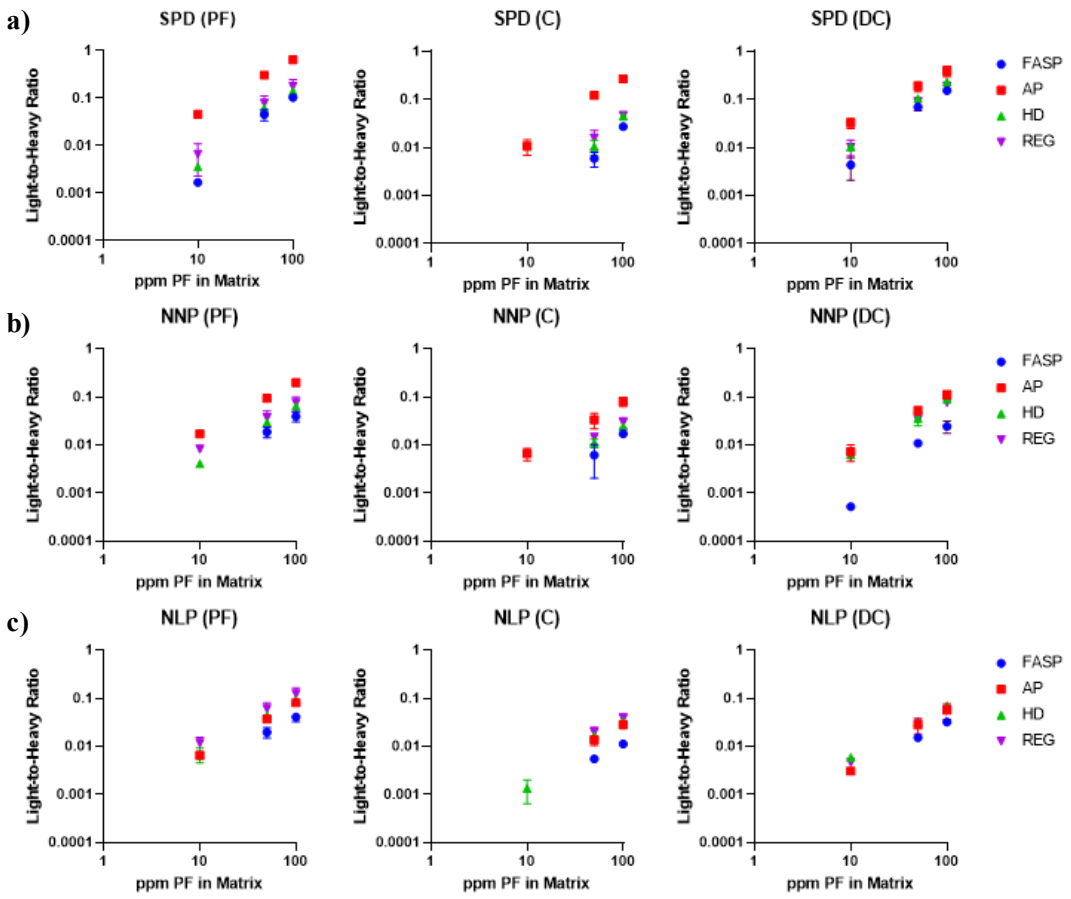

Figure 3.11: Dilution Curves for Three Target Peptides with FASP, AP, HD, or REG Sample Preparation.

Dilution curves for three representative target peptides prepared by four sample preparation strategies including FASP, AP, HD, and REG sample preparation. Peptides shown include **a**) SPD (Ara h 3), **b**) NNP (Ara h 1), and **c**) NLP (Ara h 2). Product ions used for the light to heavy ratio determinations were SPD (y9 - 977.5051+, y7 - 700.3988+, b3 - 300.1190+), NNP (y7 - 913.4567+, y5 - 669.3355+, y4 - 506.2722+), and NLP (y7 - 858.4250+, y5 - 633.3137+, y7 - 429.7162++). *N=4. Mean ± SD.*

The evaluation of different sample preparation procedures indicated an apparent effect of the procedure used on the light-to-heavy ratio recorded for peptides, as well as the overall sensitivity for some peptides. The three peptides shown in Figure 3.10 originated from each of the three peanut proteins targeted by the method, Ara h 1, Ara h 2, and Ara h 3. The protein to which a peptide is from appeared to have affected which

sample preparation procedure provided the most optimal response by MS. For SPD (Ara h 3) and NNP (Ara h 1), AP recorded the greatest peak area at most PF concentrations evaluated and for all sample types (PF, cookie, and dark chocolate). The other six peptides from Ara h 3 mirrored the result of AP outperforming the other sample preparation procedures. On the other hand, NLP (Ara h 2) did not show the same trend. For NLP, the REG sample preparation resulted in the greatest light-to-heavy ratio for the PF and cookie samples, while AP may have been the most effective method for the dark chocolate samples. The results suggested that AP of the sample extracts is an effective method for increasing the recovery for a large number of target peptides. This was likely due to the efficient concentration of protein, by precipitation, immediately after extraction, though it did have differing affects based on the type of protein. For all peptides, FASP protocols appeared to result in the smallest light-to-heavy ratios in all sample types, though only slightly compared to HD and REG protocols. With respect to variability, AP also appeared to be the superior sample preparation method as the SD for AP samples was generally less than that for the other sample preparation procedures. AP also resulted in the most sensitive levels of detection in ppm PF, as shown in Table 3.8. Additionally, HD and REG had better sensitivity of the target peptides compared to FASP. Based on the results of the improved sensitivity and increase in light-to-heavy ratio, plus the decrease in variability, AP was utilized in subsequent analyses using the targeted MS method.

Table 3.8: Lowest Detected Concentration for FASP, AP, HD, and REG Preparation Protocols in PF, Cookie, and Dark Chocolate Samples in ppm PF

Peptide and Matrix		FASP	AP	HD	REG
NLPQQ[C]GLR	**PF**	50	1	10	10
	C	50	50	10	50
	DC	50	10	10	10
SSNPDIYNPQAGSLR	**PF**	10	1	10	10
	C	50	10	10	10
	DC	10	10	10	10
TANDLNLLILR	**PF**	10	1	10	10
	C	50	10	10	10
	DC	10	10	10	10
NNPFYFPSR	**PF**	50	1	10	10
	C	50	10	50	50
	DC	10	10	10	10
TANELNLLILR	**PF**	10	1	10	10
	C	50	10	10	10
	DC	10	10	10	10
SPDIYNPQAGSLK	**PF**	10	1	10	10
	C	50	10	50	50
	DC	10	10	10	10
QQPEENA[C]QFQR	**PF**	50	50	50	50
	C	100	50	50	50
	DC	100	100	50	100
FNLAGNHEQEFLR	**PF**	10	1	10	10
	C	10	1	10	10
	DC	10	10	10	10
RPFYSNAPQEIFIQQGR	**PF**	10	1	1	1
	C	10	1	1	1
	DC	10	1	1	1

ix. Method Optimization: Addressing Method Sensitivity

The scaled-up sample preparation procedure was completed with the intention of improving method sensitivity to achieve the desired method performance. The approximate factor that the concentration of peptide was increased between the previous protocol and the scaled-up protocol was 9.4. Results indicated a large increase in method sensitivity, however, the fold changes observed for some peptides were unexpectedly greater than 9.4. Fold changes were calculated by taking the light-to-heavy ratio as determined for the scaled-up protocol and dividing it by the light-to-heavy ratio determined in the previous experiment. The fold changes calculated based on the 50 ppm PF in matrix sample concentration, for each target peptide and sample type are shown in Table 3.9.

Table 3.9: Fold Changes Between Scaled Up and Previous Sample Preparation Protocol Based on 50 ppm PF in Matrix Sample

Peptide	**PF**	**C**	**DC**
NLPQQ[C]GLR	6.63	3.86	11.54
SSNPDIYNPQAGSLR	4.85	6.32	5.13
TANDLNLLILR	8.19	9.15	8.38
NNPFYFPSR	5.95	7.11	7.58
TANELNLLILR	9.68	9.77	9.59
SPDIYNPQAGSLK	4.96	5.05	5.53
QQPEENA[C]QFQR	6.23	4.79	nd*
FNLAGNHEQEFLR	17.30	17.97	19.50
RPFYSNAPQEIFIQQGR	15.23	18.98	25.17

*not detected

The fold changes for a majority (seven) of the target peptides were within the expected range for the scaled up protocol. However, two peptides (FNL and RPF)

recorded unexpectedly high fold changes, greater than the theoretical 9.4x increase in peptide concentration of the sample. This suggested some impact of their physiochemical properties on their detection by MS. These peptides had been demonstrated to be variable in previous experiments, perhaps due to their hydrophobic characteristics. However, the unexpected fold change indicated a greater recovery than expected which points to resuspension or behavior of these peptides in the instrument. One possible explanation is that a greater solvation of these two peptides at resuspension compared to the other seven peptides occurred, perhaps due to buffer capacity. Another possibility was the effect of the chemical nature of these peptides at high concentrations either in LC or MS/MS. One peptide, QQP, was not detected in the dark chocolate sample at 50 ppm PF in matrix which suggested the peptide is not as sensitive as the other eight peptides and may not achieve the desired method performance in dark chocolate. The lowest levels of PF in matrix which were detected in this experiment are shown in Table 3.10.

Table 3.10: Lowest Levels of Detection for Each Peptide and Sample Type Using the Scaled Up Protocol to Address Method Sensitivity in ppm PF

Peptide	**PF**	**C**	**DC**
NLPQQ[C]GLR	10	10	1
SSNPDIYNPQAGSLR	10	0.5	1
TANDLNLLILR	0.5	10	0.5
NNPFYFPSR	0.5	10	10
TANELNLLILR	0.5	10	0.5
SPDIYNPQAGSLK	1	10	0.5
QQPEENA[C]QFQR	10	10	50
FNLAGNHEQEFLR	1	1	1
RPFYSNAPQEIFIQQGR	1	1	1

x. Method Optimization: Calibrant Extraction

In the previous experiment with the scaled up sample preparation protocol, large %CVs were observed between PF extracts. The extraction %CV for PF samples ranged from 42.98-68.94%. As the PF samples will be used to create a calibration curve for the quantification strategy of this method, further optimization of the PF calibrant extraction was pursued. Greater extraction concentrations for PF in buffer were analyzed based on the hypothesis that some of the extraction variability observed was due to issues in accurate weighing of the PF material or the small sample size of the PF itself. After the various extraction concentrations, the resuspension buffer volume was proportionally changed to result in the same theoretical peptide concentrations prior to injection for MS.

Though four different extraction concentrations were evaluated (0.060 g PF/30 mL buffer, 0.120 g PF/30 mL buffer, 0.150 g PF/30 mL buffer, and 0.300 g PF/30 mL buffer), there was no observable trend for the %CV of the light-to-heavy ratio for each of

these different sample types (data not shown). No extraction concentration appeared better than another with respect to the %CV between extract replicates. Based on the principle that a higher weighing volume should, in theory, reduce weighing inaccuracies and gather a large sample size of the PF material, the 0.150 g PF/30 mL buffer was selected for the extraction concentration for the PF calibrant samples. It is important to consider that only two extract replicates were taken to evaluate each of these extraction concentrations. More extraction replicates may have provided a better understanding of the %CV between extractions at various concentrations.

xi. Quantitative Method Evaluation

The quantitative method, which had been developed and optimized throughout the previously described work, was evaluated for its efficacy in analyzing incurred cookie and incurred dark chocolate matrices. The purpose of evaluating the quantitative method was to determine its ability to detect and accurately quantify peanut protein at low levels in processed food matrices. Prior to this analysis, the method had been challenged only on post-digestion dilutions of the incurred matrices at high peanut concentrations (10,000 ppm PF). This evaluation investigated the method against matrices that had been incurred with PF at 2.38 ppm in cookie, 11.91 ppm in cookie, 4 ppm in dark chocolate, and 40 ppm in dark chocolate. With respect to peanut protein, the cookie samples were 1.24 and 6.21 ppm peanut because the PF used to incur this matrix was 52.1% protein (as determined experimentally by the Dumas method). The manufacture of the dark chocolate was designed in ppm peanut protein and therefore these samples were 2 and 20 ppm peanut protein in matrix.

Overall, the method achieved the needed method performance with respect to sensitivity and detection limits to ensure the safety of food products for peanut-allergic consumers. This is because the method was able to robustly detect peanut-incurred matrices, even at very low levels of peanut contamination (1.24 ppm peanut protein in cookie and 2 ppm peanut protein in dark chocolate). Eight of the nine target peptides detected these low concentrations of peanut protein in 100% of samples analyzed (*N=18* for each matrix). The QQP peptide detected the 1.24 ppm peanut protein cookie in 100% of analyzed samples (*N=18*), but only detected the 2 ppm peanut protein dark chocolate in 77.78% of replicates (*N=14*). Representative calibration curves are shown from the second experiment and both days of analyses for three peptides (Figure 3.12).

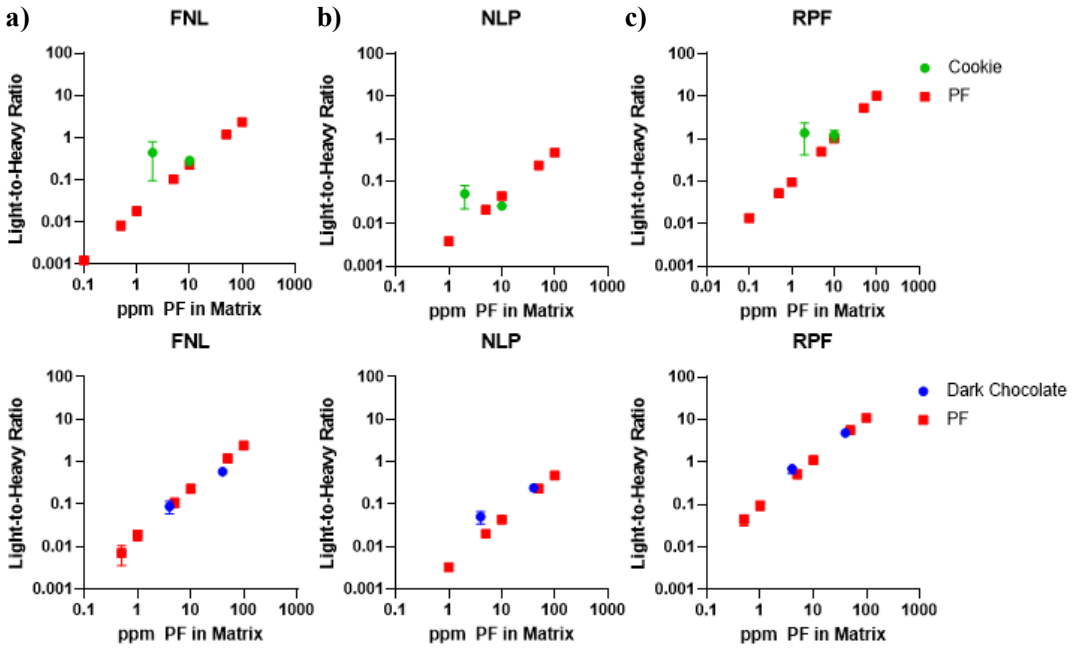

Figure 3.12: Calibration Curves for Incurred Cookie and Incurred Dark Chocolate Samples for Quantitative Method Evaluation.
Representative calibration curves for analyzed incurred cookie and incurred dark chocolate shown for three peptides. Peptides shown include **a)** FNL (Ara h 3), **b)** NLP (Ara h 2), and **c)** RPF (Ara h 3). Quantification of these peptides continued with the outlined quantification strategy for this method to determine peanut concentration in ppm peanut protein and the reported value shown is the peptide average within one experimental day. *N=9, Mean ± SD.*

As shown in Figure 3.12, the light-to-heavy ratio of the analyzed incurred matrices samples were relatively linear in their response compared to the same-day PF calibration curve. It can be visually observed that the incurred matrices, both cookie and dark chocolate, have greater variability in the lower incurred levels than with the samples of greater peanut concentrations. Further, the lower levels appeared to have light-to-heavy ratios greater than the PF calibrant in the cookie matrix and to some degree for the NLP peptide dark chocolate matrix. However, overall, the robustness of this method was demonstrated through the effective detection of peanut protein at low levels in incurred matrices and in the relatively accurate ability of the PF calibrant to quantify the presence of peanut.

The quantification of peanut protein in the analyzed matrices was completed based on the light-to-heavy signal ratio and the interpolation of the ratio to the PF calibration curve, as generated by Skyline. A summary of the three quantitative reported values for the method is shown in Figure 3.3. The results for the average peptide reported value for measured peanut protein in the analyzed incurred cookie matrix is shown in Table 3.11.

Table 3.11: Quantification, Variation, and Recovery of Peanut in Two Incurred Cookie Levels in ppm peanut protein

	1.24 ppm peanut protein Cookie			**6.21 ppm peanut protein Cookie**		
	Average ppm peanut protein *N=18*	%CV	% Recovery	Average ppm peanut protein *N=18*	%CV	% Recovery
FNL	5.67*	121.03%	456.81%	7.01	37.89%	112.95%
NLP	3.54	86.04%	285.40%	2.63	20.59%	42.43%
NNP	3.36	108.55%	270.62%	3.94	37.34%	63.51%
QQP	4.50	105.76%	362.39%	5.80	50.66%	93.38%
RPF	4.07	108.77%	327.79%	5.48	35.00%	88.20%
SPD	3.85	122.53%	310.34%	3.09	34.57%	49.77%
SSN	5.19	116.81%	418.47%	7.32*	41.11%	117.88%
TAND	3.90	116.64%	314.62%	3.59	37.11%	57.81%
TANE	3.71	119.93%	298.96%	3.11	37.64%	50.15%

* maximum peptide average value for each analyzed matrix concentration

The method detected peanut with all nine target peptides and at both concentrations of peanut contamination, even at the level which surpasses the needed method performance outlined for this method (2 ppm peanut protein in cookie). The third reported value, the maximum extract average for any peptide, was 20.60 ppm peanut protein in the 1.24 ppm peanut protein cookie (FNL) and 12.80 for the 6.21 ppm peanut protein cookie (SSN). The maximum reporting peptides for all of these samples were either SSN or FNL, both Ara h 3 peptides. This exceptional recovery of Ara h 3 peptides

was congruent with both untargeted MS results and with targeted MS results during target filtration. The maximum reported peanut protein concentration is considered based on a food safety perspective. By assuming the maximum reported concentration to be the contamination level, any resulting risk assessment and risk management decisions will be as conservative as possible and will increase food safety for allergic consumers.

These results indicated great variability of the method, as observed by the high %CVs shown. Particularly for the 1.24 ppm peanut protein sample, the method seems to be over-recovering and quantifying a concentration much higher than the known concentration. With an in depth view of the data, there are particular extraction replicates which appear to be contributing greatly to the high average ppm peanut protein values observed. This may be due to hot spots of peanut contamination within the cookie matrix itself. Furthermore, this variability and over-recovery or quantification effect was amplified at the lower peanut protein concentration, even reporting maximum contamination values greater than that of the 6.21 ppm peanut protein cookie.

The results for the quantification of peanut protein in the analyzed incurred dark chocolate matrix is shown in Table 3.12.

Table 3.12: Quantification, Variation, and Recovery of Peanut in Two Incurred Dark Chocolate Levels in ppm peanut protein

	2 ppm peanut protein Dark Chocolate			**20 ppm peanut protein Dark Chocolate**		
	Average ppm peanut protein *N=18***	%CV	% Recovery	Average ppm peanut protein *N=18*	%CV	% Recovery
FNL	2.59	51.80%	124.08%	10.83	17.13%	51.95%
NLP	6.00*	37.03%	288.02%	21.49*	25.73%	103.07%
NNP	3.53	57.13%	169.12%	16.03	22.55%	76.91%
QQP	2.73	46.42%	131.17%	9.26	19.48%	44.40%
RPF	4.60	54.52%	220.51%	19.60	19.53%	94.02%
SPD	2.99	47.42%	143.44%	13.52	20.68%	64.83%
SSN	4.37	52.62%	209.51%	17.55	22.14%	84.19%
TAND	3.24	42.59%	155.26%	14.58	21.08%	69.95%
TANE	3.27	44.85%	156.73%	15.14	19.14%	72.62%

* maximum peptide average value for each analyzed matrix concentration
**QQP, *N=14*

The method was able to effectively detect low ppm peanut protein values in a complex incurred dark chocolate matrix. All but one target peptide was detected 18 of 18 times in the 2 ppm peanut protein dark chocolate. The QQP peptide was observed in only 14 of 18 replicates which analyzed the low level of peanut-incurred dark chocolate. The third reported value, the maximum extract average for any peptide, was 9.98 ppm peanut protein in the 2 ppm peanut protein dark chocolate (RPF) and 29.79 for the 20 ppm peanut protein dark chocolate (NLP). Commonly, the highest reporting peptide was NLP (Ara h 2), which was expected to be such in the dark chocolate as this peptide was observed to be highly sensitive throughout method development stages.

As previously mentioned, the variability observed in the quantification of peanut protein in the analyzed incurred matrices was higher than desired for a quantitative food allergen detection method. Variation analysis was completed to quantify several factors

with possible contribution to the observed variation, including method variation, extraction variation, and injection variation. The variation analysis for one representative peptide is recorded in Table 3.13 and the approach to estimating these values is described in the table caption.

Table 3.13: Quantification of the Method, Extraction, and Injection Variation in %CV for TANELNLLILR

TANELNLLILR	**Cookie**		**Dark Chocolate**	
	6.21 ppm peanut protein	**1.24 ppm peanut protein**	**20 ppm peanut protein**	**2 ppm peanut protein**
Method Variation: Overall	37.64%	119.93%	19.14%	44.85%
Method Variation: Within Day (Day 1)	40.57%	16.06%	11.46%	31.23%
Method Variation: Within Day (Day 2)	33.21%	79.40%	9.77%	42.80%
Method Variation: Day to Day	8.95%	71.46%	15.99%	27.15%
Extraction Variation: Within Day (Day 1)	40.48%	14.88%	11.34%	31.20%
Extraction Variation: Within Day (Day 2)	33.06%	79.38%	9.70%	42.75%
Extraction Variation: Day to Day	37.52%	119.91%	19.09%	44.82%
Injection Variation	0.20-4.81%	0.77-9.32%	0.55-2.26%	0.40-4.21%

Method variation = %CV of quantified values from all data points collected for the analyzed concentration and matrix overall, (*N=18*) or within day (*N=9*). Day-to-day method variation equated to the %CV between the average values for each day (*N=2*).
Extraction variation = %CV between three extract averages (*N=3*) per sample, calculated for each day. Day-to-day method variation the %CV between the extraction values of any day (*N=6).*
Injection variation = %CV of quantified values from injection replicates (*N=3*). A range is shown to indicate the minimum and maximum injection variation calculated.

The variation quantified for the TANE peptide is comparable to that of the majority of the method's target peptides. The overall variation is generally acceptable for

the incurred matrices, except for the 1.24 ppm peanut protein, which generally recorded a very high method variation for most peptides. Further, the extraction variation seemed to play a particular role in the variation observed in the quantitative output of the method. Extraction variation within day and between day is somewhat high. Extraction may be a highly variable procedure in sample preparation, as indicated for TANE and for other peptides. This may suggest a need for improved extraction procedures to reduce the variability observed between extracts. Injection variation did not impact the overall variation to a large extent, as the observed injection variations were minute compared to other variation measures. Overall, further optimization of the method, including extraction procedures, may be necessary to decrease the variability observed with the final reported peanut protein concentration.

Overall, evaluation of the quantitative method on the incurred cookie and incurred dark chocolate provided support for the efficacy of the developed MS method. The method appeared to be highly sensitive and robust in its detection of low-level peanut contamination. The method was able to robustly detect and to reasonably quantify levels of peanut contamination that were lower than the needed method performance. In order to protect the most sensitive 1% of peanut-allergic individuals, calculated method sensitivity desired was 2 ppm peanut protein in cookie and 4 ppm peanut protein in dark chocolate. This quantitative MS method achieved detections and quantifications of peanut protein in incurred cookie at 1.24 ppm peanut protein, a level more sensitive than the desired 2 ppm peanut protein level. For the dark chocolate, the MS method was able to detect and quantify an incurred matrix of 2 ppm peanut protein, which is more sensitive than the

desired 4 ppm peanut protein concentration. Though the method is highly sensitive to detect peanut protein in cookie and dark chocolate matrices, the variability of the method indicated a need for further optimization to increase the repeatability and quantitative accuracy.

V. SUMMARY

A quantitative LC-MS/MS method for the detection and quantification of peanut protein in cookie and dark chocolate matrices was developed based on nine target peptides identified as sensitive and robust in these matrices in previous work. Method optimization was completed for several components of the method including extraction, calibrant preparation, chromatography, and MS instrument parameters. Method sensitivity was improved through method optimization procedures, as well as inclusion of an acetone precipitation of extract protein and an increase in protein digested and subsequent peptide loaded on column. The quantification strategy used for this method relied on SIL peptides and signal light-to-heavy ratios, plus an external calibration curve using PF material. The maximum quantification value in ppm peanut protein was considered as the final reported value for the method to maximize food safety.

The developed and optimized MS method achieved the desired method performance with respect to sensitivity. The method robustly detected low levels of peanut contamination in incurred food matrices, including 1.24 ppm peanut protein in cookie and 2 ppm peanut protein in dark chocolate. Based on risk assessment principles, the method sensitivity is sufficient to detect levels of peanut protein which would be relevant for the most sensitive peanut-allergic individuals.

For the incurred cookie matrix, the 1.24 ppm peanut protein level was quantified by target peptides with averaged quantified values ranging from 3.36 - 5.67 ppm peanut protein (270.62 - 456.81% recovery) and for the 6.21 ppm peanut protein level the reported quantification ranged from 2.63 - 7.32 ppm peanut protein (42.43 - 117.81% recovery). For the dark chocolate matrix, the 2 ppm peanut protein level, as quantified by target peptides, averages ranged from 2.59 - 6.00 ppm peanut protein (124.08 - 288.02% recovery). The 20 ppm peanut protein level reported quantification averages that ranged from 9.26 - 21.49 ppm peanut protein (44.40 - 103.07% recovery). Though the method indicated high variability, the developed MS method is highly sensitive and is generally robust in its detection of low levels of peanut protein in incurred cookie and dark chocolate matrices.

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CHAPTER IV: SUMMARY AND FUTURE WORK

I. SUMMARY

Because of the prevalence of peanut allergy and the severity of elicited allergic reactions, the United States (US) requires the labeling of peanut and ingredients derived from peanut on food products. To comply with labeling regulations and to monitor the unintended presence of peanut in food products, the food industry utilizes a number of allergen detection methods, such as immunoassays, to detect peanut protein. However, antibody-based detection methods may struggle to detect or accurately quantify peanut protein in complex or processed food matrices. Mass spectrometry (MS) provides the opportunity for an alternative, orthogonal peanut detection method that does not maintain the same deficits when analyzing food matrices. Thus, a quantitative mass spectrometry method for the detection and quantification of peanut protein in processed food matrices, namely cookie and dark chocolate, was developed.

Peanut-incurred cookie and peanut-incurred dark chocolate matrices were generated at various concentrations of peanut protein for target selection, method development, and method evaluation strategies. Untargeted MS analysis of incurred matrices, blank matrices, and peanut flour (PF) was completed for target selection purposes, followed by the identification and quantification of peanut peptides. Based on criteria established to select peptides which were both abundant and minimally affected by the matrix, a subset of the identified peptides was selected as candidates for inclusion in the final method. Selected candidate target peptides for the cookie and dark chocolate matrices numbered 32 and 67, respectively.

Peptides that were selected as candidate target peptides were subsequently filtered using iterative rounds of targeted MS analysis, specifically parallel reaction monitoring (PRM). PRM was used to analyze post-digestion dilutions of incurred matrices to empirically determine the best performing peptides for each matrix. A number of target peptides were determined to be robust and sensitive in the cookie (six peptides) and in the dark chocolate (seven peptides). The final target peptides were combined, and nine target peptides were identified for the detection and quantification of peanut protein in these matrices. Of the nine target peptides, seven were from Ara h 3, one was from Ara h 1, and one originated from Ara h 2. Four of the nine target peptides were determined as final target peptides in both matrices. The discovery-based target selection approach appeared to be successful in its identification of peptides that are sensitive in their detection and robustly detected in the incurred matrices, on which the untargeted MS was completed.

Using the nine target peptides determined to be sensitive and robust in the analyzed food matrices, a quantitative LC-MS/MS method was developed (PRM). Peptide detection criteria for were formalized and included the following: detection of three of the three pre-established product ions, library dotp value ≥ 0.80, a 5.0 ppm mass error tolerance for the average mass error of measured product ions, and peak found ratio (PFR) equal to one. Detected peptides were quantified using their ratio to a constant amount of stable isotope labeled (SIL) peptides, with an external calibration to PF. A maximum value for the quantification of peanut protein by the nine peptides in the

analyzed sample was reported based on food safety principles and conservative estimates for food allergen risk assessment.

The sensitivity and variability of the quantitative method was optimized through a number of strategies. Method parameters, such as the automatic gain control (AGC) and injection time (IT) were tested to determine optimal settings of 1e6 and 110 ms, respectively. The addition of non-analyte background protein to dilutions of samples prior to injection decreased the variability observed for SIL and analyte peptides for calibrant samples. Chromatography was optimized by decreasing the slope of the acetonitrile (ACN) gradient. This provided the expected increase in analyte separation and improved points across the peak for peptides in the method. An acetone precipitation (AP) of sample extracts was shown to increase the response and decrease the variability of peptide peak areas. The final peptide concentration of samples was increased through scaled up sample preparation, primarily at the digestion stage. This resulted in improved method sensitivity. The variability of the PF calibrant was addressed through optimization of the PF extraction concentration.

The developed and optimized method was evaluated with the peanut-incurred cookie (1.24 and 6.21 ppm peanut protein) and peanut-incurred dark chocolate matrices (2 and 20 ppm peanut protein) to assess the sensitivity, quantitative accuracy, and precision of the method. The method detected peanut protein in the analyzed matrices, even the lowest levels of peanut contamination (1.24 ppm peanut protein in cookie and 2 ppm peanut protein in dark chocolate). This sensitivity surpassed the needed method performance based on the reference dose for peanut and typical consumption of the food

product, which was calculated at 2 ppm peanut protein in the cookie and 4 ppm peanut protein in the dark chocolate.

The method achieved quantification of peanut protein in the incurred food matrices using the nine target peptides. Reported values for the quantification included peptide averages, the maximum of peptide averages, and the maximum extract average across any peptide.

Of all nine peptide averages (*N=18*), the average with the maximum quantified value was reported for each analyzed matrix sample and concentration. The peptide maximums were determined to be as follows: 5.67 ppm peanut protein (1.24 ppm peanut protein cookie), 7.32 ppm peanut protein (6.21 ppm peanut protein cookie), 6.00 ppm peanut protein (2 ppm peanut protein dark chocolate), and 21.49 ppm peanut protein (20 ppm peanut protein dark chocolate).

Next, the maximum for an extract average was included to provide a conservative estimate of the peanut contamination to inform risk assessment and increase food safety for allergic consumers. The maximum for any extract average was reported as follows: 20.60 ppm peanut protein (.24 ppm peanut protein cookie), 12.80 ppm peanut protein (6.21 ppm peanut protein cookie), 9.98 ppm peanut protein (2 ppm peanut protein dark chocolate), and 29.79 ppm peanut protein (20 ppm peanut protein dark chocolate).

It is recommended that the maximum of any extract average be used to inform risk assessment as it provides the most conservative quantification of the peanut contamination in the matrices. When carried through risk assessment principles, this value will provide increased food safety for peanut-allergic consumers.

The method demonstrated considerable variability in the quantified values for the concentration of peanut in the incurred matrices. This is an issue which will need to be addressed in future work. Additionally, the method reported values which are considered over recovery of peanut protein based on the known concentration of peanut protein in the analyzed matrices. Thus, future work regarding the recovery of peanut protein in the incurred food matrices may be needed to improve the quantitative accuracy of the method.

II. FUTURE WORK

The primary aspects of the developed quantitative LC-MS/MS method for the detection of peanut protein in cookie and dark chocolate that need to be addressed in future work include further determination of method recovery, consideration for quantitative variability, evaluation of method extensibility, and transfer of the method to additional MS instrumentation.

One area in which future work regarding this method may be necessary is the additional determinations of the recovery of the method compared to the known concentrations of analyzed samples. First, additional replication of the method in its analysis of the incurred matrices is important to determine more accurately the average or expected method recovery. This is because the over-recoveries observed may be an artifact of the small number of replications completed for this work. A greater number of recovery data points for each matrix and level may confirm whether recovery of this method is consistently higher than expected for such a method. Second, spike and recovery experiments will help to better understand the recovery of the method by

determining the role that the matrix components and the processing of the matrix play in the recovery of peanut protein by the method. To complete this analysis, blank cookie and blank dark chocolate matrices spiked with PF should be analyzed using the method in tandem with the true, incurred matrices (at the same PF concentration as the spiked materials) as generated for this work. Third, comparison against commercially available ELISA kits will provide perspective on the recovery of peanut protein observed for this method. ELISA kits that target different peanut proteins should be included so that the recovery comparison is not biased based on matrix or processing effects. Suggested ELISA kits for this comparison are the Veratox® for peanut allergen (Neogen®) kit, Peanut Protein ELISA Kit II (Morinaga Inc.), and the BioFront MonoTrace Peanut ELISA kit. Comparison will indicate whether there is support for the use of MS over ELISA when evaluating these matrix types for the presence of peanut.

The next step in future work regarding the method would be to address the high variability of the method observed in the quantitative evaluation of incurred matrices. As with method recovery, an increased number of replicates analyzed by this method would allow for a better understanding of the variation measure observed. More replication of samples extracted and digested on the same and different days would provide a greater understanding of the sources of variation in the method. It may also be necessary to monitor the variation across an extended period of time. The reason additional replications may be advisable is because the high variability observed in this work may be due to hotspots of peanut contamination within the generated incurred matrices. A greater number of samples analyzed from the incurred matrices would allow for the

determination of whether the variability is a result of heterogenous contamination of peanut in the matrices. One way to improve the variability may be to target the digestion variability. To complete this, digestion standards can be added to samples prior to digestion and by including target peptides from the standard proteins in the method, normalization of the other peptide responses can be completed to control for digestion variability. Additionally, extraction variability should be addressed as it appears to contribute greatly to method variation.

An additional component of future work would be to analyze the extensibility of the method in its evaluation of other food matrices. One acknowledged limitation of this method is that it was developed specifically for two matrices, cookie and dark chocolate. Therefore, until evaluated, it has uncertain efficacy for analysis of peanut protein in matrices other than cookie and dark chocolate. Thus, to extend the application of the method, it should be tested on other food matrices. Further, other matrices that have the potential to be contaminated by peanut due to allergen cross-contact should be evaluated. These may include pie crusts, muffin, milk chocolate, ice cream, or other complex and processed food matrices. The rationale behind evaluating the extensibility of the developed method is primarily based on the four target peptides (NLPQQCGLR, SSNPDIYNPQAGSLR, RPFYSNAPQEIFIQQGR, TANDLNLLILR) that were determined to be robust and sensitive in both matrices during PRM filtration. There were four target peptides that performed well in two very different matrices, one of which contains a gluten network that is extensively thermally processed, while the other is high in fats and polyphenols and endures multiple thermally processed steps. If four targets

succeed in these dissimilar matrices, perhaps they may perform sensitively and robustly in other matrices. Thus, this potential should be explored. This evaluation will also give insight to the contribution of the effect of the matrix on the recovery of target peptides included in the method.

Additionally, the method could be transferred to other MS instrumentation. As the method was developed using one specific MS instrument (Thermo Scientific™ Q Exactive™ Plus hybrid quadrupole-Orbitrap™ mass spectrometer), transferring the method to another instrument will allow for more expanded application of the method. After transferring the method to an additional instrument, the quantification, recovery, and variation of the method should be determined and compared to the same measures for this method. One reason this work would be important is to evaluate whether instruments with lower resolution can effectively utilize this method to detect and quantify peanut protein in incurred food matrices, as a decrease in resolution may increase the observed matrix interference.

Overall, future work concerning this method should be centered around further determination of both method recovery and variability, followed by strategies to improve these measures. Additionally, the extensibility of the method to evaluate other food matrices should be explored, as well as transferring of the method to other MS instrumentation to increase future application and use of the method in the food industry.