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ASSESSING THE QUANTIFICATION OF SOY PROTEIN IN INCURRED MATRICES USING TARGETED LC-MS/MS

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by

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Soy-derived ingredients are commonly added as auxiliary components to a diverse range of food products. The versatile end-applications of commercially processed soy ingredients is concerning for the safety of allergic consumers. Immunological detection of soy proteins in food matrices has some drawbacks, including loss of epitope binding and matrix masking. Therefore, the aim of this study was to assess naturally incurred matrices with an existing liquid-chromatography tandem mass spectrometry (LC-MS/MS) workflow to determine the recovery of total soy protein.

The existing LC-MS/MS workflow was time intensive. The peptide responses of five soy protein targets were compared between two dilution methods and two trypsin digestion conditions. The tryptic digestion was shortened by 3 hours from the addition of subsequent additions of trypsin at 1:100 (trypsin:protein) to a single addition at 1:50. External standards were prepared with an optimized serial dilution method after reconstitution (AR). This workflow requires less time and reagents. Three peptides, LSA, VFD, and NIL, had reproducible peptide responses among all conditions and thus were chosen as the final quantifying peptides.

Absolute quantification of total soy protein was achieved with a combination of internal and external standards. Several replicates of external standards were prepared for

the optimized serial dilution (AR). The variation of the external standard peptide responses was insignificant among replicate standard curves whether prepared on the same day or different days. Therefore, all replicate curves were able to be pooled into an average, or "master" standard curve. The total soy protein in incurred bread and frankfurter matrices was then estimated from the master curve. Several factors were assessed for the effect on protein recovery: soy ingredient type and concentration, heat treatment, and matrix. The effect of the matrix was determined to have the most appreciable impact on protein recovery. The lowest percent protein recoveries, less than 50%, were calculated for all uncooked matrices. The cooked matrices had percent recoveries between 50-150 % for both matrices. Soy ingredient type was also an issue for texturized vegetable protein (TVP), where total soy protein recoveries were low and variable among replicate extracts. With the LC/MS-MS method, detection of TVP was low but above the limit of detection. Further work is needed on the total soy protein recoveries of these matrices with immunochemical methods.

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CHAPTER 1: LITERATURE REVIEW

I. INTRODUCTION

Soybeans have been cultivated and consumed for thousands of years in a variety of standalone products. More recently, the protein fractions have been concentrated to create several soy-derived ingredients. The end-applications of the protein-rich ingredients vary depending upon the biological (e.g. enzyme activity) and chemical (e.g. emulsification) functionalities. Soy protein is also capable of triggering allergic reactions. The presence of low total protein levels, milligram amounts, is enough to trigger an allergic reaction in the most sensitive individuals. Therefore, accurate detection and quantification of soy proteins is important for the safety of allergic consumers. The presence and concentration of soy protein can be determined by several DNA- and protein-based methods. However, it is challenging to detect soy protein especially after product manufacturing. There is a need for robust allergen detection methods capable of accurate quantitative recovery for highly denatured proteins in complex food matrices. In this review, mass spectrometry (MS) is presented as a protein-based analytical method capable of improving the detecting and quantifying highly processed soy ingredients compared to other commonly used analytical methods.

II. SOYBEANS

A. Raw Commodity

The taxonomical classification of legumes is referred to by several names: Fabaceae, Leguminosae, or pea family. Legumes are characterized as mature seeds surrounded by a protective pod (*1*). The surrounding pods can range in color from black, brown, or tan (*2*). Each pod can contain up to four seeds with seed coat colors ranging from black, brown, green, or yellow (*2*). The pod is commonly removed before

consumption. Fabaceae contains many important food crops including beans, lentils, peas, chickpeas, peanuts, and soybeans (*1*). A common agricultural characteristic of legumes is the ability to fix nitrogen in the soil, with soybeans contributing most of the nitrogen fixed compared to other legumes grown worldwide (*3*). This is due to a symbiotic relationship with the bacteria *Bradyrhizobium japicum*.

Soybeans are classified in the subgenus Soja, within the genus Glycine (*4*). It is unclear how many taxa are classified as part of this subgenus. The United Stated Department of Agriculture (USDA) Natural Resources Conservation Center indicates five and other sources list fewer (*4*). The modern cultivated soybean is descended from a wild-type soybean crop*, Glycine soja*, with taxonomical evidence of the first domestication in China (*5*). The natural genetic variation over the course of hundreds of years has since given rise to domesticated soybeans, *Glycine max*.

Soybeans were first introduced to the western hemisphere in the southern and eastern parts of the United States and Canada. The USDA Natural Resrouces Conservation Center lists the species of soybean that is cultivated as a major food crop worldwide: *Glycine max* (L.) Merr. The U.S. has been a top producer of soybeans worldwide since the 1960s accounting for more than 50 % of the production up until the 1980s (*6*). The USDA Natural Resources Conservation Center lists two cultivars of *Glycine max* (L.) Merr.: Bobwhite and Quail Haven. There are many more soybean cultivars cited elsewhere in the literature without specific taxonomical designations (*7*).

Foreign trade statistics provided by the U.S. Department of Commerce of major oil seed crops estimate soybeans to have the largest production, import, export, consumption, and thus profit, in the world (*8*). The total trade value is estimated at two

billion U.S. dollars (*1*). There has been an overall steady increase in the metric tons of soybean exported from the U.S., as well as soybean protein and oil, from 2011-2018 (*8*). Despite the low levels of sulfur containing amino acids, soybeans have been considered as a quality source of protein compared to animal-based proteins such as eggs and milk (*9, 10*). Heat treatments have shown to increase the digestibility by inactivating anti-nutritional factors, such as lectins and trypsin inhibitor proteins (*11*). Therefore, soybeans have an increasingly high value for domestic and worldwide trade as a quality and inexpensive protein source.

Legume food crops are split into two groups known as pulse and non-pulse crops. The distinction between the two groups is the part of the crop that is harvested. Pulse crops are grown with the intent to harvest only the seed (*12*). Soybeans have been categorized as non-pulse crops because historically they have been grown for the oil fraction. Soybeans are composed of mostly protein (38 %) followed by oil (18 %), soluble carbohydrates (15 %) and fiber (15 %) (*13*). Soybean cultivars can be selected for high oil or high protein contents (*14*). The protein and oil content in the soybean seed have been observed to inversely correlate with each other (*15*). The different concentrations of oil and protein are influenced by agricultural factors (e.g. drought), geographical factors (e.g. region specific climates), and selective breeding (*15, 16*).

The oil fraction is made up of mostly unsaturated 18-carbon chain triglycerides (*17*). A novel application of soybeans is that the oil fraction has been converted to biofuel, with over one billion gallons projected to be produced each year (*18*). Soybean and peanut oil are the only U.S. export commodities projected to not decrease in value as the global competition in the world trade market increases (*18*).

The protein fraction of soybeans is equally as profitable as the oil fraction. In the early 1900s, the US shifted interest from soybean oil production to the harvest of the protein fraction, specifically for animal feed (*19*). Today this shift remains apparent as only a small portion of the total soybean protein harvested is intended for human consumption (*19*). However, the supply of animal-based protein is challenging in a limited environmental capacity and to meet the demands of the increasing growth of the global population. A solution to find more sustainable protein sources may cause a worldwide shift to more frequent plant-based protein consumption, especially for soybeans (*20*).

B. Soy-Derived Functional Ingredients

As a result of hundreds of years of cultivation, soybeans have been used to prepare a variety of foods, and thus, have become entrenched in cultures around the word. The native soybean can be processed in many traditional and novel ways to create a variety of soy-derived products. Fermented soybean foods (sufu, tempeh, miso, natto, soy sauce) are custom dishes in Eastern Asian countries. More recently, soybeans have processed into vegetarian alternatives for dairy (soy-based beverages) and meat (imitation of fibrous texture) products. The rise in demand for vegetarian and vegan alternatives has influenced novel soy-derived foods to be introduced into the market. For example, texturized vegetable protein (TVP) is a fibrous product, similar in texture to animal protein, made possible by high pressure and temperature extrusion processing (*21*). Additionally, proteases can be added to hydrolyze proteins for functionality, hypoallergenicity, or reduction in the bitter, beany taste that is characteristic of beans (*22*).

Soybeans may be consumed due to the highly publicized health benefits. In the U.S., a health claim, approved for packaged food products since 1999, indicated that regular soy consumption results in the lowing of the risk of cardiovascular disease (*23*). Health benefits have been associated with the different bioactive molecules in the soybean protein fraction such as isoflavones, lignans, phytic acid, and sterols (*24*). There is a long list of potential health effects tied to the progressively studied isoflavones including lowering the risk of several types of cancers, obesity, and osteoporosis (*24*). There is ongoing research investigating the health effects of isoflavones as estrogen-mimicking compounds to alleviate menopausal symptoms (*25*).

Soybeans are unique compared to other oil seed crops because of the high oil and protein content. Soybeans have a higher protein content compared to other food legumes, as well as other protein-rich foods, such as eggs, milk, beef, and chicken (*10*). The industrialization of soybean products is in part due to the fact that they contain the highest protein content on a dry basis (35-40%) compared to other food legumes (*1*). Soybean protein is added as a minor ingredient to a range of food products for desirable organoleptic and functional properties.

Soy-derived protein ingredients can be grouped into three general categories based on the final protein concentration: flours (50% protein), concentrates (70% protein), and isolates (90% protein). The protein fraction of the seed is concentrated in a general workflow. The soybean pods and hulls are removed, the seeds are finely ground, and the oil is extracted with a non-polar solvent (e.g. hexane). The resulting defatted soy flakes can be directly marketed as soy flour or heat treated to different degrees and marketed as toasted soy flour. Protein can be concentrated further using alkaline

extractions, isoelectric precipitation, or centrifugation to produce soy protein concentrates and isolates (*26*). A schematic of the processing and application of several types of soy-derived ingredients is shown in Figure 1-1.

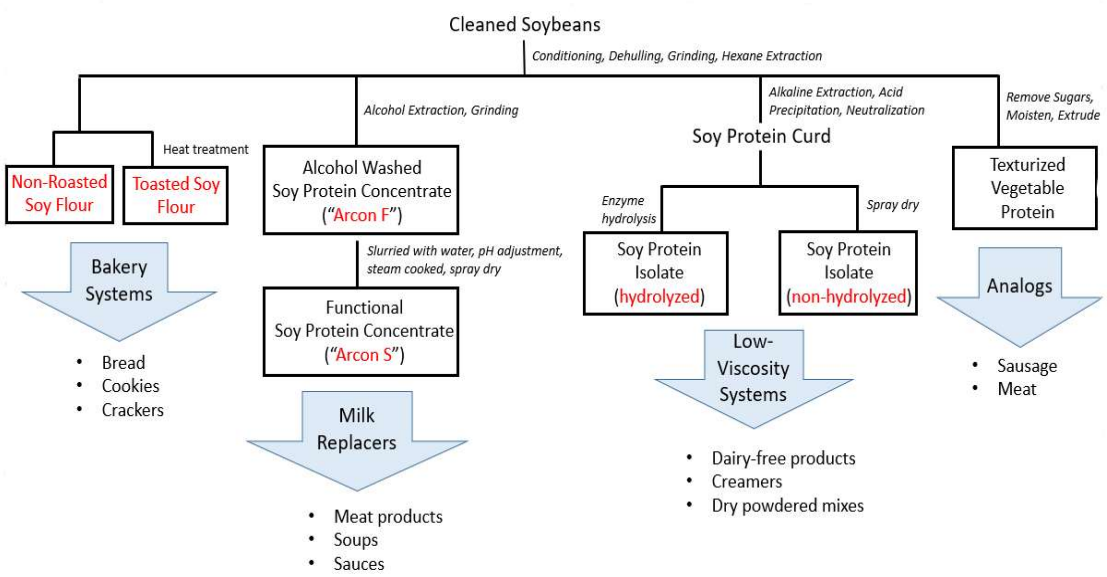

Figure 1-1. General schematic of selected commercially-processed soy ingredients: flours, concentrates, isolates and textured. Red font indicates the different sub-types of ingredients. Figure adapted from product information obtained from Archer Daniel Midland Company (ADM) (*27, 28*).

The biological and chemical properties of the proteins can be manipulated to achieve different functionalities in a wide range of food systems (*29*). Products containing soy-derived ingredients include bakery items (bread, cookies), sauces, soups, meats, and desserts (*30*). Minimally processed soy ingredients, such as non-roasted soy flour, can be used for its biological properties. For example, NRSF contains the enzyme type 2- lipoxygenase, and under acidic conditions, can bleach carotenoids in wheat flour improving the white color of the product (*31*). Chemical properties of other soy ingredients, such as isolates or concentrates, include emulsification, water and oil binding, foaming capacities, gelation, and viscosity (*32*).

The widespread use of soybeans is attributed to numerous factors including low agricultural cost, high nutritive value, perceived health benefits from short and long-term consumption, and protein functionalities for a wide range of food products. However, an increased consumption of the oil seed crop is not necessarily devoid of any consequences. Soybeans are recognized as a major food allergen worldwide. As the use of soy-derived protein ingredients increases, the quality of life for soy allergic individuals is decreased without reliable food allergen quantification methods.

III. SOY PROTEIN

There are several forms of nomenclature for soy proteins based on the physical, taxonomical, and immunological characteristics. In early research of soy protein characterization, molecular weight was a physical characteristic used to resolve a heterogeneous mixture of biological molecules in a sample (*32*). Analytical methods can measure proteins based on the molecular weight of macromolecules, such as SDS-PAGE, size exclusion chromatography, and mass spectrometry (MS). Ultracentrifugation is a method used to characterize proteins by the rate of sedimentation. This is related to the size of the molecule and can be expressed in sedimentation coefficients (Svedberg units) (*33, 34*). In early soybean protein characterization, four major fractions were identified and measured at 15S, 11S, 7S, and 2S which constitute approximately 5-11%, 31-52%, 35-37% and 8-22% of the total protein respectively (*29, 35*). The heterogeneity in the expression of specific proteins are based on the cultivar (*36*). Therefore, the total percentages of proteins discussed here are an approximation. A major limitation in molecular weight-based identification is the generalization of protein groups with actual fundamentally different biological, structural, and immunoreactivity characteristics.

There are even differences between proteins coded from the same gene. These protein variants are referred to as isoforms or "protein species" and differ based on the types of post-transitional modifications made after translation of the protein from mRNA (*37*). Clearly, categorizing proteins based solely on the sedimentation coefficients is not very informative to understanding the immunochemical mechanisms of allergens and does not account for other important biological determinants.

The biological functions of these proteins can be generally categorized as metabolic, structural, or storage proteins (*38*). Seed storage proteins are estimated to make up over 70% of the total protein fraction in soybeans (*39*). More recently they have been described as nutrient reservoir proteins (InterPro O22120). The most abundant proteins within this fraction are known as β-conglycinin and glycinin from the 7S and 11S protein fractions, respectively. These proteins serve as a nitrogen source for developing seed embryos (*40*). The nitrogen source becomes available to the seeding after germination when the proteins are hydrolyzed into peptides and amino acids (*41*). Seed storage proteins are common targets for food allergen detection methods because of their high abundance and allergenicity.

Analysis of proteins at the genetic level is based on evolutionary and structural similarities. This may be more informative for answering questions associated with the degree of allergenicity between protein groups (*42*). Proteins can be grouped into families and superfamilies based on the percentage of amino acid sequence similarity and biological function (*43*). The major soy proteins have been classified into several groups including cupins (β-conglycinin and glycinin), prolamins (2S albumin), and pathogenesis-related proteins (Kunitz-type protease inhibitor). Additionally, soybean

proteins have genetic variability between the cultivated type (*Glycine max*) and the wild type (Glycine soya) that may give rise to proteins with different biological functionalities (*44*).

The Allergen Nomenclature Sub-committee formed jointly by the World Health Organization (WHO) and the International Union of Immunological Societies (IUIS) created a naming system for allergenic proteins. These names are based on the taxonomic classification (e.g. *Glycine max*) followed by a successive number based on the time of identification as an allergen (e.g. Gly m 1). According to IUIS, there are currently allergen designations for eight soy proteins (Gly m 1-8) and there are 17 variants of these proteins (e.g. Gly m 1.0101). Proteins subunits from β-conglycinin have been identified as major allergens: Gly m Bd 60K, Gly m Bd 30K, and Gly m Bd 28K (*45, 46*). Gly m Bd 60K is part of the alpha subunit and is considered as the major allergenic protein from β-conglycinin (*47*).

Glycinin, β-conglycinin, and protease inhibitors are considered the major allergenic soy proteins (*48*). There are several online databases housing thousands of other protein accessions for allergens including Allergome and AllergenOnline (*49, 50*). According to Croote et al., β-conglycinin, glycinin, and 2S albumin have publicly available MS data (*51*). Therefore, classification based on the seed storage protein nomenclature will be used in this review.

A. Glycinin

The major protein in the 11S fraction and the most abundant seed storage protein in soybean is known as glycinin (*52*). It is classified as a legumin protein and lacks post-transitional modifications that are present in β-conglycinin (e.g. glycoslylation). Glycinin

is made up of several protein subunits. Each subunit consists of an acidic and basic polypeptide linked by disulfide bonds. The subunits can be classified as G1, G2, G3, G4, and G5 with an overall sequence similarity of 42-45 % (*44*). As the primary sequence of the protein subunits was further studied, the subunits were grouped based on the percent of sequence similarity. Group 1 consists of subunits A1aB1b, A1bB2, A2B1a and Group II consists of subunits A3B4, A5A4B3 with sequence similarity of each group between 82-86% (*44*).

The hexameric protein consists of different combinations of these five possible protein subunits. In the quaternary structure, the subunits converge in a barrel-like formation. Three subunits (consisting of a basic and acidic polypeptide) form a disk-like shape. Two of the disks interact non-covalently with one another to form a stacked structure (*53*).

B. β-Conglycinin

The major protein in the 7S fraction is β-conglycinin, a type of glycoprotein also known as a vicilin. The quaternary structure is composed of three proteins, or subunits, that form a disk-like shape (*43*). The protein subunits are classified as alpha, alpha prime, and beta with molecular weights of 68 kDa, 72 kDa, and 52 kDa respectively (*39*). The different combinations of subunits form a trimeric protein that can range in size from 150-200 kDa (*54*). The primary sequence lacks sulfur containing amino acids and thus disulfide bonds do not stabilize the secondary structure. The subunits are more susceptible to denaturation by heat and pH changes compared to glycinin and protease inhibitors (*48*). The beta subunit is classified as a bicupin because of the two conserved regions of β-sheet folding at the core of the tertiary structure. The beta subunit has two

known isoforms as designated by Gly m 5.0301 and Gly m 5.0302. The core regions of the amino acid sequences share sequence similarity. The highest similarity between alpha and alpha' (86.8 %), followed by alpha and beta (75.5 %), and alpha' and beta (71.4 %) (*54*).

IV. FOOD SENSITIVITIES

Antigens are any substance determined to be foreign by the immune system. Common examples of antigens are microorganisms including fungi, helminths, bacteria, and viruses. Some antigens have evolved to combat the immune system with sophisticated methods (e.g. HIV) to deceive cells. The evolution of foreign invaders has pushed the evolution of human immune systems. An evolutionary race between antigenic substances and immune cells, to some extent, contributes to an immune system composed of a complex network of cells and biological chemical signals that work together to recognize and eliminate antigens. The types of cells and the mechanisms with which the immune system responds typically fall into two branches, referred to as innate and adaptive immunity.

Innate immunity is the initial barrier of protection of the immune system. This branch of the immune system relies on the commonalities of antigens, such as pattern recognition receptors on bacteria, to recognize foreign invaders (*55*). The types of cells involved, broadly categorized as granulocytes, are basophils, eosinophils and neutrophils (*56*). The collective cell response is the same for a range of antigens. Adaptive immunity is a more complex system of cells that actively recognize a reoccurring or persistent foreign invader. The cells involved in adaptive immunity are the B and T lymphocytes which recognize foreign protein expressed on antigen presenting cells (APCs) (*57*). This

branch of immunity becomes more specific and efficient after an initial exposure to the antigen. In other words, these memory cells accumulate over the course of an infection and mount a stronger immune response. The longer the system is fighting the foreign invader the more tailored the immune response becomes as mutations on the surface protein receptors of B and T lymphocytes increase specificity through V(D)J rearrangement (*58*). Immune cells specific to an antigen can remain present in the immune system for up to several years, serving as a source of memory in the event of reinfection. While this is effective at mounting an immune response to a harmful substance, the immune system may mount a response against substances usually considered harmless such as food.

An immune response actively mounted against a substance that is generally considered nonthreatening is a hypersensitivity reaction. The overstimulation of the immune system can be categorized into four types of hypersensitivity reactions (*59*). Type 1 reactions are mediated by IgE antibodies and is the immune mechanism for food allergy reactions. This type of reaction is triggered by the crosslinking of IgE antibodies linked to surface receptors (FceR) on mast cells and basophil cells from the binding of a foreign protein. Symptoms are caused by the release of potent mediator molecules (e.g. histamine) from an immune cell biological cascade, also known as degranulation. The mediator compounds are biological chemicals that are vasoactive, capable of increasing blood flow to the infection site and acting as a recruitment signal to initiate other cells to congregate and respond. Type II is an IgG and IgM response to complement, a type of protein which activates immune cells, associated with autoimmune diseases. Type III is an inflammatory response caused by deposited antigen-antibody complexes. Type IV is

an immune response generated from potent mediator molecules (i.e. cytokines) released by sensitized T cells. Although there are categories of hypersensitivity reactions the reaction is more likely part of a spectrum. The type of reaction depends upon many factors including the symptoms, including severity, time of manifestation (immediate and delayed hypersensitivity) and the cell types mediating the immune reaction (*60*).

Hypersensitivity reactions triggered by food are known collectively as food sensitivities (*61*). Food sensitivities are broadly categorized as food intolerances and food allergies. The distinguishing factor is the type of response, either caused by non-immunological or immunological mechanisms respectively (*62*). The type of food allergy can be defined by the type of immunological mechanisms: cell-mediated and IgE-mediated reactions (*61*). A common example of a cell-mediated food allergy is celiac disease, where long-term exposure to gluten causes inflammation in the digestive tract. Food allergies, such as to peanut, are IgE-mediated and require total avoidance of the offending food (*61*).

A. Food Intolerance

A food intolerance is a reproducible, non-immunological adverse reaction after the ingestion of a food or food component (*63*). It is the most common type of adverse reaction to a food and limited amounts of the offending food can be tolerated (*62*). An idiosyncratic response is an adverse response with an unknown mechanism (e.g. sulfite sensitivity). Metabolic food disorders can affect the cells responsible for the metabolism of a food component, usually decreasing the nutritive value. This incomplete digestion can cause mild effects, such as discomfort, or more severe and life-long complications, such as malnutrition.

One of the most common metabolic disorders is lactose intolerance, which affects the breakdown of milk sugar into monosaccharides. Lactose is a natural disaccharide found in milk composed of glucose and galactose. The monosaccharides are linked by a β-1,4-glycosidic linkage. Lactose intolerance occurs when an individual has an overall decreased activity of the enzyme β-galactosidase (lactase) to effectively break down the sugar into the constituent monosaccharides (*64*). Inadequate absorption of the disaccharide results in fermentation by bacteria in the gut into lactic acid and other by-products such as carbon dioxide, methane and hydrogen gas. This imbalance results in uncomfortable symptoms such as flatulence or diarrhea (*64*). Genetic predisposition, relative consumption of milk products, and age influence the decrease in activity of lactase among different populations. Therefore, some ethnic groups are more susceptible than others (*65*).

B. Non-IgE Mediated

Cell-mediated reactions to foods are also known as a delayed hypersensitivity reactions. A common example is celiac disease, which affects 0.5-1% of the total population (*66*). This is a chronic condition where the ingestion of gluten will trigger inflammation in the gastrointestinal tract, specifically in the small intestine, damaging the villi over time. Gluten-containing cereals including wheat, rye, and barley must be avoided. The adverse digestive symptoms, such as diarrhea, are a consequence of incomplete absorption in the small intestine.

Early introduction of soybeans or cow's milk can result in a non-IgE mediated reaction known as food protein-induced enterocolitis syndrome (FPIES). Infants experience vomiting, diarrhea, and dehydration after the consumption of soy- or milk-

based formulas (*67*). The gastrointestinal symptoms can be resolved from removing soy or milk from the diet, and the condition can be outgrown into adulthood in a few months to years (*68*).

C. IgE-mediated

Food allergy can be defined as reactions that are IgE-mediated, cell-mediated (non-IgE-mediated), or exhibit both types of mechanisms (mixture of IgE- and non-IgE mediated) (*69*). A food allergy is a reoccurring immunological response after the exposure of a certain food substance (*63*). A "true" food allergy is an IgE-mediated immune response triggered after the ingestion of an allergenic food and can also be an exercise-induced response (*61*). Theoretically, all food proteins have the potential to trigger an allergenic response, and over 160 different allergenic foods have been identified (*70*). The range of symptoms as defined by the anatomical systems of the body affected include: respiratory (shortness of breath), gastrointestinal (vomiting, diarrhea), cutaneous (hives) and the cardiovascular system (weak pulse).

A fatal reaction, known as anaphylactic shock, is general or systemic. Anaphylaxis (Greek for against protection) was first defined jointly by Paul Portier and Charles Richet by observing an increase in the severity of reaction in dogs (*71*). Epinephrine can be administered, through hand-held devices called epinephrine autoinjectors, in an emergency situation for temporary alleviation during a severe reaction. The diagnosis of anaphylaxis differs among medical professionals and affects the reporting and response to patients with symptoms (*72*). The Joint Task Force of the American Academy of Allergy, Asthma and Immunology and the American College of

Allergy, Asthma, and Immunology have defined anaphylaxis as the life-threatening and unanticipated symptoms of an IgE-mediated allergic reaction (*73*).

IgE antibodies are part of adaptive immunity and play a role in cell memory. Cells involved as part of the adaptive immune system are able to increase specificity to a specific antigen and mount an immune response over several exposures. The same mechanism is observed in IgE-mediated immune responses to food allergens. The exposure to an allergenic food protein is known as sensitization (*57*). In this stage, the food protein stimulates cells of the adaptive immune system, B and T lymphocytes, to start the production of IgE antibodies (*57*). IgE antibodies will then be present on the surface of mast cells and basophil cells (*74*). Epitopes are regions on a protein, or antigen, which bind to the specialized light chain on the antibody (*75, 76*). More specifically, an allergenic epitope is a protein region with the ability to initiate an allergic reaction (*77*).

A subsequent exposure to an allergen is known as elicitation. In this phase, IgE antibodies reactive to specific allergenic proteins are present on the surface of mast cells and basophil cells (*57*). Binding of the protein must occur on at least two antibodies. This cross-linking activates the immune cells to initiate a biological cascade releasing mediator molecules (histamine, prostaglandins, tryptase, and inflammatory cytokines) (*78*). These mediator molecules have been historically associated with the fight against large parasites such as helminths (*57*). Therefore, these are potent biological molecules evolutionarily intended to kill large microorganisms, too large for phagocytosis. This release of chemicals initiates more intense immune responses including vascular permeability, smooth muscle contraction, and stimulation of nerve endings (*78*).

Soybean protein has been well-documented to cause IgE-mediated hypersensitivity reactions in sensitive individuals (*79, 80*). To date, there is no cure for food allergy and the individual is usually advised to adhere to a complete avoidance diet to one or multiple allergenic foods. The first case of documented soy allergy occurred in the 1930s in a soy flour factory (*81*). The workers were exposed to respiratory allergens later identified as Gly m 1 and Gly m 2 (*82*). Allergic individuals can be sensitive to a specific soy protein or several (*28*). The allergenicity may be a product of certain characteristics such as abundance, resistance to harsh environmental conditions (pH, temperature, enzymes), and structural characteristics (*21*). Soy protein in processed food has been documented as the cause of fatal anaphylaxis reactions such as in hamburger, sausage on pizza, crab sticks, and kebabs (*83, 84*).

There is evidence that small amounts of allergenic protein can be tolerated by allergic individuals (*85*). Long-term oral immunotherapy trials under professional care attempt to desensitize allergic patients by gradually exposing them to larger doses of allergens (*86*). Quantitative risk assessment methods estimate levels of allergenic protein that will likely not cause adverse reactions for 95-99% of the allergenic population (*87*). Data from double-blind placebo-controlled food challenges, which were first proposed in 1976, continue to the gold standard to diagnose a food hypersensitivity (*62, 88*).

In spite of the research progress, individuals must continue to adhere to a strict diet, free of any traces of the offending food. There is currently no cure for food allergies, and due to the potentially fatal reactions from exposure of small amounts of the offending food, complete avoidance continues to be the primary treatment of allergic individuals.

This makes the reliability, sensitivity, and reproducibility of allergen detection methods important to maintain consumer safety.

V. ALLERGEN REGULATION

Food allergies are a worldwide health concern, and the prevalence seems to be increasing (*80*). The prevalence of food allergies is 2-2.5% worldwide, with a higher prevalence in children ranging from 5-8% (*74*). Soybeans are one of the most common food allergies and require labeling on foods worldwide (*89*). Soy is known to have a higher eliciting dose compared to other food allergies (such as peanut) and children diagnosed with soy allergy commonly outgrow the allergy (*90*). Soy allergy prevalence is estimated to below 1% in the total population and 1.4-2.7 % among children (*28, 77*).

The WHO and the Food and Agriculture Organization (FAO) jointly created guidelines outlining a set of voluntary food standards for the labeling of food allergens (*91*). The work is intended to be applied internationally. The guideline encompasses the labeling of foods associated with causing food sensitivity reactions including food intolerances and IgE-mediated food allergies. However, these are only guidelines and legal jurisdiction lies with the national regulatory bodies because food allergen prevalence differs internationally. The different consumption patterns of different populations may be a factor in the different proportion of allergy prevalence to specific foods. The increased consumption of a food within a population is correlated with an increased prevalence of that specific food allergy. Although countries have different rates of food allergies, the source of variation may be a result of the differences of the study designs which report the prevalence rather than true differences among the populations (*92*).

In the United States, the Food, Drug and Cosmetic Act of 1906 was pivotal in the establishment of government oversight in the production of consumer goods with the intent of consumer protection. The Nutrition Labeling and Education Act (NLEA) of 1990 was an extension of the Food, Drug, and Cosmetic Act and required for the first time a nutrition facts panel to be presented on packaged foods in the US (*93*). The required information on the nutrition facts panel includes information on total fat, saturated fat, cholesterol, sodium, total carbohydrates, complex carbohydrates, sugars, total protein, and dietary fiber as well as certain vitamins and minerals (*93*). The scope of NLEA extends beyond the nutrition information and regulates health claims to avoid misleading consumers. Since this law was enacted, US national regulatory bodies continue to modify and add on requirements to food labels in order to provide consumers with the most complete and accurate information.

In 2004, the Food Allergen Labeling and Consumer Protection Act (FALCPA) was the first law enacted that required the labeling of allergenic foods to be included as part of the ingredient labeling (*93*). Additionally, the scope includes not only packaged food but also dietary supplements, infant formula, and medical foods (*93*). In the U.S., the allergenic foods required on the label were at least in part determined from the majority, 90%, of all allergic reactions to foods in the population (*93*). The group of allergenic foods is collectively known as the “Big Eight” and is as follows: crustacean shellfish, tree nuts, fish, wheat, milk, eggs, peanuts, and soybeans. Some of these allergenic foods are actually a diverse collection of species specifically tree nuts, finfish, and crustacean shellfish. Common names within an allergenic food group can be declared (e.g. bass, flounder, or cod). Any nationally mandated allergenic food or ingredients

derived from these foods must listed as part of the ingredients statement, including the common name (e.g. milk following sodium caseinate), or in a separate "contains" statement. As of 2019, the Illinois General Assembly passed legislation requiring the labeling sesame seeds on packaged foods (*94*), but this requirement is not recognized at the national level.

It is well known and has been previously discussed that the protein fraction of foods causes allergic reactions. Therefore, when other portions of the food are used, such as the oil fraction, the allergenic potential is uncertain. In the case of soybeans, the oil fraction is a valuable commodity. Vegetable oils can be mechanically separated, in equipment such as an expeller-press, or chemically extracted, using a non-polar solvent (*95*). The oil fraction will contain some amount of protein and will be higher in the mechanically separated oil (unrefined oils) (*96*). Highly refined oils have many more processing steps that remove water soluble substances such as proteins and carbohydrates. The process includes a series of thermal and chemical treatments such as degumming, neutralization, bleaching, and deodorization to improve organoleptic properties and shelf stability (*97*). The low protein content of highly refined oils has sparked research in the allergic potential of these allergen-derived ingredients (*98*). One study demonstrated the safe ingestion of all soybean oil products by oral food challenges of three types of soybean oils, partially hydrogenated, non-hydrogenated, and cold-pressed soybean oil, to patients with a history of allergic reactions to soy (*99*). Other studies have found similar results for highly refined peanut oils (*100*). FALCPA does not require labeling of highly refined oils derived from the major allergens or any ingredients derived from these oils (*93*).

Some allergen-derived ingredients can be exempt from the labeling requirements if there is substantial evidence of low allergenic risk. Ingredients derived from allergenic foods can become exempt through a petition process which requires substantial scientific evidence of the safety of the ingredient. There are two methods which a company can take to exempt an ingredient from labeling by submitting a petition or notification. A petition provides scientific evidence to support the hypothesis that it is not a risk to sensitive individuals. The notification provides scientific evidence of the absence of allergenic protein in the product. To date, there have been only two approvals for soy-derived products, both of which were for soy lecithin, when used in specific applications. Lecithin is part of the oil fraction, chemically defined as a phospholipid, and can contain low amounts of protein (*95*). In 2007, the Solae company was approved to use soy lecithin as a "processing aid". Many functions were listed including release agent, anti-stick, and preventions of oil absorption. In 2015, Archer Daniels Midland Company (ADM) was approved to use soy lecithin as a "releasing agent". One of the major functions of the processing aid listed was as a release agent, preventing products from sticking to equipment or clumping.

Around the world, a mandated list of allergenic foods is required to be explicitly stated on packaged food labels. These allergens are determined by the appropriate regulatory bodies. The Big Eight are a group of regulated allergenic foods in the USA, Canada, Australia, New Zealand, and the European Union (*89*). Buckwheat has been identified as a major allergen in Japan and South Korea (*101*). Europe recognizes additional allergenic foods which list mustard, celery, lupine, sesame seeds, sulfites, and

gluten-containing cereals as major allergens. This list was expanded to included mollusks and lupine (*102*).

Allergic consumers rely on the accuracy of food labels to avoid foods containing allergenic ingredients. Unintentional allergens in a packed food product are considered misbranded and can be subjected to a mandatory food recall under Section 403 of the FD&C Act. The current legislation is not based on estimated threshold levels of food allergens and is therefore considered a zero-tolerance policy (*103*). However, proving a level of zero allergenic foods would be impossible and certainly would not be measurable. This remains a challenge in a globalized food industry where the sharing of equipment at each step in the processing system can increase the risk of cross-contamination (*95*). A zero level of allergens in large facilities is difficult to control because of cross contact between product lines and rework (*104*). Additionally, fatal accidents can happen outside of packaged food products such as in restaurants, schools, travel, or large gatherings (*105, 106*). An allergic individual must manage the avoidance diet in all social settings, restaurants, schools, and planes, which leads to increased anxiety (*77*). Food products with allergenic foods added as substitutions for more expensive ingredients may be the biggest risk for consumers. The intentional addition of allergenic sources of protein, such as soybeans, may happen if it is being substituted as a cheaper ingredient in a product. For example, soybeans may be used to adulterate animal-based products and are thus excluded from the label (*107*).

A study done on the accuracy of food labels, specifically false or misleading claims, on packaged foods found 21-28% of the products (domestic and imported respectively) to be outside of the FDA regulations (*108*). Among these foods, one of the

most common sources of the violation was undeclared ingredients (*108*). Interestingly, this same problem applies to the labeling compliance of food allergens. Allergenic foods found in mislabeled products are classified as undeclared allergens and is the primary cause of food recalls in the US (*109*).

The consumer, business, and regulatory risks associated with undeclared allergens have influenced the increased use of precautionary allergen labeling (PAL). These are labels with statements such as “may contain”, “processed in the same facility as”, or “processed on the same equipment as”. However, this often confuses the consumer leading to more risky behavior and higher anxiety thus lowering the quality of life for these individuals (*110*). A risk assessment tool known as Voluntary Incidental Trace Allergen Labeling (VITAL) was developed to decrease the use of PAL statements. VITAL includes guidelines on the quantitative risk of unintended food allergens. VITAL uses double-blind placebo control food challenge threshold clinical data to predict finite levels of allergic protein tolerated by a specific portion of the allergic population (*111, 112*). The overall goal is to minimize the use of PAL statements if the unintended allergen is below this threshold.

VI. ALLERGEN DETECTION METHODS

The cross-contact among different food products makes for a unique challenge in the detection of low amounts of allergenic protein. The current methods of detection differ primarily based on the target analytes: protein or DNA. Method accuracy depends upon the composition and processing of the sample food matrix and verification with incurred reference materials (*113*). In general, sensitivity, specificity, and ease of use are among the most important requirements for an allergen assay (*114*). There is a need for

accurate and robust methods capable of detecting and quantifying residual amounts of allergenic protein in complex food matrices. The general types of quantification methods discussed here are DNA-based and protein-based.

A. DNA-Based Methods

Polymerase chain reaction (PCR) is a detection method that amplifies a target region of DNA to identify a certain food or group of foods. Target sequences may be from nuclear, mitochondrial, and chloroplast sections. The amplification response is measured from fluorescent nucleotide tags which produces a sigmoidal curve: background, exponential, linear, plateau (*115*). The standard curve is the linear portion of that response in DNA derived from a calibrant material. Absolute quantification of sequence biomarkers in real-time PCR is determined by the linear amplification range and efficacy is assessed with the R^2 value and slope (*115*).

PCR methods for soybeans in incurred food matrices have reported LOD ranging from 0.16-10 ppm (mg soy/kg matrix) (*116*). However, this is not a widely applied method in the food industry because PCR does not directly measure the allergenic component of foods. Commercially processed soy ingredients are concentrated from the protein fraction of the native bean and contain lower amounts of DNA (*113*). DNA may be damaged from the food processing environment, such as elevated in heat or change in pH. The total DNA extraction of several soy-derived ingredients found the total amount of DNA was highest in soy flours, the ingredient which was the most similar to the native soybean (*117*). The most processed soy-derived ingredients, soy protein isolate and toasted soy flour, had comparably lower total DNA levels, and soy fiber had no

measurable DNA (*117*). Therefore, protein-based methods may be more appropriate for the detection of soybeans and soy-derived ingredients.

B. Protein-Based Methods

Enzyme-Linked Immunosorbent Assays

Immunochemical detection methods are the most widely applied methods for allergen analysis in the food industry (*118*). Enzyme-linked immunosorbent assay (ELSIA) is a protein analysis method that relies on the affinity between antibodies and specific antigens to measure concentrations of analytes in a sample. There are several protocols of ELISA assays differing in the steps at which the antibodies and the substrate are combined such as indirect, direct competitive, antibody-sandwich, double antibody-sandwich, direct cellular, and indirect cellular (*119*). The most popularized and recognizable formats of ELISA include direct, indirect, sandwich, competitive with labeled antibody, and competitive with labeled antigen (*120*). The sandwich ELISA format is the most widely used for commercial food allergen analysis.

Commercialized ELISAs are sandwich assay kits with many advantages such as rapid analysis, minimal training, and large sample size capabilities. The workflow has similar steps for different commercialized kits and the protocol steps are well known. Sandwich ELISA starts with an antibody-coated microwell plate. The antibody is of class A (from chicken eggs) or G (from sera of animals such as rabbits or from mouse monoclonal antibody cultures). The antibody coated and absorbed onto the wells is known as the capture antibody. A capture antibody is the first to bind to the target antigen. After the antibody-antigen complex is formed, a second antibody is added and binds to the same antigen. This antibody is the primary antibody. This primary antibody

is complexed with an enzyme. The absorbance from the enzymatic reaction occurring after the addition of a substrate is measured at a certain wavelength. The concentration of protein, or concentration to a reference material (e.g. NRSF), in unknown samples is then interpolated from a standard curve. The more intense the color, the higher the concentration of analytes. The intensity of the resulting color follows a similar pattern to DNA-based methods with a background, exponential, linear, and plateau regions. Commercial kits are commonly coupled with proprietary analysis software that converts the spectrophotometer units to a final protein concentration. Conversion factors are sometimes provided to calculate the concentration of ingredients (e.g. soy protein isolate) depending upon the reference material used in the standard curve. Although commercialized ELISAs have many advantages, the lack of standardization among different kits due to differing sample preparation, reporting results (total protein vs specific proteins), and reference proteins is a major limitation to comparing results (*121*).

Another immunoassay that has been popularized due to its portable nature and fast results is lateral flow devices (LFDs), commonly known as dipsticks. LFDs are a semi-quantitative method composed of antibodies immobilized on handheld devices with similar advantages to commercial ELISA kits such as fast results and easy handling (*89*). These can be easily be transferred to food manufacturing settings and current work has investigated the qualitative results for presence or absence of allergens in processed food products and especially on equipment surfaces after cleaning (*122-124*). ELISA formats have also been adapted to detect DNA components, knowns as enzyme-linked oligonucleotide assay (ELOSA), and methods have been developed for lupine, gluten, peanut, and egg (*123*).

ELISA faces a challenge known as cross-reactivity. Cross-reactivity is the shared affinity of an allergenic epitope between two or more homologous proteins. Soybeans are part of the legume family among other commonly consumed food crops such as peanut, lima beans, pea, garbanzo bean, and green bean all which may have homologous protein regions. In the development of an ELISA method, foods containing no known traces of the target analytes are tested for a positive response to determine cross-reactivity. An ELISA format using β-conglycinin as the target protein showed cross-reactivity (false positive) results for chickpeas in a sandwich ELISA, and for several tree nuts (walnut, Brazil nut, pecan nut), chickpea, chicken, and cocoa in a competitive ELISA (*125*). Chickpea was also shown to be cross-reactive for two commercial ELISA kits: Tepnel Biosystems kit and ELISA Systems kit (*126*). The drawbacks for soy ELISA kits are food matrix interferences and cross-reactivity.

As mentioned previously, ELISA depends on the affinity between an antibody and a protein. The loss of the native integrity of the allergenic epitopes, linear or conformational, will decrease the accuracy of the ELISA assay. The potential loss of native epitopes on proteins will cause two major issues with ELISA results in weakened immune-recognition. The protein may be subjected to many forms of food processing before it interacts with the antibody. Food products can be subjected to a variety of processing techniques: thermal (e.g. moist and dry), hydrolysis (e.g. fermentation), pressure, and physical (e.g. mashing) treatments, which all cause some degree of denaturation (*127*). The constituents within a food undergo different chemical reactions such as browning reactions (Maillard reaction, non-enzymatic) that can change the chemical composition of the proteins. The decrease in immunoreactivity of fermented soy

products has been shown with tempeh, miso, and yogurt (*128*). The potential loss of epitopes from the hydrolysis of soy proteins into peptides decreased the immunoreactivity 77-89% when compared to unfermented soybean meal (*128*). Processing changes the structure of proteins, leading to aggregation and protein-protein interactions (*129*). The extraction of these processed proteins may be lowered and decreases the proteins that are available for analysis.

The robustness of a method depends upon the effectiveness of the target protein extraction. This may be especially limiting for the detection of residual amounts of protein in more complex food matrices. The protein recovery of Gly m Bd 28 K was determined to be significant in highly processed products (soy protein isolate and soy milk) but undetectable in others (sausage and hamburger) (*130*). An ELISA method developed with monoclonal antibodies raised against Gly m Bd 30k showed lower recovery of the protein in high fat foods such as sausages because of the interaction between the protein and oil bodies (*131*). Protein extracted from several soy products, including sausage, sweet potato cake, and tomato sauce, at 10 ppm total soluble soy protein showed recoveries from 89.7-98.7% (*131*).

Preliminary experiments, as part of an undergraduate research project, compared the extraction of six different soy ingredients in reducing and non-reducing buffers (unpublished). The soy ingredients extracted were non-roasted soy flour, toasted soy flour, hydrolyzed and non-hydrolyzed soy protein isolate, and two types of soy protein concentrate (Arcon S and Arcon F). The effectiveness of the extraction of soluble proteins was dependent upon the temperature (60 and 100 C) and time (10, 15, and 20 minutes) of extraction. Extracted soy protein was quantified with two commercial soy

ELISA kits: Veratox Soy Allergen ELISA (range of quantification 2.5-25ppm) and the r-Biopharm RIDASCREEN ® FAST Soya ELISA (range of quantification 2.5-20 ppm soy protein). The protein recovery was higher in buffers with reducing agents compared to buffers without, as shown in Table 1-1.

Table 1-1. Percent total protein recovery of six soy -derived ingredient extracts as measured by two commercial soy ELISA kits.

Soy-Derived Ingredient	Initial Total Protein Concentration (Dumas)	Extracts in 0.01 M PBS with 0.1 M Sodium Sulfite & 1% SDS[A]		Extracts in 0.01 M PBS[B]	
		60 °C for 20 min	100 °C for 10 min	60 °C for 15 min	100 °C for 10 min
Non-Roasted Soy Flour	52	31	110	160	4.5
Toasted Soy Flour	51	158	129	2	5
Hydrolyzed SPI	90	15	9	0.5	0.5
Non-Hydrolyzed SPI	90	172	146	80	7.5
SPC (Arcon F)	66	141	104	40	5
SPC (Arcon S)	70	93	106	12	2

[A]Extracts measured by r-Biopharm RIDASCREEN® FAST Soya ELISA
[B]Extracts measured by Neogen Veratox® Soy Allergen ELISA

The Veratox ELISA failed to recognize the highly processed ingredients such as toasted soy flour and hydrolyzed soy protein. The toasted soy flour had the same protein concentration as the non-roasted soy flour, demonstrating the potential risk of "hidden" soy proteins which are often subjected to some form of processing. Both ELISA kits had low percent recoveries of the hydrolyzed protein products. This is likely from the native epitopes of the protein being destroyed during processing as mentioned previously. Additionally, sandwich ELISAs require the recognition of two protein epitopes, which

lowers in highly processed foods. The low recovery of the direct extraction of highly processed soy indicates equal or less recovery of these ingredients in highly complex food matrices containing only a residual amount of protein that has undergone some form of processing.

Mass Spectrometry

The sequencing of entire genomes has been extensively studied in a field known as genomics (*132, 133*). Over many years, entire genome sequences from single-celled organisms to more complex life forms, such as soybeans have become available (*134*). Following was a natural progression to determine the expression of an entire set of proteins within an organism, defined as proteomics (*135*). However, compared to genomics, proteomics has many unanswered research questions because the number of proteins expressed is not equal to the number of genes they are encoded from. Proteins expressed in an organism can change depending on the environment. The dynamic nature of proteins expressed at a given time in an organism presents a unique challenge. Mass spectrometry (MS)-based methods have been used as proteomic tools for characterizing and measuring large sets of proteins.

MS is a chemical technique that does not rely on the interaction between antibodies and proteins. More specifically, it is the analysis of the relationship between mass and energy. The transition of molecules from a ground state, the lowest state of energy, to an excited state requires energy. MS first ionizes analytes then filters the product ions into a series, or spectrum, depending upon the charge and mass. The relative abundance of these ions is dependent upon the chemical structure of an analyte, sample preparation (e.g. digestion) and the sample purity (*136*). The mass spectra can be

searched against a sequence database to identify peptides that are present (*136*). This is a form of identification, like a fingerprint. MS-based methods rely on the specificity of both parent and product ion spectra for the detection and identification of target analytes, such as proteins.

MS was not a tool used to study large biological molecules up until the 1990s because of the conventional ionization techniques (*137*). Proteins are nonvolatile and thermally unstable and were unable to be ionized into gaseous particle (*138*). The widely used ionization techniques, referred to as "hard ionization", introduce energy high enough to fragment the peptide bonds, rendering spectra from a peptide or protein less useful for identification purposes. Novel ionization techniques, such as matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI), were developed and successfully applied as "soft" ionization for intact proteins and even intact microorganisms (*139*). Soft ionization techniques allowed new research opportunities for MS-based analysis of large biological analytes.

MS instruments all consist of three main components: an ionizer, a mass analyzer, and a detector. Conventionally, analytes were first introduced into the ionizer as gaseous particles. Soft ionization techniques can introduce samples in liquid or solid forms. Electrospray ionization (ESI) is a common type of ionizer for protein analysis. ESI has many advantages, such as a sensitivity (because of nanoliter flow rates), ability to produce multiple charges on a single molecule, and online-coupling to a liquid chromatography system that allows a continuous ionization flow (*140*). Peptides are first introduced in a liquid form as an aerosol stream. As the droplets travel to the inlet of the mass analyzer, desolvation can take place in either a vacuum or in a flow of inert gas

(*141*). A voltage is then applied resulting in free ions (*141*). Another common ionization source for the analysis of large biological molecules is matrix-assisted laser desorption/ionization (MALDI). In this workflow, the sample is ionized from a solid, crystallized form. Metal ions can be added to the solid matrix and ionized simultaneously with the protein (*137*). Energy is introduced to small sections of the sample by using a laser, commonly ultraviolet light, that excites molecules and produces gaseous, ionized peptides (*142*). MALDI typically produces low-charged (often singly-charged) species, expressed as $[M + H]^+$, and is often more useful for studying proteins rather than peptides (*143*).

The mass analyzer filters volatilized, ionized particles in time and space based on the mass to charge (m/z) ratios. The types of mass analyzers have varying resolutions, mass accuracy, sensitivity, and scan rates and are applied depending on the research setting (*144*). Common types of mass analyzers are time-of-flight (TOF), quadrupole (Q), Orbitrap, linear ion trap, (LIT) and Fourier-transform ion cyclotron resonance (FT-ICR). Orbitrap mass analyzers, especially the high resolution and accurate mass (HR/AM) measurements, have been applied to quantitative and qualitative workflows (*145*). Mass analyzers differ based on the separation of ions in space such as the type of energy applied (magnetic, electrostatic) and the measurement (continuous or pulsed) (*146*). A single analyzer, or a hybrid of two or more types of analyzers, can be used. The use of two mass analyzers in succession is referred to as tandem MS, or written shorthand as MS/MS.

Lastly, the detector measures the relative abundances of the m/z ratios of the ionized fragments. As fragment ions are scanned, the signal intensity is proportional to

the rate at which ion species are detected. Electron capture detectors measure ion abundance by the rate at which ions are sensed by the detector. The ions are sensed by an amplification of electrons, creating an electrical current that is translated into an ion spectrum (*147*).

There are two general workflows for protein-based MS methods. A common MS approach, known as "bottom up", infers the presence or concentration of proteins by measuring peptide (*148*). The other approach, known as "top down", measures intact protein masses directly, both with and without fragmentation, and can generate information on the primary structure (*149*). The most common route for allergen quantification has been through the bottom up method (*150*).

In a bottom-up workflow, MS-based detection of peptides does not rely on the structural integrity of the protein. Therefore, MS methods are able to have harsher extraction techniques (use of detergents, chaotropic, and reducing agents) compared to ELISA. Peptide fragments are fractionated by methods such as gel electrophoresis or liquid chromatography (*151*). Techniques to enrich the protein in a sample prior to fractionation have improved the detection of low abundant proteins (*152*). Enzymatic or chemical protein digestion then generates peptides. In-gel digestion was the pioneering method that has since been used less frequently in place of fractionation using liquid chromatography and direct digestion of sample peptides in solution. MS directly coupled with liquid chromatography systems (LC-MS) has high sensitivity for the detection of minute amounts of target peptides (*153*). This is especially advantageous for target peptides with low abundance in complex food matrices. Liquid chromatographic

separation of peptides followed by a tandem fragmentation of peptides and fragments of peptides is referred in shorthand notation as LC-MS/MS.

The sensitivity of MS-based methods may be compromised at later sample preparation steps when the protein in the sample may be at low ppm (µg/mL) concentrations. Loss of sample protein by nonspecific binding of peptides to equipment is expected, especially for hydrophobic peptides on plastic surfaces (*154*). To minimize this effect an alternative source of protein is added to the sample preparation to preferentially bind to the surroundings and minimize sample loss. This protein is known as a carrier protein. Currently, there are no recommendations for the concentration of carrier protein to add to a sample for the greatest sample recovery (*155*).

MS is a technique with high-throughput and multiplexing analytic capabilities for monitoring and characterizing thousands of proteins (*144*). It is a crucial tool to study an entire set of proteins expressed in an organism, known as the proteome, and in the study of food proteins, known as 'foodomics'. Technological advancements, growth of protein sequence databases (Uniprot, BLAST) and allergen databases (AllergenOnline) and public availability of analysis software (e.g. Skyline) have all aided in the increased application of MS-based methods for clinical, pharmaceutical, and food-processing settings.

Proteins are identified by the unique spectrum of fragment ions that is based on the intensities and m/z ratios. In targeted MS, peptide transitions are pre-selected, and the mass-to-charge ratio profile of these peptides can be determined through a combination of in-silico and experimental observations. Once a list of the most robust target peptides has been selected, the transitions of the fragments are monitored. The ability to monitor

more than one protein in a single run is one major advantage of MS-based methods compared to immunological methods. Selected reaction monitoring (SRM) (sometimes known as Multiple Reaction Monitoring (MRM)) pre-selects specific fragments of a peptide to monitor. Monitoring only selected fragments can increase speed and limit the noise in the peptide response because of the narrow parameters used to isolate the fragment. Parallel reaction monitoring (PRM) measures all fragments of a peptide within the experimental parameters. These fragments are measured in tandem so the intensities of multiple fragments can be monitored. Therefore, the fragment ions are monitored either by prior selection of specific transitions (SRM) or measuring all peptide fragments in tandem with fragment selection occurring during data processing (PRM).

SRM has been the gold standard for proteomic workflows for food allergens due to the high sensitivity and selectivity (*150*). SRM has been the method of choice for protein analysis, and only recently have PRM methods gained popularity because of the equivalent performance of detection of peptides compared to SRM methods. PRM can have selectivity, dynamic range, and sensitivity advantages compared to SRM methods (*145, 156, 157*). A series of studies measuring transitions with SRM compared to PRM has shown equal and better sensitivity and selectivity of fragment ions with PRM, especially on high resolution accurate mass (HR/AM) spectrometers (*138, 142, 143*). PRM acquisition on a HR/AM Orbitrap instrument had similar quantitative parameters (dynamic range, linearity, and precision) when compared to SRM on a QqQ instrument (*158*). PRM was also shown to provide more selectivity for targeted proteins in a complex matrix background (*144*).

VII. QUANTITATIVE METHODS FOR FOOD ALLERGENS

MS-based methods in the context of food, known as foodomics, aims to improve consumer health (*159*). MS has emerged as a method for the identification, characterization, and detection of food allergens (*160*). As mentioned previously, immunoassays are the primary analysis method of food allergens. However, MS has some advantages compared to antibody-based method including sensitivity, pre-fractionation prior to peptide detection to improve detection of proteins at low abundance, and a direct measure of the peptide of interest whereas ELISA indirectly measures the response through a chemical reaction (i.e. an enzyme and a substrate).

MS-based methods are time intensive for sample preparation and data analysis as well as require expensive equipment. This hurdle must be overcome in order to apply this method for routine allergen analysis. For analysis, one of the disadvantages of MS proteomics, as mentioned previously, is the imbalance of the number of genes to proteins is due to different protein isoforms (*37*). Protein modifications vary in vivo, such as glycosylation, phosphorylation, acetylation, and in vitro chemical modifications are possible during the sample preparation such as carbamidomethylation (*161*). These modifications will change the physical and chemical properties of the protein. This can affect the detection of a target protein because a change in mass due to these modifications will affect the m/z. In contrast, this can be an advantage if there are shared sequences among the same protein species (e.g. Gly m 5 isoforms). Therefore, the detection of a single peptide indicates the presence of all the protein variants.

Quantification methodology of MS-based methods measure signal intensities of sample and standard peptides. Isotopically-labeled peptides are the gold standard for

quantification methods. Heavy peptides are identical to the target peptide except for atoms with extra neutrons compared to the most abundant found in nature (e.g. carbon-12 and carbon-13). Heavy peptides are synthetically made by tagging or incorporating isotopic atoms (e.g. ^{15}N, ^{13}C, ^{18}O) or isotopic amino acids (e.g. (^{15}N)methionine) (*162*). The isotopic atoms are incorporated into proteins using several methods: metabolic stable-isotope labeling (isotopically enriched or depleted cell media), isotope tagging (reagents containing isotopes), and stable-isotope incorporation (^{18}O incorporated after proteolysis) (*144*). The difference in mass results in unique isotopic distribution of spectra, and the ratio between the intensities of the two species can be measured. The use of an internal standard or heavy-labeled peptide can be used to compare the response of protein abundance in samples (*163*). Other methods of quantification omit the use of heavy peptides. Label-free quantification relies on the relative abundance of peptides between samples that are under different conditions (*164*).

Absolute quantification (AQUA) of sample peptides can be determined with isotopically labeled peptides (*165*). The ratio of the response of sample ("light") peptides to the isotopic ("heavy") peptides can be plotted as a linear curve. An external calibration curve can be plotted using a series of known protein concentrations and the ratio of the target peptide to a heavy-isotopically labeled internal standard. The linear response of target soy peptides have been used to estimate the recovery of soy protein in multiple incurred matrices (chocolate, cookie, ham, etc.) (*166*).

Linear dynamic range is one of the most important concepts in developing a quantitative method. The linearity of a calibration curve may be affected at higher concentrations as the ion detector reaches its saturation limit. The response of the mass

spectrometer is expected to be linear. The variance of the peptide response may be more variable at higher or lower concentrations. In one study researchers found that variability in higher peptide intensities was correlated with higher variances (*167*). There seems to be evidence of a correlation between concentration and variance. Linearity may not always be clear between two methods. A correlation coefficient, or R^2, is often reported in data to demonstrate the viability of a standard curve. Although R^2 measures the correlation of data points, it does not measure the agreement of the data to the linear regression. This may make residuals more useful when determining the behavior of linearly correlated data (*168*).

MS methods for the detection of soy protein have been gaining traction. There is a larger gap between the methods for the detection of soy protein and the quantification of soy protein. Quantitative LC-MS/MS methods have been developed for soybeans as part of multiplexing experiments. Several soy protein peptide markers have been detected in incurred food matrices including meat, cookies, bread, and chocolate (*169*). Additionally, the detection of extensively processed proteins, such as hydrolyzed proteins for hypoallergenic or functional products, likely have destroyed epitopes that are unrecognizable by antibodies. MS methods may offer better detection and quantification of these highly processed ingredients that still have allergenic potential. However, the detection of target peptides in routine analysis of soy remains a challenge due to variation of the concentration of proteins across processed soy ingredients (*36*).

VIII. SUMMARY

Food allergy is a life-long immunological response managed in daily life by a complete avoidance diet. These strict diets require accurate and complete food labels to

prevent any accidental exposure to allergenic proteins. The current legislation reflects this zero-tolerance threshold. However, proving zero is neither more protective nor more practical because sensitive individuals have finite threshold levels. The presence of undeclared allergens in packaged food products is more easily determined compared to the risk associated with the quantity present.

Food processing affects the extraction and immunoreactivity of target proteins. The methodology of commercially available immunological methods depends on antibody-antigen binding. The epitopes of these proteins may be destroyed during food processing but remain allergenic. MS-based methods do not rely on the integrity of proteins and have shown to be capable of detecting highly processed food ingredients in incurred matrices. These detection methods have comparable sensitivity to ELISA with multiplexing abilities. The quantification of processed ingredients in relevant food products using MS-based methods still needs to be determined.

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CHAPTER 2

STANDARD CURVE OPTIMIZATION AND SELECTION OF ROBUST QUANTIFYING PEPTIDES FOR AN EXISTING TARGETED LC-MS/MS METHOD

I. ABSTRACT

Soybeans are an important oilseed crop. The protein fraction can be concentrated into different functional ingredients. These ingredients are commonly added as auxiliary components to a diverse range of food products. The potential for the cross-contract between soybeans and other various food products is concerning because of the widespread use of these protein-rich ingredients. This presents a unique analytical challenge that requires a robust quantification method capable of detecting processed soy proteins in complex food matrices.

External standards can be used as part of a quantification workflow for food allergens using mass spectrometry (MS). An existing targeted LC-MS/MS method developed by Chen et. al (2019) was modified to shorten the total sample preparation time. Internal standards were spiked into samples to normalize the peptide response. The ratio of the external to internal standards was used as the measured peptide response of each dilution method. Optimal digestion time and trypsin concentration were modified for shorter sample preparation steps. Two dilution methods, after extraction (AE) and after reconstitution (AR), were compared to optimize the external standard preparation. The peptide response was measured using linear regression analysis (slope, R^2), variance, and linearity.

The AR method was determined to be the more robust method. AR external standards had linearity from 1-400 mg/kg total soy protein and similar peptide responses between different digestion conditions and background protein concentrations. The quantifying peptides with later elution in the RP-HPLC chromatography gradient were determined to have greater variability in peptide response than earlier eluting peptides. The greater variability of the heavy peptide standards for the hydrophobic peptides suggests the hydrophilic peptides are more suitable for quantification. Future work will focus on quantifying soy protein using the AR method and hydrophilic peptides.

II. INTRODUCTION

Soybeans are one of the most common causes of food allergies in the United States. The prevalence of soy allergy in North America is estimated at 0.4% of children and 0.3% of adults (*1*). Allergic consumers rely on accurate food labels for complete avoidance of the offending food. Research suggests a measurable amount of protein can be tolerated by allergic individuals (*2*). Therefore, quantifying the possible presence of soy protein in a range of food products is important for allergen risk assessment.

Immunoassays have been the most widely used technique to detect and quantify soy proteins for quality control purposes. Enzyme-linked immunosorbent assays (ELISAs) have many analytical advantages such as the ease of use, fast results, sensitivity, and sampling capacity (*3*). However, the challenge with ELISA is the reliance on the integrity of antibodies and proteins (*4*). This limits sample preparation steps as well as the detection of highly processed target proteins. DNA-based methods, namely polymerase chain reaction (PCR), are less commonly used because these assays target DNA, rather than protein. Similar to immunoassays, food processing can damage DNA and weaken detection (*5*). The major drawback of both methods is detecting processed target analytes.

Mass spectrometry (MS)-based methods have emerged as a method for food allergen analysis. Although MS methods do not rely on the integrity of the protein, process-induced modification can hinder detection and quantification. However, comparatively MS may more effective for the detection of food proteins after extensive processing (*6*). Sensitivity is increased by coupling to liquid chromatography systems, fractionating the sample before analysis (*7*). Selectivity can be increased with parallel

reaction monitoring (PRM) workflow because many proteins can be monitored at the same time (*8*).

Quantification methods may use external standards, internal standards, or a combination of both. Internal standards in targeted MS workflows are identical to the sample targets except with the addition of isotopically-labeled atoms ("heavy" peptides) and are considered the gold standard for absolute quantification (*9, 10*). The sample peptide responses are then normalized to the heavy peptide responses to control for instrumental variation.

The major drawback to MS-based methods is the intensive time needed for the sample preparation and data analysis. ELISA is more easily applied to food processing settings because of the availability of commercialized kits and the minimal training required. There remains a need for more adaptable MS methods intended for food industry use. Therefore, this work intends to optimize a sample preparation workflow for targeted LC-MS/MS of soy protein and apply the workflow for robust quantification.

The method optimized is a quantitative targeted LC-MS/MS developed for the absolute quantification of soy protein (*11*). The method was developed to be used to quantify soy protein in a wide range of complex food matrices. Sample preparation time can be significantly shortened by optimizing the dilution of the standard curve. The first objective of this work is to compare the existing dilution of external standards to a streamlined method. The most robust standard curve preparation method was determined by the sensitivity, linear dynamic range, and variance. The second objective of this work was to determine the most robust quantifying peptides to be used in future quantitative

studies. In future work, the most robust peptides will be used to quantify soy protein in model food matrices.

III. Materials and Method

A. Materials

Urea was purchased from Bio-Rad. Trizma base, iodoacetamide (IAA), and ammonium bicarbonate were purchased from Sigma. Dithiothreitol (DTT) and polyvinylpolypyrrolidone (PVP) were purchased from Acros Organics. Trypsin was purchased from Promega. The protein assay used was the Pierce 660 nm protein assay (cat # 2260). The albumin standard (for the protein assay) and trifluoroacetic acid (TFA) were purchased from Thermo Scientific. Formic acid, acetonitrile, and MS-grade water were purchased from Fisher Scientific. External standards were diluted using MiniSorp polyethylene tubes purchased from Thermo Scientific. Centrifugal filters for filter-aided sample preparation were Amicon 10 kDa filters purchased from Millipore. Reconstituted samples were transferred to polypropylene LC vials for MS analysis. Non-fat dry milk was purchased from a local grocery store in Lincoln, NE. The non-roasted soy flour (NRSF) was kindly provided by Archer Daniel Midlands Company.

B. Targeted LC-MS/MS Workflow

Sample preparation for parallel reaction monitoring (PRM) liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis was followed as previously described in Chen et al, 2019. Briefly, 1 g of each sample was extracted in 20 mL buffer containing 50 mM TRIS-HCl (pH 8.6), 20 mM DTT, 6 M urea, and 0.5 % NFDM. A final concentration of 1.0% PVPP (w/v) was added directly added to bind to polyphenols

present in the sample. After centrifugation, the protein concentration of extracts was estimated with the 660 nm assay (Pierce 660 Protein Assay, Thermo Scientific). Bovine serum albumin (BSA) was used as the reference standard. Samples were then reduced with 100 mM DTT (starting concentration) and alkylated with 100 mM IAA (starting concentration) for 20 min in the dark. Sequencing Grade Modified Trypsin was added on a weight basis for overnight digestion at a final concentration of 1:50 (µg trypsin to µg protein) (Promega, cat # V5111). Protein was enriched using filter-aided sample preparation (FASP). Samples were digested on Amicon 10 kDa centrifugal filters (Millipore, cat # UFC501096) and centrifuged at 14,000 g for 15 min prior to desalting with Pierce C18 spin columns (max 30 µg protein) (Thermo Scientific, # 89870). Samples were freeze dried at -80 °C overnight then stored at -20 °C until further analysis.

A total of five absolute quantification (AQUA) peptides were synthetically made and obtained from Thermo Scientific (Table 2-1). The isotope modifications were on the C-terminal lysine ($^{13}C_6{}^{15}N_2$) and C-terminal arginine ($^{13}C_6{}^{15}N_4$). These are referred to as internal standards. All heavy peptides were mixed in equal concentrations to prepare a 5 fmol/µL solution. All samples were reconstituted in 0.1% formic acid (FA) and 3% ACN then spiked 1:1 (v/v) with the heavy peptide mixture. All samples were injected at a volume of 5 µL and eluted over a 60-minute gradient. The first 5 minutes of the chromatography gradient was 98% (solvent A) (0.1% formic acid in water), peptides were then eluted over a 20-40 % gradient (solvent B) (0.1% formic acid in acetonitrile) for 30 minutes followed by a methanol wash for 10 minutes, and a final wash with 98 % (solvent A) for 10 minutes. Data was collected in targeted mode on a Q Exactive™ Plus Orbitrap™ mass spectrometer. Precursors were monitored with a full MS1 scan from

400-2000 m/z at a resolution of 17,500, ACG target of 3×10^6, and maximum IT of 50 ms. In tandem, pre-selected fragment ions were monitored with PRM scans at a resolution of 140,000, ACG at 2×10^5, automatic max IT, and with an isolation window at 1.4 m/z with 0.4 offset. Raw data files were then imported in Skyline (version 20.1) for further analysis (*12*).

Table 2-1. Target soy peptides.

Quantifying Peptide	Protein Accession No.	Mass to Charge (m/z)[1]	Positive Charge State (z)	Retention Time Window (min)	
				Start	End
LITLAIPVNKPGR	P13916	464.6293	3	19.66	24.66
NILEASYDTK	P13916	577.2904	2	10.9	15.9
LSAQYGSLR	P04405	497.7694	2	8.07	13.07
SQSDNFEYVSFK	P04405	725.8279	2	16.85	21.85
VFDGELQEGR	P04776	575.2804	2	8.97	13.97

[1]Experimental m/z (*11*)

C. Digestion

Modifications to the existing digestion protocol were compared (*11*). The existing method consists of subsequent additions of trypsin at 1:100 (trypsin: protein) (w/w). The first addition is followed by a 3-hour digestion, and the next addition is followed by an overnight digestion. Complete digestion with a single addition of trypsin at 1:50 was investigated. A 1:20 (w/v) NRSF extract was diluted in blank extraction buffer (0.5% NFDM) to a final concentration of 20 mg/kg total soy protein. Digestion of light peptides was observed with 9 subsamples over a 20-hour time period. These subsamples (based on hour) were as follows: 0, 0.5, 1, 2, 3, 4, 14, 16, 18, and 20. Replicate digest samples were prepared for each time point (18 samples total).

D. SDS-PAGE

One digest replicate at each time point was retained on the centrifugal filters and frozen at -20 C to stop digestion. These samples were brought to room temperature before analysis by sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE). One part sample was mixed with 5 parts of 6x Laemmli buffer and heated at 95 °C for 8 min. Samples were loaded into Mini-Protean TGX Precast 4-20% gradient gels. The gel was run at 200 V for 45 min in Tris/Glycine buffer, prepared as recommended by the manufacturer. Precision Plus Protein molecular weight markers (10-250 kDa) were used to determine the size of the protein bands and loaded at the appropriate volume. Each sample was loaded with equal volumes (12 µL). Two staining solutions were applied to the same gel following the manufacturer's instructions (Bio-Rad). The destain procedure was followed according the manufacturer. Coomassie Brilliant Blue R-250 Staining Solution was first applied overnight followed by a 2-hour destain. Silver Staining solution was then applied.

E. Standard Curve Preparation

The reference material for the external standards is non-roasted soy flour (NRSF) obtained from Archer Daniel Midland Company (ADM) (Decatur, IL, USA). The initial protein concentration of NRSF was determined to be 51.5% and was determined using the LECO Dumas method. A 1:20 (w/v) extract of NRSF was extracted and centrifuged at 13,000 g for 10 minutes. The supernatant of the NRSF extract was then serially diluted in blank extraction buffer (without PVPP). The concentration of soy protein in each dilution was based on the total soy protein concentration on a weight basis (mg total soy

protein/ kg NRSF). Two methods of dilution at different steps in the sample preparation were compared (Figure 2-1). Each external standard is then spiked 1:1 (v/v) with internal standards (4 fmol/µL). Each sample was then injected in duplicate and analyzed using the LC-MS/MS workflow as described previously.

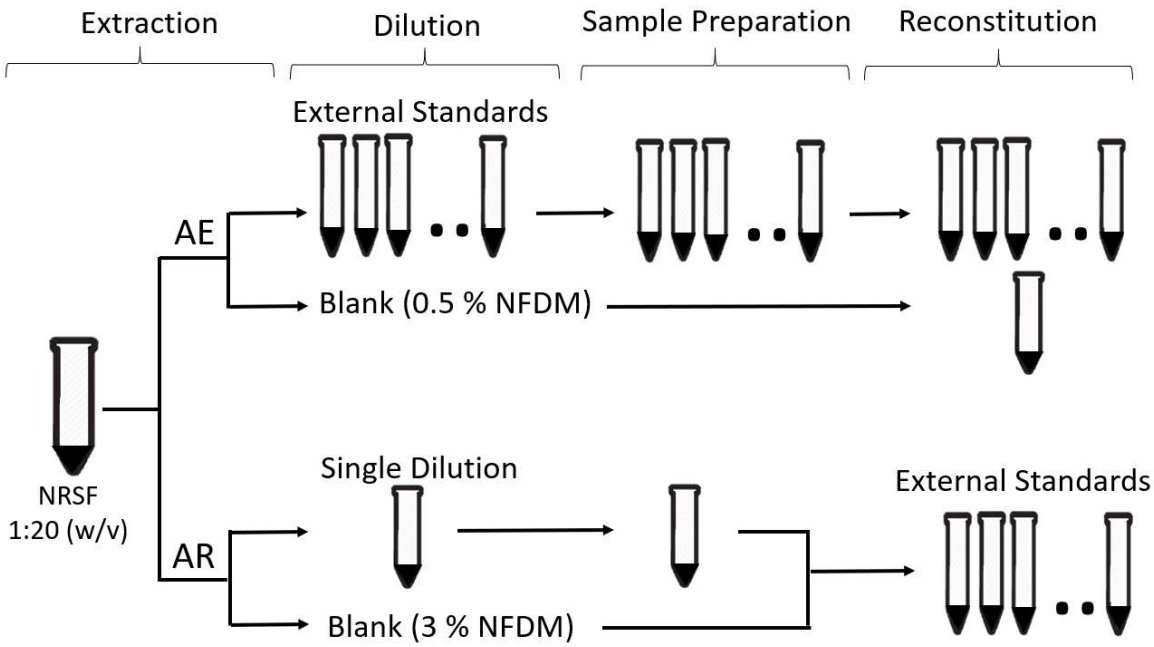

Figure 2-1. Two dilution methods for external standard preparation. After extraction (AE) serially dilutes samples immediately after extraction. The standards are then processed in tandem with a blank sample of extraction buffer (0.5 % NFDM). After reconstitution (AR) dilutes a single, higher concentration of total soy protein. This sample is then processed in tandem with a concentrated milk buffer (3 % NFDM) as a blank. The concentrated milk buffer is then used as a diluent for the external standards after reconstitution.

The methods are referred to based on the step in which serial dilution takes place: after extraction (AE) and after reconstitution (AR). Each method dilutes to the same theoretical total soy protein concentration (mg/kg). In the AE method, the NRSF supernatant is serially diluted in extraction buffer (0.5 % NFDM) into seven total soy protein concentrations (1, 2, 5, 10, 50, 100 mg/kg total soy protein) prior to digestion. These external standards are then processed in tandem through the end of the sample workflow without any modifications to the sample preparation. In the AR method, the NRSF extract is diluted to a single higher concentration (500 mg soy protein/kg NRSF). In tandem, a solution identical to the extraction buffer is prepared containing a higher concentration of NFDM (3 %) referred to as the concentrated milk buffer (CMB). The external standards and CMB sample are prepared in tandem. Small C18 spin columns (max 30 µg) were used for cleanup for the external standards. A large Pierce C18 spin column (max 5 mg) was used for the CMB to maximize the digested milk protein recovered for reconstitution (cat# 89852). The concentrated soy sample was reconstituted in 20 µL 0.1% FA and 3 % ACN prior to dilution with CMB.

The CMB is reconstituted to a known protein concentration. The background protein concentration for maximum light peptide recovery is unknown. Therefore, several background protein concentrations were compared: 0.05, 0.1, 0.25, and 0.5 µg/µL (µg digested milk protein/ µL reconstitution buffer). Replicate 500 mg/kg total soy protein samples were then diluted to the same seven total soy protein concentrations as the AE method with the different background protein concentrations. The AR external standards were determined to have theoretically equivalent total protein concentrations compared to the AE method.

F. Dilution Method

The maximum external standard in the existing protocol is 100 mg/kg total soy protein. The linearity of the two external standard dilution methods were compared with higher concentrations of total soy protein. For each method, ten dilutions of total soy protein were prepared. The dilutions for AE included 1, 2, 5, 10, 20, 50, 100, 200, 300, and 400 mg/kg. The dilutions for AR included 1, 2, 5, 10, 20, 50, 100, 150, 300, and 400 mg/kg. The AR method was prepared with one different concentration because of the limited starting volume after reconstitution. Therefore, 150 mg/kg was substituted for 200 mg/kg for sufficient volume to dilute down to 1 mg/kg.

Two digestion conditions of the external standards were compared. A higher amount of trypsin was compared to investigate any variability from the possible underestimation of the protein content. The two amounts of trypsin added for digestion was based on either the 660 nm assay (2 µg) or a theoretical maximum (4 µg). Both are based on a final digestion ratio of 1:50. Replicate standard curves of the dilution methods were prepared and digested with the two amounts of trypsin for a total of four external standard curves.

G. Analysis

Raw files were imported into Skyline for chromatography-based quantification analysis (version 20.1) (*12*). A set of pre-determined acceptance criteria was applied manually to each target peptide chromatogram in Skyline: average mass error < 2 ppm, dotp value >0.85, and a qualitative check for all three predetermined co-eluting fragments. Because multiple protein fragments are measured, the dotp value was part of

the criteria set to measure the similarity between the relative intensities of the peptide fragments to the predicted intensities. Peaks that fulfilled the entire set of criteria were considered a positive detection. These positive detection peaks were used to calculate the ratio of light peptide to heavy peptide peak areas in Skyline. Results from Skyline include linear regression analysis (slope, R^2 values) and peptide abundance (total peak area, ratio of total peak areas). Skyline reports were exported into several supporting software packages including Excel, SAS (version 9.4), and GraphPad Prism (version 8.0.0).

IV. RESULTS AND DISCUSSION

A. Digestion

Digestion efficiency is important to quantification workflows for the complete and consistent generation of target peptides. The optimal time and trypsin concentration for complete digestion of external standards has yet to be investigated for this method. The existing protocol digests samples with subsequent additions of trypsin at 1:100. A single addition of a higher ratio of trypsin to protein will streamline the protocol by eliminating the 3-hour incubation period between subsequent additions of trypsin. The first objective was to investigate the light peptide generation from a single addition of trypsin at a ratio of 1:50. A single extract of 20 mg/kg soy protein over the period of 20 hours was observed to determine the necessity of an overnight digestion.

The progression of the protein digestion was determined by the disappearance of higher molecular weight proteins. The digestion of higher molecular weight proteins was determined using SDS-PAGE. This was observed both by the generation of lower

molecular weight protein bands and the disappearance of higher molecular weight protein bands as shown in Figure 2-2.

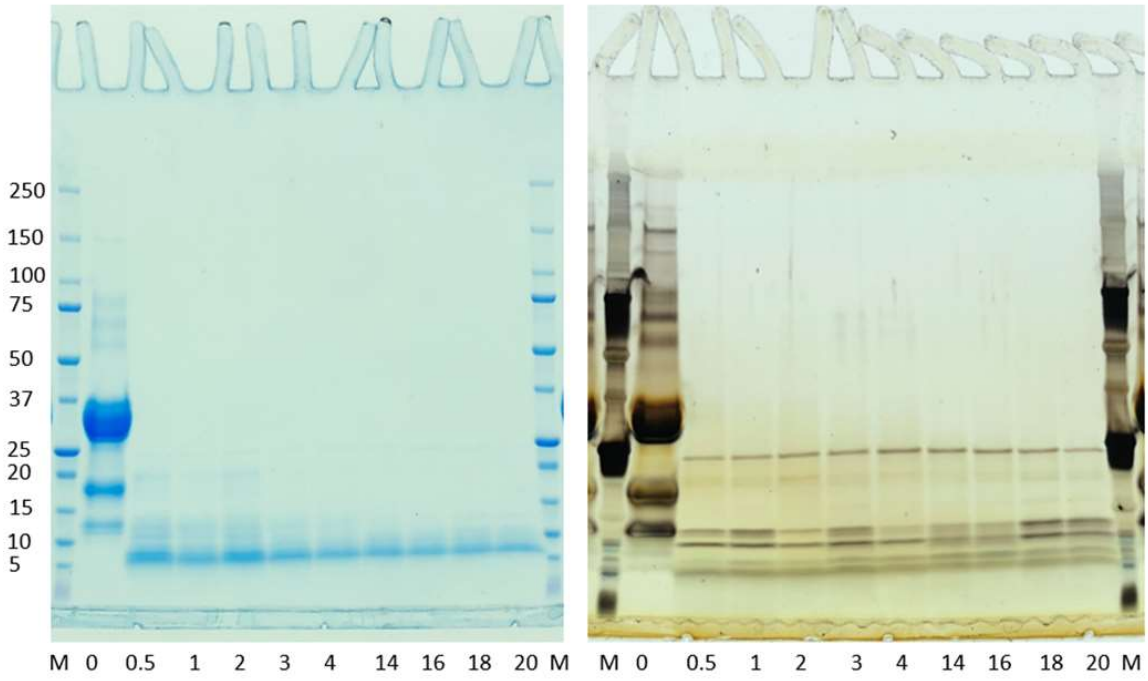

Figure 2-2. SDS-PAGE of a digested 20 mg/kg total soy protein NRSF extract at 9 time points over a total of 20 hours. Each lane is loaded with equal volumes of 12 µL of sample in 6x Laemmli Buffer. The M lane is the protein ladder. The 0 lane is the extract before digestion. The digestion time points (in hours) are labeled from 0.5-20. The gel on the left was stained overnight with Coomassie Blue Staining Solution (R-250) (Bio-Rad). The same gel is pictured on the right and stained following the Silver Staining procedure (Bio-Rad).

It is evident that there was an immediate disappearance of all higher molecular weight protein bands above 25 kDa immediately after 30 minutes of digestion. Some abundant higher MW bands remain in the extract time points between 10-25 kDa. The protein bands in this range had a general decrease in staining intensity as the digestion time progressed from 30 minutes to 20 hours. Coomassie blue staining solution has a sensitivity for micrograms of protein and is useful to visualize proteins at a higher abundance in the extract. The protein bands stained were suspected to be from the NFDM protein present in the buffer. The theoretical maximum soy protein concentration is estimated as 0.001 µg/µL. The protein concentration of blank extraction buffer (0.5%

NFDM) is estimated at 0.975 µg/µL with the 660 nm assay. Therefore, the milk protein concentration is approximately 1000-fold higher than the soy protein concentration in a 20 mg/kg total soy protein NRSF extract. The gel stained with Coomassie blue staining solution provides evidence of the complete digestion of milk protein. However, soy protein may have different rates of digestion and needs to be further investigated.

Analysis of lower abundant proteins that may originate from soy was observed with a more sensitive staining procedure. The gel stained with silver staining solution is sensitive enough to detect nanograms of protein. The SDS-PAGE gel does not allow the observation of protein bands below 10 kDa, which may be indicative of target peptide generation. Although, it does provide evidence of complete digestion of all extracted proteins above 20 kDa. The stained gel showed an indigestible band around 20 kDa. The identification of the protein band is unclear based solely on the molecular weight. The centrifugal filters have a cutoff at 10 kDa, so the indigestible protein band will be retained on the filter. Interestingly, the trypsin inhibitor protein is approximately 20kDa. Partially digested trypsin inhibitor proteins are possible but were not considered a concern. The harsh extraction conditions, chaotropic and reducing agents, likely disrupt the structure of most proteins, and the temperature may inactivate the trypsin inhibitor activity. Therefore, the indigestible protein band at 20 kDa was not a concern for the detection of target soy proteins.

The length of time allowed for digestion can compromise the abundance of light peptides in the sample. Enough time must be allowed for the complete digestion of proteins. Continuing digestion after the complete generation of peptides can result in the degradation of peptides in the sample. Additionally, overnight digestion is the longest

step in the sample preparation workflow, and shortening this step can streamline the overall sample preparation time. The digest replicate retained on the centrifugal filter was analyzed with SDS-PAGE for complete digestion of higher molecular weight proteins. The flow through of the other digest replicate was processed at each time point (30 minutes-20 hours) to the end of the LC-MS/MS workflow.

LC-MS/MS measured the change in target peptide abundance over the course of the digestion as shown in Figure 2-3. The abundance of light peptides was measured by the total peak area of the target fragments at each time point. A single data point represents the average of the total peak area for duplicate injections. The highest rate of digestion was within the first four hours of trypsin digestion. The increase in light peptide abundance plateaued overnight from 4-14 hours, with a maximum light peptide abundance at 18 hours for three of the five quantifying peptides. A slight decrease in intensity was seen for all peptides after 20 hours of digestion. This type of digestion kinetics, a slow increase to the maximum abundance from 16-18 hours, followed by degradation has been observed previously (*13*). The longer digestion time may result in enzyme degradation (nonspecific cleavages) or chemical modifications (carbamylation from urea) to the protein resulting in a decreased abundance (*14*). The maximum increase in intensity at 18 hours followed by a slight decrease was observed for all peptides. Therefore, 18 hours of digestion was interpreted as the optimal digestion time.

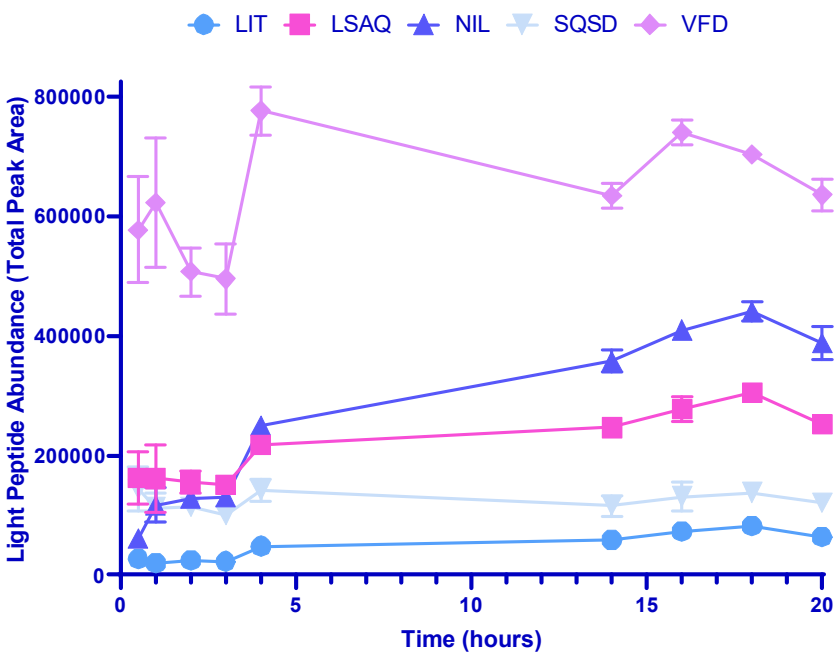

Figure 2-3. Light peptide abundance of five quantifying peptides from 0.5 to 20 hours of digestion using trypsin at 1:50 (w/w). Each data point represents the mean of duplicate injections. The error bars represent the standard error of the mean of the technical replicates.

The two peptides with slight changes in peptide intensities over the 20 hour digestion were SQS and VFD. The intensity of the total peak area of VFD increased 1.2 fold between 30 minutes and 18 hours. There seems to be an immediate digestion at 30 minutes, followed by a maximum peptide peak area intensity at 4 hours. The decrease in intensity from 4 hours to 18 hours may be from some degradation of the high concentration of peptide in solution. SQS had an immediate digestion, however, the abundance at 30 minutes was only positively detected in one of the duplicate injections. This peptide had a slight decrease in light peptide total peak area intensity from 30 minutes compared to 18 hours. A likely factor for the observed fluctuation for both VFD and SQS is the small sample size. The slight changes in peptide abundance may be more of a reflection of sampling error rather than a true indication of peptide degradation. A

single replicate was measured with a duplicate injection. Therefore, the variation of the total peptide peak area intensities between 30 minutes and 20 hours may be larger because there was only one replicate measured.

Three peptides had a steady increase in intensity from 30 minutes to 18 hours: LIT, LSA, and NIL. The abundance increased in varying orders of magnitude for each peptide as follows: NIL by 7-fold, LIT by 3-fold, and LSA by 2-fold over the course of digestion from 30 minutes to 18 hours. The abundance of LIT was determined to be near the limit of detection until after 2 hours of digestion because only one replicate from each injection gave a positive detection. Peptides NIL and LSA were positively detected in both replicate injections after 30 minutes.

NRSF is the least processed soy ingredient investigated and most closely resembles the native soybean. Therefore, the abundance of the light peptides may be explained by the initial concentration of protein in soybeans. All target peptides are from the seed storage proteins β-conglycinin and glycinin, with glycinin having a higher concentration in the native soybean (*15*). Interestingly, NIL is from the protein β-conglycinin and at a higher total peak area than two peptides from glycinin, LSA and SQS. Therefore, the peptide response in the sample may be more based on the accessibility of the peptide to trypsin digestion.

Peptides from the same protein subunit had different rates of digestion. The greater abundances of certain peptides compared to others within the same protein subunit may be explained from the variation of protein isoforms. LIT and NIL are part of the same alpha subunit in β-conglycinin with a higher abundance of NIL. LIT is present in one isoform (Gly-m-5.0101) and NIL is present in two isoforms (Gly-m-5.0101 and

Gly-m-5.0201) (*15*). Another possible difference between the peptide digestions is the presence of post-transitional modifications. β-conglycinin is a glycosylated protein which lowers the digestion efficiency (*13*). SQS and LSA are part of the same glycinin basic subunit B1a with a higher abundance for LSA. However, SQS is present in three isoforms (Gly-m-6.0101, Gly-m-6.0201, and Gly-m-6.0301) and LSA is present in one isoform (Gly-m-6.0201) (*15*). The different abundances of peptides from same protein suggests the digestion to be peptide specific. In the case of glycinin subunit B1a, LSA may be preferentially digested compared to SQS. However, the rate at which the proteins are digested may suggest otherwise if they increase proportionally at the same rate. Protein-based digestion was investigated with the correlation between the increases in abundance of target peptides over time (Figure 2-4).

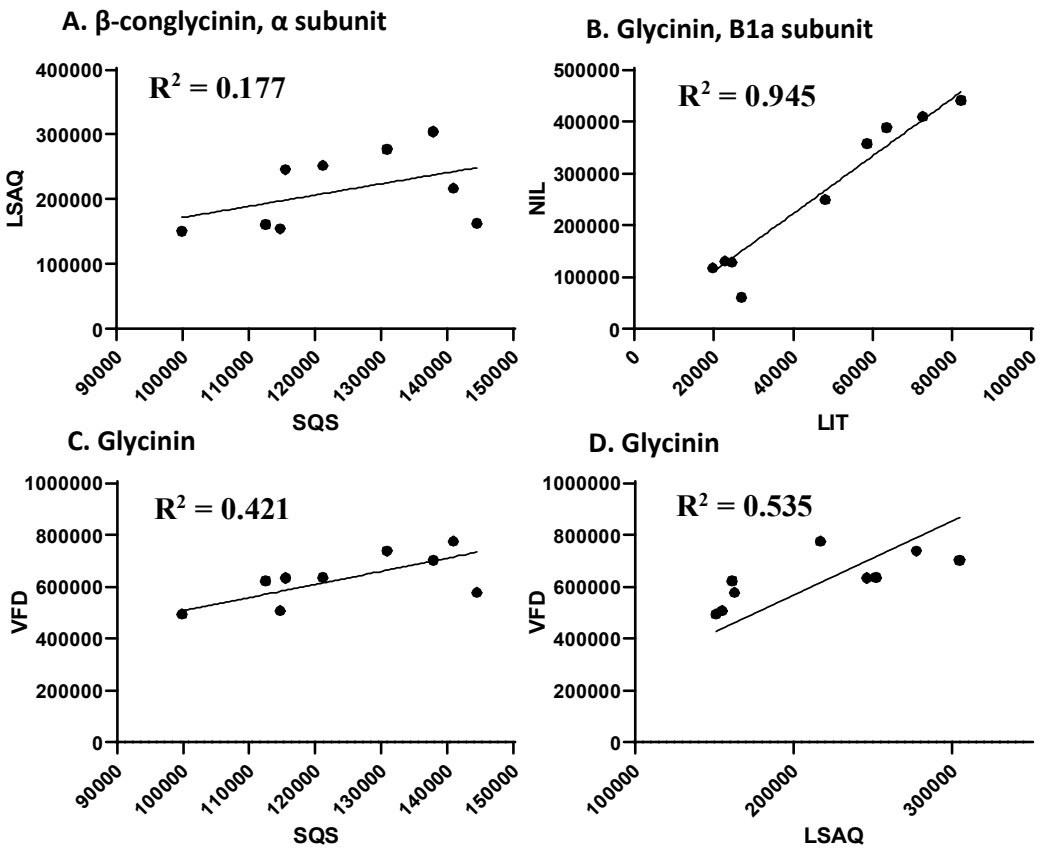

Figure 2-4. Correlation of light peptide abundance from 0.5 to 20 hours digestion. The correlation was measured between the target peptides in the same protein subunit, β-conglycinin subunit alpha (A), glycinin subunit B1a (B), or within the same protein, glycinin (C, D).

The generation of light peptides was determined to be either protein or peptide dependent. The digestion of the two target peptides from β-conglycinin had evidence of protein-dependent digestion with a linear correlation of 0.94. Both target peptides increased in intensity from 30 minutes to 18 hours with a slight decrease at 20 hours. The high correlation suggests digestion rates are similar for the protein. This was expected because both peptides are present in the protein isoform Gly-m-5.0101 (*15*). The digestion of the two target peptides from glycinin subunit B1a, SQS and LSA, had a poor correlation of 0.18. These peptides are both present in the isoforms Gly-m-6.0201 and Gly-m-6.0301, indicating the digestion is peptide specific. The peptides within the

glycinin B1a subunit had poor correlation to the peptide in the glycinin A1a subunit, VFD. The correlation between SQS and VFD was slightly lower than the correlation between VFD and LSA with correlations of 0.42 and 0.53 respectively. The poor correlation between peptides within the same subunit and peptides within the same protein further suggests the digestion of peptides from glycinin are peptide dependent. Agger et. al (2010) observed similar peptide dependent digestion abundances from the correlation between peptides within the same protein (*16*).

The digestion step is crucial to the generation of light peptides in the sample. The complete digestion of target proteins depends upon the properties of the protein (accessibility to enzyme, protease inhibitor), time, concentration of trypsin, denaturing solvents (detergents, chaotropic agents), among other factors (*17, 18*). The rapid increase in the light peptide generation in the first four hours is reflected in both the dramatic decrease of higher molecular weight bands in the SDS-PAGE gel and the abundance of the light peptide measured by LC-MS/MS. Overnight digestion remains necessary to generate the maximum peptide abundance, especially for three of the target peptides. From preliminary digestion experiments, it was determined that subsequent additions of trypsin were equivalent to a single addition of trypsin for the light peptide generation. The single addition of 1:50 trypsin ratio and a maximum overnight digestion time of 18 hours was determined to be the parameters for complete digestion. These digestion conditions will be applied to the sample preparation workflow of the external standards. The peptide dependent digestion requires the further investigation of complete digestion of proteins in complex matrices, especially with other food proteins present, to determine if these parameters are sufficient for unknown samples.

B. Carrier Protein Concentration

A carrier protein minimizes nonspecific adsorption losses of peptides throughout the MS workflow (*19*). This is especially important with samples containing low protein concentrations of target peptides. NFDM is added to the extraction buffer (0.5 %) to act as a carrier protein. Theoretically, at low concentrations of total soy protein, the milk protein present in the buffer will preferentially bind to the hydrophobic plastic surfaces and minimize nonspecific protein absorption of soy peptides. Currently, there are no recommendations on the carrier protein concentration in samples to minimize the loss of nonspecific binding (*20*). Therefore, background milk protein concentrations were compared to investigate nonspecific protein losses of external standards diluted with the AR method. The initial concentration of background protein was 1.5 µg/µL. This was based on the background milk protein determined to be in the blank extraction buffer as shown in Table 2-2.

Table 2-2. Estimated background protein concentration at each step in the sample preparation workflow.

Initial Protein Concentration (µg/µL)[A]	Protein Added to Digestion (µg)	After Digestion (µg/µL)	Before Desalting (µg/µL)	C18 Column (30 µg)	After Reconstitution (µg/µL)
0.975	102.375	0.511875	0.383906	51.05953	1.5

[A]Based on the 660 nm assay

The light peptide performance of the AR curve diluted in 1.5 µg/µL background protein was compared to the AE curve. Three curves were prepared for analysis: replicate curves prepared with the AR method (AR_1 and AR_2) and one replicate with the AE method. Seven concentrations of total soy protein were serially diluted in both methods (1, 2, 5, 10, 20, 50, and 100 mg/kg total soy protein). The lowest concentration that was

determined to have a positive target peptide detection in each curve is shown in Table 2-3.

Table 2-3. The lowest total soy protein concentration (mg/kg) determined to have a positive target peptide detection with AR or AE methods.

Peptide	External Standard Curve					
	AR_1		AR_2		AE	
	mg/kg[1]	Slope	mg/kg	Slope	mg/kg	Slope
LITLAIPVNKPGR	5	0.090	5	0.099	10	0.030
LSAQYGSLR	1	0.024	5	0.023	5	0.024
NILEASYDTK	1	0.021	1	0.021	1	0.019
SQSDNFEYVSFK	2	0.060	2	0.065	2	0.054
VFDGELQEGR	1	0.023	1	0.024	1	0.019

[1]mg/kg total soy protein

Three quantifying peptides had consistent detection at the lowest concentration for all curves: SQS, VFD and NIL. The most sensitive peptides, VFD and NIL, had a positive light target peptide detection at the lowest total soy protein concentration reference sample for all three curves. The other peptides, LIT and LSA, had variable detection between the two dilution methods. The peptide LIT was positively identified at 5 mg/kg in reference standards prepared AR and at 10 mg/kg in reference standards prepared AE. However, the AR 5 mg/kg reference standards with positive identification had only one replicate injection with a positive identification. Therefore, there is not sufficient evidence that the AR method has a higher sensitivity. It is more likely the total soy protein concentration is near the limit of detection for that particular peptide. The same trend was observed for the peptide LSA. One sample injection was positively identified in the AR_1 sample, while both injections at 5 mg/kg in the AR_2 and AE reference curves were positively identified. Considering the limit of detection of the peptides, the dilution methods gave similar sensitivity results. The slopes values for the more hydrophobic peptides, LIT and SQS, were determined to have a larger divergence

in slopes among the digestion conditions and dilution methods. Higher abundances of these peptides were observed in the AR method because of the higher slope values. The increase in light peptide abundance in the AR method may be from the starting concentration of soy protein. As seen in the digestion, these peptides are at the lowest abundance in the sample. The AE method may be more prone to losses of these peptides at each step of the sample preparation. The AR method starts with a higher concentration of total soy protein which may increase the recovery for dilution. Therefore, the AR method was determined to be robust for further sample preparation analysis because of the similar sensitivity to the AE method and the increased peptide response of two quantifying peptides.

AR external standards require a certain volume for complete dilution. A larger volume requires more protein to achieve the desired background protein concentration. The current concentration of background protein, 1.5 µg/µL, required a higher concentration of NFDM as a starting sample (3%) for sufficient protein recovery after reconstitution. At this concentration, the digestion required 20 times more trypsin compared to the blank extraction buffer (0.5% NFDM) for a 1:50 digestion. The parameters for C18 also required adjustment to recover enough protein to prepare 1.5 µg/µL solution after reconstitution. Higher capacity C18 columns (5 mg) were used to increase the total protein. The CMB not be a truly representative blank of the AR external standards because of the differences in digestion and desalting conditions. Therefore, lower concentrations of background NFDM were investigated.

A blank extraction buffer sample with a starting concentration of 0.1 % NFDM was reconstituted to 0.05, 0.1, 0.25, and 0.5 µg/µL. Four replicate concentrated soy

protein samples (500 mg/kg) were diluted each with a different background protein concentration. The same dilution was carried out but only 1, 5, 10, 50, and 100 mg/kg concentrations were measured which were representative of the light peptide responses of an entire standard curve. Figure 2-5 shows the ratio of the light to heavy peptide responses at each concentration for each peptide in replicate external standards prepared with the AR method with different concentrations of diluent background protein. Duplicate injections were averaged and treated as a single data point with error bars indicating the standard error of the mean for the technical replicates.

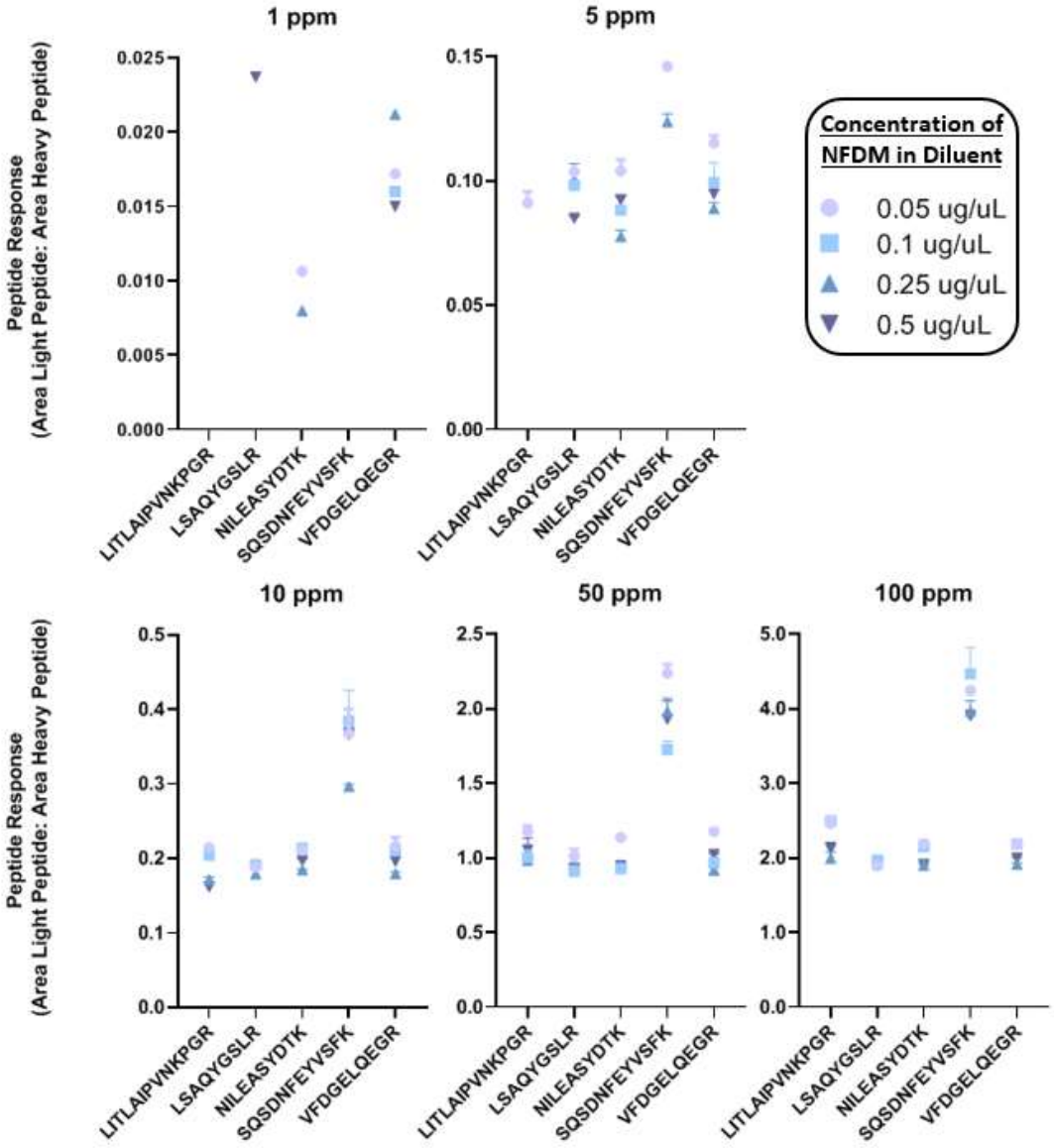

Figure 2-5. The mean peptide response of each concentration of external standards prepared with the AR method and diluted in 0.05, 0.1, 0.25, and 0.5 µg/µL background milk protein digest. SEM bars are shown for all points unless the range is smaller than the point on the graph.

Interestingly, the 0.05 µg/µL milk background had the highest peptide responses for all five quantifying peptides at 50 mg/kg, as well as at least one of the highest peptide responses for all of the other external standard concentrations. This was unexpected because higher concentrations of carrier protein may be more protective for hydrophobic peptides (*19*). The peptide performance was not considered to be dependent upon the concentration of background protein. At each concentration, light peptide responses were similar for all quantifying peptides regardless of the protein concentration. The lowest peptide response for NIL was observed for samples diluted in 0.05 and 0.25 µg/µL. Although this is near the limit of detection for this peptide and was considered random.

The lowest concentration of background protein, 0.05 µg/µL, is considered the most efficient for the current method. The maximum protein recovery expected is 30 µg because of the binding capacity of the C18 columns. The recovery of 30 µg milk protein produces a sufficient volume at 0.05 µg/µL to dilute out AR standards which can be obtained using the smaller C18 spin columns. As calculated previously, the recovery of 30 µg can be achieved with a background NFDM of 0.5%. These parameters prepare a blank sample that is more representative of the background protein in the NRSF external standards.

AR standards diluted in 0.05 µg/µL milk protein concentration digest was determined to produce robust peptide responses. The NFDM diluent can be prepared from a starting concentration of 0.5 % NFDM. Sufficient protein recovery is possible with the lower capacity C18 columns (30 µg) and requires lower amounts of trypsin compared to a higher stating concentration of NFDM (3%). These conditions allow the NFDM diluent to be more representative of the background protein of the external

standards. This preparation method of AE samples was considered more robust than the AR method because of the sensitivity and the streamlines sample preparation workflow.

C. Selection of dilution method

The accuracy of external calibration curves has a significant influence on the absolute quantification of unknown samples in MS workflows (*21*). The robustness of peptide responses were compared for the two standard curve methods, AE and AR. The sample preparation workflow used was as previously described with the following modifications: a single addition of trypsin (1:50) followed by an 18-hour overnight digestion (*11*). The external standard dilutions were prepared for AE as previously described. The AR external standards were diluted in 0.05 µg/µL background milk protein following reconstitution. In the previously mentioned workflow, trypsin was added at a ratio of 1:50 based off of the 660 nm assay of the protein extracts. However, a majority of the protein in the buffer is milk protein which masks the concentration of soy protein, even at high total soy protein concentrations (500 mg/kg). Therefore, total protein amounts estimated for 1 mg/kg and 500 mg/kg are the same. An underestimation of the protein at the higher soy protein concentrations will result in an incomplete digestion. This will skew the peptide response according to the type of dilution method used. Trypsin amounts were estimated for a 1:50 of a theoretical maximum total soy protein. This digestion condition was analyzed to assess any variability due to possible underestimation of total protein, especially for the higher concentrated soy protein samples (500 mg/kg). Therefore, two amounts of trypsin were used, one based on the 660

nm assay and one based on a theoretical maximum, to digest replicate standard curves prepared for each method.

Samples were injected in duplicate. The ratio of the total peaks areas of light to heavy peptide were averaged for each technical replicate. Each sample average was treated as a single data point. Standard curves were plotted with the total peak area ratios from 1 mg/kg to 400 mg/kg total soy protein. A linear regression line was fit to each curve and R^2 value were calculated in Skyline (Table 2-4). A higher R^2 value was observed more frequently for the AR method compared to the AE method. The lower R^2 values calculated for the AE method are likely representative of the sample to sample variation. This is expected because each external standard is carried through each step of the sample preparation with increasing variability from each step (*22*). The AR method has a higher correlation because the sample to sample variation is the same because all external standards are diluted from the same sample. A majority of variation in the AR curves is likely from the error in the serial dilution.

Table 2-4. Linear regression analysis of external standards prepared by two dilution methods (AR and AE) with two digestion conditions (2 µg and 4 µg trypsin).

Peptide	AR				AE			
	2 µg		4 µg		2 µg		4 µg	
	Slope	R^2	Slope	R^2	Slope	R^2	Slope	R^2
LITLAIPVNKPGR	0.033	0.980	0.025	0.989	0.010	0.920	0.018	0.906
LSAQYGSLR	0.024	0.985	0.022	0.986	0.021	0.983	0.027	0.996
NILEASYDTK	0.029	0.987	0.025	0.991	0.030	0.979	0.036	0.996
SQSDNFEYVSFK	0.105	0.982	0.086	0.995	0.050	0.966	0.075	0.980
VFDGELQEGR	0.022	0.986	0.022	0.987	0.019	0.968	0.027	0.995

The greater amount of trypsin was expected to improve the light peptide response from the concentrated soy protein sample. However, the slopes of the AR method between the two digestion conditions were closer in value compared to the AE method. This was unexpected because there is a 5-fold higher soy protein concentration in the highest starting dilution of the AR method compared to the AE method. As mentioned previously, the AR method is expected to have a higher sample to sample correlation because of the absence of the variability introduced at each step of the sample preparation workflow. The higher range of slope values between the two digestion conditions of the AE method is likely from the variation in the sample workflow, rather than a more complete digestion of the protein. As expected, the greatest variability was with the more hydrophobic peptides, LIT and SQS. This may be because of the low abundance of these peptides in the starting sample.

Figure 2-6 shows the spread of the light to heavy peptide ratios for each method, AE and AR, from 1 to 400 mg/kg. All standard curves (4 total) were plotted and linear lines were fit to each, as can be seen in the upper left hand corner of each graph. There is a 400-fold difference between the lowest external standard (1 mg/kg) and the highest external standard (400 mg/kg). This wide range makes it difficult to obverse the variation for the lower total soy protein dilutions (1-100 mg/kg) when plotted as a curve. Therefore, the variation of the peptide response at each external standard concentration was shown as a boxplot.

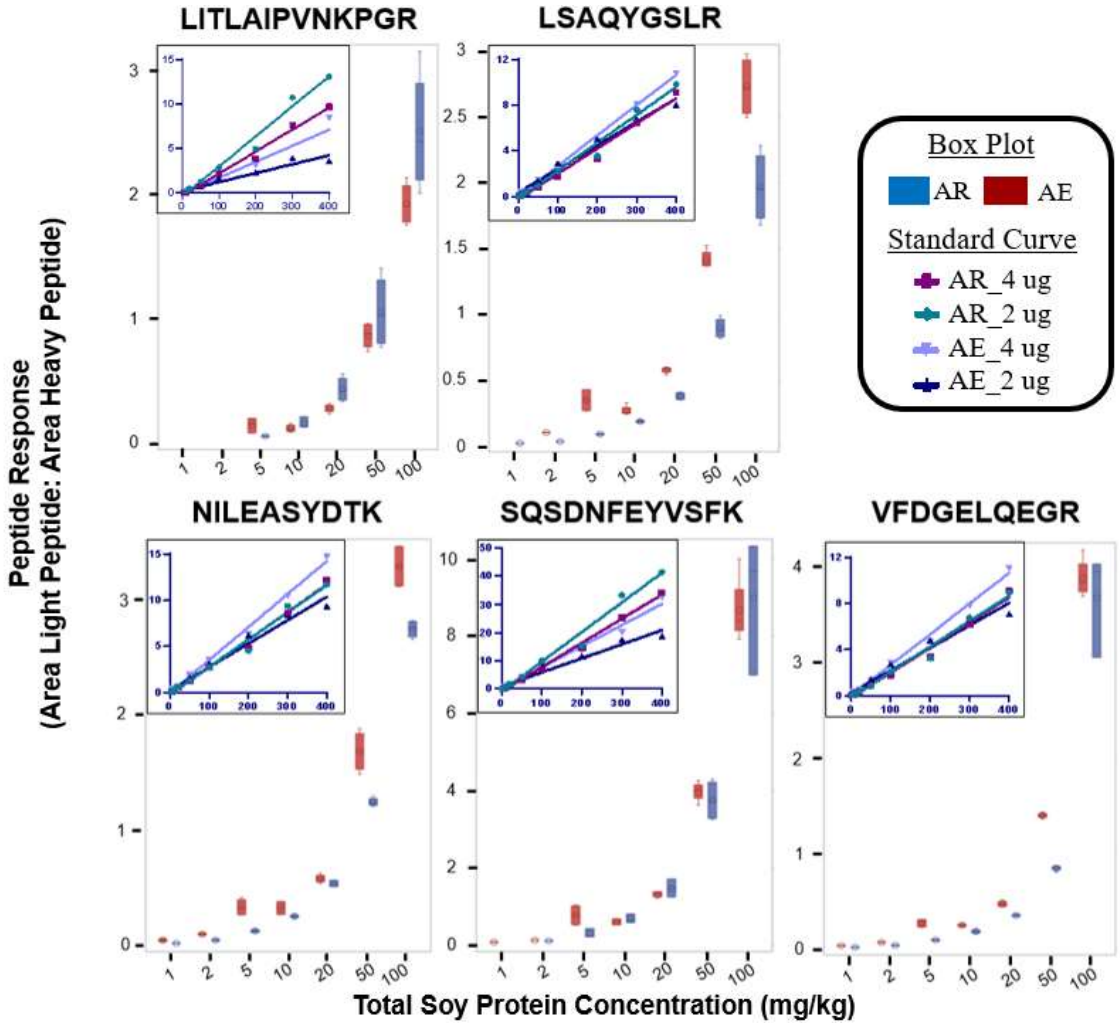

Figure 2-6. Variation of **s**tandard curves of two dilution methods (AE and AR) and two digestion conditions (2 µg and 4 µg). Curves were fit with linear regression slopes as shown in the upper left-hand corner of each graph. External standard concentrations (1-100 mg/kg) for each quantifying peptide were analyzed by the spread of peptide responses (ratio of light to heavy peptide total peak areas) within each method. The spread of ratios at each standard concentration is represented by box plots for the two dilution methods (AE and AR).

The hydrophilic peptides (NIL, LSA, and VFD) had narrower variation between and within digestion methods compared to the hydrophobic peptides (LITR and SQS). The AR method had a narrower variation at all external standard concentrations for the peptide NIL. LSA and VFD had similar variation between methods. The AE method had less variation at higher soy protein concentrations (10-100 mg/kg) using the more hydrophobic peptides, LIT and SQS. The greater spread in the hydrophobic peptides between digestion conditions and within methods indicate a more robust quantification for the hydrophilic peptides. The spread of the data for the AE method is greater than the AR method at higher concentrations of total soy protein as indicated by the greater divergence of AE standard curves from 1 to 400 mg/kg. This can be seen in the peptide VFD. There is a higher variation of AR peptide responses at 100 mg/kg, however, the standard curves plotted out to 400 mg/kg were narrower for the AR method compared to the AE method. This indicates a more robust linear response of AR curves. A general trend observed for all variables was a direct correlation between the increase in total soy protein concentration and the increase in the spread of data. One study assessing the reproducibility of a quantitative assay found a decrease in CV with an increase in analyte concentration in intra- and inter-laboratory measurements (*23*). An indirect correlation between external concentration and variance can also be observed (*24*). The variance from the current data set may be an artifact of the statistical analysis. Ordinary least square regression (OLS) is more affected by outliers compared to other weighted forms of regression (*25*). This may influence the spread of the standard curves of the AE method, which are more variable because of the sample to sample variation. The statistical analysis then favors the AE method, where there is a higher correlation among

standards. Other forms of regression may be considered because of the high influence of the upper values of the calibration curve if fit with an OLS regression. Transformation of the data may stabilize the variance, some studies plot the curves by an x and y log transformation (*26*).

The higher variability of the hydrophobic peptides, LIT and SQS, may be explained by the variability in the heavy peptide response. The light peptide response is normalized in the samples by the heavy peptide total peak area. A higher instability of the heavy peptide will greatly influence the final regression line. This variation was measured from heavy peptide responses of all injections within one standard curve as well as the overall data set. All standard curves were acquired within the same instrument calibration status. Every injection with a positively detected heavy peptide total peak area was calculated as part of the coefficient of variation (CV). A percent CV less than 20 was considered acceptable (*24*). The percent CV was plotted alongside the corresponding average heavy peptide peak areas (Figure 2-7).

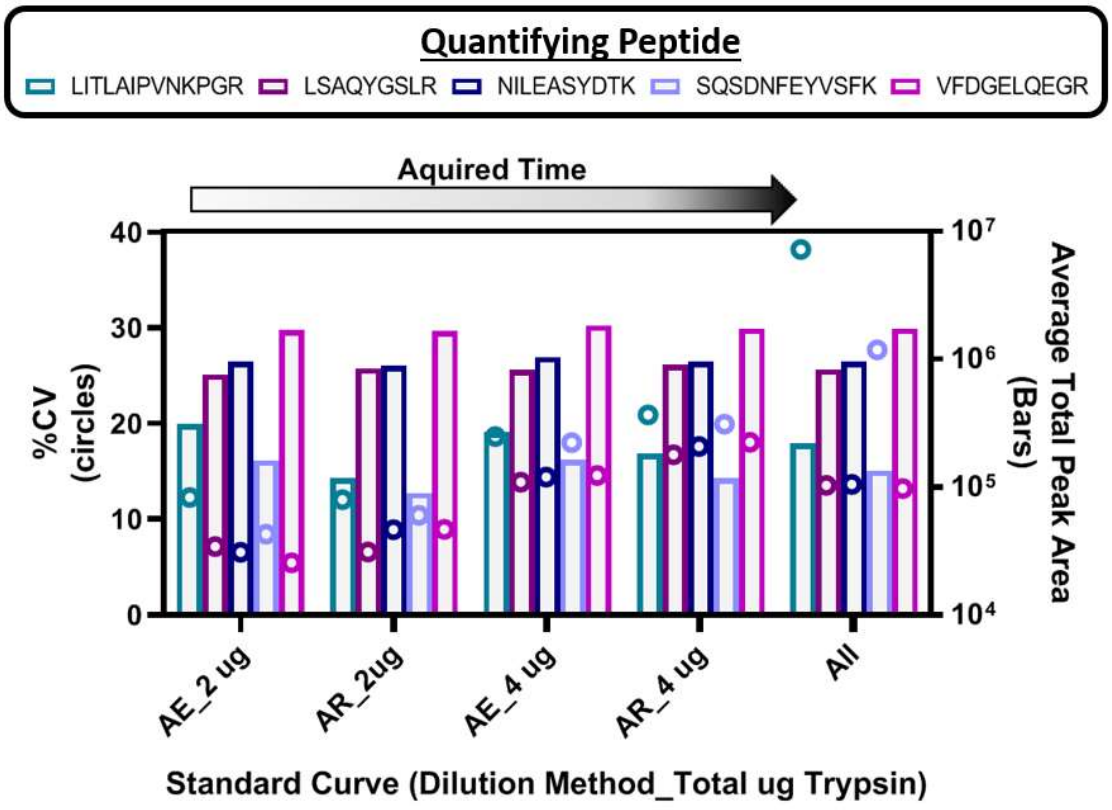

Figure 2-7. Percent coefficient of variation (CV) and average total peak area of quantifying heavy peptides for all standard curves. Circles correspond to percent CV values and bars correspond to the average total peak area for each positive injection within each standard curve and the overall sample set. Samples sizes within a curve was n=20 (except for SQSDNFEYVSFK with n=18 for AE_2 µg and n=19 for AE_4 µg) and all values n =80 (except for SQSDNFEYVSFK with n=77).

The heavy peptides from the three hydrophilic peptides, NIL, LSA, and VFD, seemed to produce the most stable response (< 20 %). The average heavy peptide total peak area within a curve was consistent compared to the average of all injections. The same trend was observed for the percent CV within a curve compared to the overall CV. The hydrophobic peptides, LIT and SQS, showed greater instability. These peptides had larger CV values within a curve as well as larger variations for the entire acquisition. The CV values were less than or equal to 20% within a curve but range from 30-40% overall. Interestingly, there was a general trend of an increase of variance as the time of

acquisition increases for all peptides. However, the variability of the heavy peptide increases faster for the hydrophobic peptides. It is possible these peptides are less stable over time after reconstitution in the autosampler. The variation from the hydrophobic peptides may be evidence of instability of the light peptide.

D. Selection of robust quantifying peptides

A common source for variability of the peptide response is bias in the ionization of peptides with electron spray ionization (ESI). There is preferential ionization of hydrophobic, basic, and larger MW peptides (*27*). This may not contribute much to the variability of the external standards because the sample background consists of the blank extraction buffer containing digested milk protein. It is unknown whether the hydrophobic milk peptides, present at a higher concentration than target soy peptides, could preferentially ionize compared to the target peptide.

A more likely source of variation is the hydrophobicity of peptides. Nonspecific binding of hydrophobic peptides throughout the sampling preparation results in loss of peptides due to the contact with hydrophobic surfaces (plastic) (*28*). This can continue after reconstitution and negatively affect the chromatographic elution of peptides from the reverse-phase (RP) LC column. The hydrophobic peptide targets elute after 10 minutes on the acetonitrile gradient and the hydrophilic peptides elute before 10 minutes. “Sticky peptides” are peptides with nonspecific binding to parts of the LC system (e.g. autosampler, column) that result in the loss of signal and can “carryover” to other samples in the queue (*28*). LIT is the most hydrophobic quantifying peptide composed of 64 % of nonpolar amino acid residues. In comparison, LSA, SQS, and NIL are composed

of more than 75 % polar residues. Interestingly, one of the more robust peptides, VFD is composed of 50 % nonpolar amino acid residues. This peptide is one of the most sensitive with consistent positive detection down to 1 mg/kg total soy protein. However, there is high abundance in the sample which is evident even after 30 minutes of digestion. The high peptide abundance likely influences the sensitivity of the peptide. It may be more likely to be detected at low total soy protein concentrations because of initially high abundance at the start of dilution and is therefore less affected by losses to nonspecific binding.

SQS can be considered one of the more hydrophobic peptides because it elutes later in the RP chromatography gradient. The final light peptide abundance is low, after 18-hour digestion, and as the dilution decreases the total soy protein concentration this peptide may fall below the limit of detection for the LC-MS/MS method. This peptide has higher sensitivity compared to LIT with consistent detection as low as 2 mg/kg total soy protein. Considering the high performance of VFD and SQS, the hydrophobicity does not fully explain the loss of sensitivity and increase in variability of the peptide LIT.

One study by Houston et al, (2011) studied 10 soy allergens for the development of an absolute quantification of soy allergens in different soybean varieties (*29*). The peptide LIT was found to have a low peak area and designated as a qualifier instead of a quantifier (*29*). A major limitation of this LC-MS/MS is the optimization of peptide performance using one set of ingredients. The abundance of the different soy proteins as well as the abundance of the individual protein subunits varies among soybean varieties (*30*). This could limit the universal application of this method on soy ingredients manufactured by different suppliers and made from raw ingredients obtained during

growing seasons from other cultivars. In a study by Plaque et. al, LIT was a peptide marker for the detection and quantification of soybean protein in different commercial food products including chocolate, ice cream, tomato sauce, and cookies (*31*). It has been observed that LIT has the same limit of detection, 5 mg/kg, for our experiment as observed in more complex matrices (*31, 32*). Therefore, the variability of LIT in our method may a combination of physiological and chemical properties. It may a sticky peptide that does not consistently elute into the retention time window. The similarity in physiochemical properties of the light and heavy peptides may indicate instability of the heavy peptide. The low abundance may be below the limit of detection for this LC-MS/MS workflow because of the initial low light peptide generation and additional nonspecific binding losses. The variance of the method is expected and has been shown in the development of other quantitative workflows (*33*).

V. SUMMARY

The external standards diluted with the AR method were determined to have comparable results to the existing method AE. The AR method successfully streamlines the protocol by reducing the sample load from nine to two with sensitive and robust peptide responses. The higher sample to sample variance of external standards prepared with AE may be a consequence of a complex sample preparation workflow, such as digestion efficiency and desalting, rather than the instrumental variation (*23*). The most robust quantifying peptides were determined to be the more hydrophilic peptides. This was determined from a combination of factors such as low abundance, losses from nonspecific binding, and peptide instability after reconstitution. These combined losses may result in hydrophobic peptide responses below the limit of detection for this method.

Future work aims to quantify the variation between day and within day external standard preparation. A finalized standard curve will then be used to quantify soy protein in model incurred matrices.

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CHAPTER 3

QUANTIFICATION OF SOY DERIVED INGREDIENTS IN MODEL FOOD MATRICES WITH AN OPTIMIZED LC-MS/MS EXTERNAL STANDARD CALIBRATION WORKFLOW

I. ABSTRACT

The detection and quantification of soy protein is important for accurate food labels and to prevent the presence of undeclared soy proteins. Heat processing and matrix interactions affect the accuracy of protein-based allergen detection methods. The sensitivity of ELISA methods is compromised if the protein epitopes are destroyed during processing. Therefore, an MS-based method was evaluated for the recovery of total soy proteins in incurred matrices.

MS-based quantification of total soy protein was assessed using a combination of external and internal standards. The reproducibility of the external standard curves was investigated. Variation was compared within a set of replicates and among all sets of replicates prepared on separate days. The replicate standard curves were then pooled and calculated as an average. A set of incurred samples was prepared using bread and frankfurters as model food matrices. Several soy derived ingredients were used to spike the matrices at varying levels of total soy protein. The total soy protein of incurred bread and frankfurter samples was then estimated using the pooled standard curve and the percent protein recovery was determined.

The variation of replicate standard curves between groups and among all groups was not significant. The slopes obtained from replicate standards run in different group

sets were minimal, indicating a standard curve is not required to be run with every sample set. The most influential factor on the percent protein recovery in incurred samples was the effect of the matrix on the soy protein. The lowest percent protein recoveries, less than 50%, were calculated for uncooked matrices. The cooked matrices had percent recoveries between 50-150 % for all total soy protein levels. Other factors, such as type of ingredient and heat, were not as significant in protein recovery.

II. INTRODUCTION

Soybeans are the cause of one of the most common food allergies worldwide (*1*). Allergic consumers rely on the accuracy of food labels to avoid foods containing soy protein. In the U.S., the addition of soybeans, or soy-derived ingredients, in any packaged food product must be included in the ingredients statement as required under the Food Allergen Labeling and Consumer Protection Act (FALCPA) (*2*). Quantitative risk assessment methods estimate finite levels of allergenic protein considered safe for a portion of the allergenic population (*3, 4*). A risk assessment tool known as Voluntary Incidental Trace Allergen Labeling (VITAL) estimates these finite levels for individual allergens to implement more effective precautionary allergen labeling (*5, 6*).

Soy protein detection and quantification is a unique analytical challenge because of the widespread application of soy protein in food products. The differences in the end-applications of soy-derived ingredients require a protein assay able to detect a wide range of denatured proteins. Process-induced modifications of soy proteins can negatively affect the sensitivity and specificity of protein-based allergen detection methods. The type of heat treatment, constituents within a food, and type of soy-derived ingredient interact and drive different chemical reactions, further modifying the structure and physicochemical properties of proteins into numerous by-products (*7*). These modifications decrease the extractability of proteins and affect the overall detection and quantification. Immunological methods may be particularly susceptible due to limitations in the extraction conditions and the loss of epitope binding (*8*). MS-based methods have

been developed for the detection of soy proteins in food matrices, but further work is needed on the quantification and recovery of food allergens (*9, 10*).

Matrix effects on the accuracy of protein assays are commonly studied with spike-and-recovery methods (*10*). A spike can be defined as an addition of a known amount of protein to a food matrix. “Naturally” incurred samples more accurately model the effects of the interactions between the components of a food matrix with target proteins as would be expected in industrial processing (*11*). The detection of target soy peptides has been verified previously with several soy ingredients in a naturally incurred cookie matrix (*12*). The quantification of the same soy ingredients, as well as texturized proteins, at different total soy protein levels is yet to be evaluated using this method. The objective of this work was to investigate the quantification of several soy-derived ingredients in model food matrices with an optimized LC-MS/MS external standard calibration workflow.

III. METHOD AND MATERIALS

A. Materials

Several types of soy-derived ingredients were obtained from Archer Daniel Midland Company (ADM): soy protein isolate (SPI), soy protein concentrate (SPC), texturized vegetable protein (TVP), toasted soy flour (TSF) and non-roasted soy flour (NRSF). Varieties of these ingredients include hydrolyzed and non-hydrolyzed SPI and two commercial types of SPC: “Arcon S”, which is a functional soy protein concentrate and “Arcon F”, which is an alcohol-washed protein concentrate. The total protein content

of each soy ingredient was determined using the LECO Dumas method on a LECO FP-528 Protein/Nitrogen Determinator.

B. Incurred Matrices

Ingredients used to prepare model foods were purchased from a local grocery store in Lincoln, NE. Soy-free all-purpose baking flour manufactured in a facility without soybeans was kindly provided by Julie Nordlee. All ingredients used were tested for the absence of soy using Veratox Soy ELISA kit before use. Blank samples were prepared for both matrices.

Soy protein spikes were prepared in carrier ingredients based on the total soy protein content. Spikes were added to model foods to incur at desired final total protein concentrations (mg soy protein/kg matrix). The total weight of the carrier ingredient (e.g. flour, salt) was adjusted to account for the soy protein spike to maintain consistency between the blank and incurred matrices. A 10,000 mg total soy protein/kg carrier ingredient (mg/kg total soy protein) was prepared for higher concentration of incursion. This spike was then diluted on a weight basis to prepare lower spike concentrations. The lower concentrated spikes (2,000 or 1,000 mg/kg) were prepared to avoid adding minute amount of spike to achieve low levels of total soy protein concentrations. The preparation of spike materials and verification of homogeneity was followed as previously described (*12*). Appropriate amounts of each soy-derived ingredient and all-purpose baking flour were mixed for 25 minutes in a KitchenAid mixer on medium speed. Following mixing, the spike was spread evenly on a flat surface and six subsamples were taken from the corners and center. The variation of the soy-in-flour spike was then assessed using the

Veratox Soy ELISA kit. Homogeneity was confirmed with a percent coefficient of variation less than 20%. Lower total soy protein spike levels prepared in the same manner were assumed to be homogeneous.

Two model food matrices were chosen based on the end-application of the soy-derived ingredients as described by the manufacturer. Additionally, a higher fat matrix (frankfurter) and higher carbohydrate matrix (bread) were incurred to survey the possible matrix effects on the detection and quantification of soy protein (*13*).

White Bread

The first model food matrix prepared was white bread. Three total soy protein levels were incurred in replicate batches. The soy protein spikes were added based on the total weight of the uncooked matrix: 1, 10, and 100 mg soy protein/kg matrix. Three soy-derived ingredients were used as spike materials: NRSF, SPC (Arcon S), and TSF. The 10,000 mg soy protein/kg flour spikes were prepared by adding appropriate amounts of each soy ingredient with wheat flour. After confirming the homogeneity, the 10,000 mg/kg spikes were diluted on a weight basis with wheat flour to prepare 1,000 mg/kg spikes. All spikes were prepared following the same procedure (*12*).

The texture of the bread depends upon the percent of flour and water. Therefore, the formulation was calculated on a percent flour basis (*14*). The bread formulation consisted of 390 g of wheat flour with other ingredients incorporated on a percent flour basis as follows: 2.9% vegetable oil, 4 % granulated sugar, 2.1 % iodized salt, 1.8 % active dry yeast, and 66.7% water. The total flour is adjusted to account for the appropriate amount of soy-in-flour spike.

Model bread matrices were prepared in a Breadman (TR777SPR) on setting 3, the recommended setting for basic white bread (total time: 3 hours and 20 minutes). The Breadman is a closed system which performs mixing, proofing, and baking at specific temperatures. Opening the instrument at any time during processing can cause temperature variation. Therefore, a pair of uncooked and cooked batches were prepared in tandem as separate batches to avoid operator-induced temperature fluctuations. These batches were then considered a pair of identical uncooked and cooked matrices. A pair of uncooked and cooked batches were prepared in duplicate at each level. A total of four batches, two uncooked and two cooked, were prepared for each ingredient at each concentration (n=12 batches for each ingredient).

Incurred batches were prepared sequentially from the lowest to the highest concentration of total soy protein. All batches were prepared for each soy protein ingredient spike consecutively to avoid carryover of the ingredients. A blank batch was prepared after the highest incurred concentration of each ingredient. Cooked batches were mixed (20 minutes), proofed (2 hours), and baked (1 hour). Total weights of batches before and after cooking were recorded to determine total moisture loss. Cooked loaves were allowed to cool to RT before storage at -20 °C. The replicate cooked loaves at each spike level were stored as follows: one loaf was ground using a food processor (KitchenAid), and one loaf was cut into approximately 1-inch-slices. The sliced matrix was separated into the inner crumb and crust, and each component was ground separately using an Osterizer blender (model no. 6640, Sunbeam Products, Inc.) in glass canning jars. Uncooked batches were mixed and proofed, then immediately removed from the Breadman. These batches were allowed to cool at 4 °C before storage. The dough batches

were separated into one inch subunits and kept at -20 °C overnight. For MS sample preparation, the subunits of dough were pounded into smaller pieces using a hammer, kept at -80 °C for 30 minutes, and then pounded again into finer pieces. The final dough pieces were kept at -20 °C until further analysis.

Frankfurter

The second model food prepared was a frankfurter. The formulation used is as described: 89.5% ground beef (90% lean), 8% water, 1% granulated sugar, and 2% iodized salt (*15*). The ground beef was pre-weighed, stored at -20 °C, and thawed overnight at 4 °C prior to use. Two total soy protein levels were spiked based on the total weight of the uncooked matrix: 10 and 50 mg soy protein/kg matrix. The ingredients used as soy protein spikes were NRSF, SPC (Arcon S), SPI (non-hydrolyzed), and TVP. The TVP flakes were ground into finer particles using a food processor (KitchenAid) prior to use. Two spike levels were prepared using TVP and SPI: 50 and 10 mg/kg. One spike level was prepared using NRSF and SPC: 10 mg/kg. Soy ingredients were mixed with appropriate amounts of iodized salt to make 10,000 mg/kg spikes. Soy-in-salt spikes were homogenized in a coffee grinder (Mr.Coffee) with four pulses (approximately 20 seconds) on auto settings with scrapping between each pulse. The verification of homogeneity is the same procedure as described previously. The 10,000 mg/kg spikes were further diluted on a weight basis to prepare 2,000 mg/kg spikes using the same method. The total salt in the frankfurters was adjusted to account for the soy in salt spike additions.

The salt, sugar, and soy-in-salt spike were all mixed with water prior to adding to the meat base. The meat base was emulsified using a bowl chopper (Manica CM-14) on

the highest speed for 5 minutes. Ingredients were poured into the chopper as a homogenous solution after 1 minute. The meat and water solution were mixed for the remaining 4 minutes. The meat base was immediately stuffed into 50 mL falcon tubes using a Delta prime jerky gun (1 lb capacity). A portion of the raw frankfurter was removed from each tube so that the remaining weight was approximately 50 g (weight of frankfurter and the falcon tube). The raw portion was stored at – 20 °C until further analysis. The frankfurters were then cooked in a water bath at 40 °C for 30 minutes, transferred to another water bath at 80°C for 30 minutes, and kept in a cold room at 4°C for 30 minutes (*16*). The frankfurters were weighed before (50 g) and after cooking to determine water loss. The cooked frankfurters were ground using the same procedure as with the bread samples and stored at -20°C until further analysis.

C. Standard Curve

The standard curve method of preparation used is referred to as "After Reconstitution" (AR). This refers to the step at which standards are diluted and has been previously described in Chapter 2. Seven concentrations of total soy protein were serially diluted from a higher concentration sample into 1, 2, 5, 10, 20, 50, and 100 mg/kg total soy protein.

The reproducibility of the AR method of standard curve preparation within a single sample preparation workflow and between different sample preparation workflows was investigated. The variability within a set of replicates and among sets of replicates was determined using triplicate extracts and triplicate experimental repetitions (Table 3-1). Three replicate extracts of NRSF, 1:20 (w/v), were extracted and serially diluted using

the AR method. This experiment was repeated in triplicate on different days. Triplicate NRSF extracts were prepared in tandem and each set is referred to as a group. The entire replication has a sample size of nine standard curves. Reagents were all prepared at the same concentrations and volumes for each set of replicates. All samples within one group were digested using a single vial of trypsin at a final concentration of 1:50 (w/w).

Table 3-1. Experimental design for the reproducibility of standard curves prepared with the AR method of dilution.

		Group (Set of three replicates)		
		1	2	3
NRSF Extract (1:20) Extraction Replicate	1	11[A]	12	13
	2	21	22	23
	3	31	32	33

[A]Combination of numbers represent a standard curve replicate one part of group 1.

External standard curves were measured for three quantifying peptides: LSA, NIL, and VFD. Isotopically-labeled peptides were added as an internal standard. The ratio of the total peak areas of the external standard to the internal standard for each peptide was measured. Each external standard was injected in duplicate and the peptide response was averaged. All average peptide responses for the replicate standard curves was then plotted to determine an overall average slope. This overall average slope was used to interpolate the quantification of incurred samples.

D. Targeted LC-MS/MS Workflow

The targeted LC-MS/MS method has been previously described in Chapter 2. The digestion conditions optimized for sample preparation was experimentally determined using NRSF. Considering the digestion is peptide-dependent, the high concentration of protein in the incurred matrices was reason enough to confirm the complete digestion of

target peptides in more complex samples. The complete digestion of incurred matrices at 1:50 was confirmed by comparing the same two digestion methods tested on the digestion of NRSF. The digestion conditions differ based on the additions of trypsin either two additions of 1:100 (w/w) or a single addition of 1:50 (w/w).

The quantification of total soy protein in incurred matrices was determined on duplicate extracts of each level of incurred matrix. Blank matrices were run with the respective incurred samples to confirm the absence of target soy peptides and interfering background signal. Incurred samples were injected from the lowest to the highest total soy protein level and were randomized within each total soy protein concentration. The average peptide response for each sample was averaged. The samples were then interpolated from the "master" standard curve to determine the experimental quantification of total soy protein (mg total soy protein/ kg matrix) for each quantifying peptide. The expected total soy protein for each matrix was adjusted to account for water loss after heat treatment. A percent recovery was then calculated using the quantified total soy protein to the expected total soy protein (adjusted) using the following equation:

$$\frac{\text{Quantified Total Soy Protein}}{\text{Adjusted Total Soy Protein}} \times 100\% = \text{Percent of Total Soy Protein Recovery}$$

Quantification values were pooled for all peptides to assess reporting a single value for each incurred matrix. This value was calculated from either the average or maximum quantification value of all quantifying peptides.

IV. RESULTS AND DISCUSSION

The commercially-processed soy ingredients used in this study were obtained from one manufacturer (ADM). According to the common uses of these ingredients, NRSF, TSF, and SPC (Arcon S) are commonly used in baking products such as crackers. These ingredients were then incurred into matrices likely to be processed in the same facility, such as bread. Therefore, bread was chosen as a model food matrix for the incursion of these ingredients. Soy protein ingredients, such as soy flours, TVP, SPC and SPI, are added to frankfurters and other sausage-like products for a range of functional properties including texturizers, water and fat binding, flavor binding and gel formation (*17*). The manufacturer lists common uses of SPI, SPC, and TVP as functional ingredients of many meat products (military meat, sausages, etc.). Therefore, frankfurters were chosen as a model food matrix for the incursion of these ingredients.

A. Detection

Each incurred level of total soy protein for every matrix was extracted in duplicate and injected in duplicate (technical replicates) (n=4). Three quantifying and two qualifying peptides were monitored. Each peptide peak was then manually inspected in Skyline. A positive detection was determined with a set of predetermined criteria: i) dotp > 0.85, average mass error < 2 ppm, and presence of all three pre-determined co-eluting fragments. The maximum count of peptide peaks with a positive detection for all peptides is n=20. The total count of positive detections was determined using four injections and five peptides for each total soy protein level in bread (Figure 3-1) and in frankfurter (Figure 3-2).

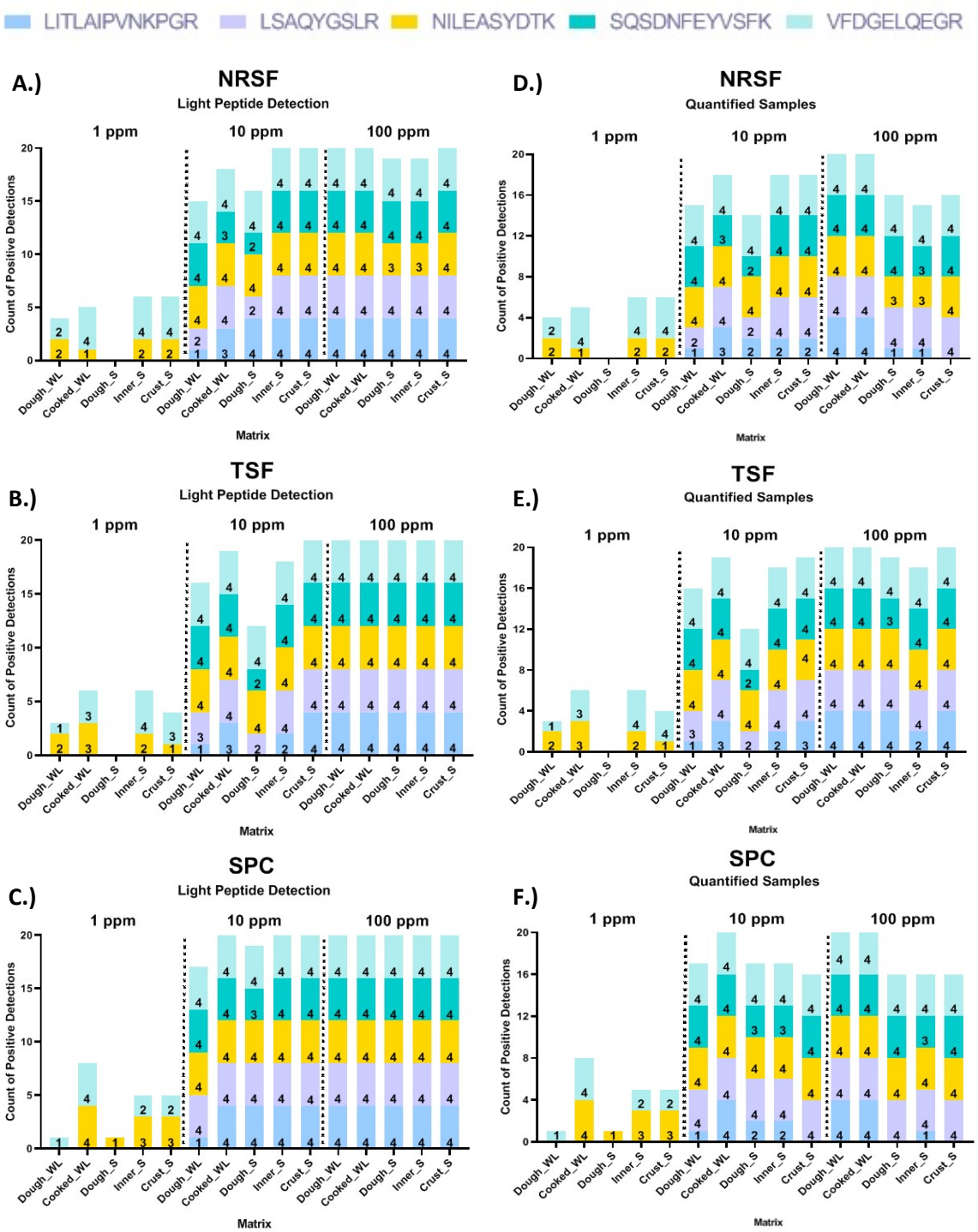


Figure 3-1. Count of positive detections for five target peptides in incurred bread matrices. The sample size is n=4 for each ingredient-matrix combination. Cooked matrices are labeled as whole loaf (WL) or slices (S). Positive detection is confirmed if all predetermined criteria are met: i) average mass ppm < 2 ii) dotp <0.85 iii) co-eluting fragment peaks. Each graph groups samples by the soy-derived ingredient used for incursion. Graphs pictured on the left (A-C) represent the total counts of light peptide detection. Graphs pictured on the right (D-F) represent the total count of quantifiable samples with positive detection of both light and heavy peptides.

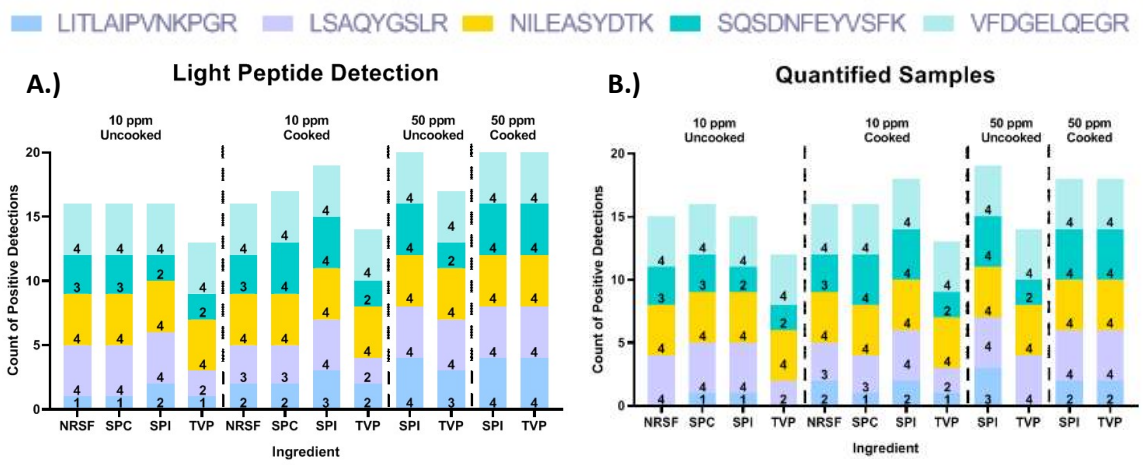


Figure 3-2. Count of positive detections for five target peptides in incurred frankfurter matrices. Sample size is n=4 for each ingredient-matrix combination. The soy-derived ingredients are non-roasted soy flour (NRSF), soy protein concentrate (SPC), soy protein isolate (SPI), and texturized vegetable protein (TVP). Each ingredient is grouped by level (10 or 50 mg/kg) and heat treatment (uncooked and cooked). Positive detection is confirmed if all predetermined criteria are met: i) average mass ppm < 2 ii) dotp <0.85 iii) co-eluting peaks. Panel A represents the total counts of light peptide detection for each ingredient and incurred matrix. Panel B represents the total count of quantifiable samples with a positive detection of both light and heavy peptides.

Three concentrations were analyzed for the bread matrices: 1, 10 and 100 mg/kg. The maximum count of light peptide detection (n=20) was observed most frequently for samples incurred with 100 mg/kg total soy protein in model bread. This was consistent across all soy-derived ingredients. Lower counts of detection at 100 mg/kg is specifically observed for the peptide NIL falling out of the predetermined specifications. Interestingly, the peptide intensity is high with co-eluting fragments and a high dotp value. For this specific peptide, higher intensities of the light or heavy peptide have been associated with higher mass errors, between 2 and 3 ppm. The exact phenomenon for this effect is unknown and the issue has been consistently observed for this peptide. However, a count of 3 out of 4 injections indicates a positive peptide peak was confidently detected in at least one of the duplicate injections for each replicate extract. Therefore, a duplicate injection for each sample is necessary to avoid false negatives due to random or

instrumental errors. A single positive peptide detection is calculated as the average peptide response for a sample.

The 10 mg/kg and 1 mg/kg total soy protein levels in incurred bread had lower counts of positive light peptide detection compared to the 100 mg/kg bread matrix. This was expected because the detection limit of LSA, LIT, and SQS is around 5-10 mg/kg total soy protein in the standard curve concentrations. The lower counts of positive light peptide detection are a result of low peptide intensities with missing fragment ions, as well as at least one other criterion out of specification. At 1 mg/kg only the two most sensitive peptides are detected: NIL and VFD. Specifically, the peptide VFD can be detected in all injections in the cooked matrices for both extraction replicates spiked with NRSF and TSF. The peptide NIL was detected likewise at 1 mg/kg in the cooked matrices for SPC. Overall, the method had higher counts of positive peptide detection in the 1 mg/kg cooked matrices compared to the 1 mg/kg uncooked matrices.

The detection of light peptides was dependent upon the concentration of total soy protein present for both matrices. Two total soy protein levels were analyzed in model frankfurter matrices: 50 and 10 mg/kg. A general decline in the count of positive detection peaks is seen in a concentration-dependent manner for the qualifying peptides LIT and SQS. The most robust peptides were determined to be VFD and NIL and were detected in all injections for all samples. The quantifying peptides, LSA, NIL, and VFD, were consistently detected at 10 and 50 mg/kg in the cooked and uncooked frankfurters.

Lower counts of the qualifying peptides, LIT and SQS, were observed in the frankfurters. At 10 mg/kg, the cooked and uncooked matrices of the frankfurter and bread

had 100% light peptide detection of NIL and VFD. The positive peptide count for each ingredient type was equal in both matrices. For example, NRSF positive light peptide counts were the same at 10 mg/kg in bread as they were at 10 mg/kg in frankfurters. This indicates the suitability of these peptides for quantification for different matrices.

The most reliable quantifying peptides are LSA, NIL, and VFD as discussed in Chapter 2. Briefly, the detection of these three light peptides are reproducible at low mg/kg levels (1-5 mg/kg) of total soy protein level and have a higher linear dynamic range compared to the peptides LIT and SQSD. Additionally, the LIT and SQS heavy peptide internal standards have higher instability over time in the autosampler. This instability was inferred by comparing the total count of light peptide detection to the total count of quantifiable samples. Quantifiable samples represent the samples with positive identification of both the heavy and light peptide peaks. The incurred samples are acquired from the lowest (1 or 10 mg/kg) to highest (50 or 100 mg/kg) total soy protein concentration. Therefore, the higher concentration peptide samples will have the longest wait period between reconstitution and injection. For LIT, the light peptide intensity is unaffected by the wait period, whereas the heavy peptide will decrease in intensity causing the fragment ions to fall below the limit of detection. This decreases the number of quantifiable samples of LIT despite the high count of positive light peptide detections. For example, at 100 mg/kg the LIT light peptide is detected in all four injections for the sliced bread matrix, and the heavy peptide is detected in 0-25 % of the injections. A similar trend was observed at 50 mg/kg in the frankfurter matrices where light peptide detection was 100 % in the cooked matrices but only 50 % of samples were quantifiable.

These samples are then unable to be quantified. Therefore, the peptides LIT and SQS are better suited as qualifiers.

B. Standard Curve

Each standard curve was prepared from the serial dilution of a single extract of NRSF as described in Chapter 2. Briefly, a single aliquot of each extract was diluted 1000-fold to a final concentration of 500 mg/kg total soy protein prior to reduction and alkylation. Several aliquots of blank extraction buffer (0.5 % NFDM) were prepared alongside the standards. All samples were carried through digestion and C18 cleanup before the final freeze/dry step. The serial dilution (1, 2, 5, 10, 20, 50, and 100 mg/kg total soy protein) is carried out after reconstitution (AR). The reconstituted 0.5 % NFDM, final protein concentration of 0.05 µg/µL, served as the diluent for the dilution of external standards. The variability of the dilution method was estimated by preparing replicate standard curves within a group of replicates and among all groups prepared on separate days. The source of variability for the AR method was determined with triplicate NRSF extracts. Each extract of NRSF, 1:20 (w/v), was prepared with the LC-MS/MS workflow in tandem and serially diluted AR into a single standard curve (1, 2, 5, 10, 20, 50, and 100 mg/kg total soy protein). A set of triplicate extracts is referred to as a group. This experimental method was then repeated in triplicate (Group 1, Group 2, and Group 3). A total of nine standard curves, three in each group, were compared.

The variability of standard curves within a group (three standard curves total) was compared to variability among standard curves between all groups (nine standard curves total). The first objective of the experimental design was to determine whether the source of variability between the peptide responses at each external standard concentration

within a group is more significant than among groups. Standard curves were then pooled, and an average regression line was fit for all replicates. The second objective was to determine the significance of the variability of the slopes on the quantification of known incurred samples.

Variation of Peptide Response

The variation of the peptide response between standard curves prepared on different days was evaluated. The peptide response of three quantifying peptides, LSA, NIL, and VFD, was measured in tandem for each external standard curve dilution (1-100 mg/kg). For the purposes of statistical analysis, the total soy protein levels are fixed treatments and are assumed to be randomly assigned. For each total soy protein concentration, the peptide response ratio is assumed to have a normal distribution. Therefore, the variance of each total soy protein concentration among replicates within a group and between all replicates for all groups were calculated using the mean square (MS) following one-way ANOVA (Table 3-2).

Table 3-2. Mean square of peptide response ratios for each concentration of the external standard curve using one-way ANOVA.

Peptide	Source of Variation	Mean Square of Peptide Response					
		Total Soy Protein Concentration (mg/kg)					
		2	5	10	20	50	100
LSA	Within[A]	6.66E-05	8.53E-05	3.15E-04	1.44E-03	1.52E-02	6.83E-02
	Across[B]	1.47E-05	4.72E-04	1.70E-03	4.05E-03	3.33E-02	2.66E-01
	F Test	NS	NS	S	NS	NS	NS
NIL	Within	1.03E-04	5.67E-04	1.12E-03	7.23E-03	5.48E-02	1.97E-01
	Across	5.05E-05	8.12E-05	8.44E-04	3.23E-03	3.38E-03	3.42E-02
	F Test	NS	NS	NS	NS	NS	NS
VFD	Within	8.53E-05	3.97E-04	7.11E-04	3.81E-03	2.99E-02	6.65E-02
	Across	4.02E-05	3.42E-04	4.57E-04	2.68E-03	1.31E-02	1.30E-01
	F Test	NS	NS	NS	NS	NS	NS

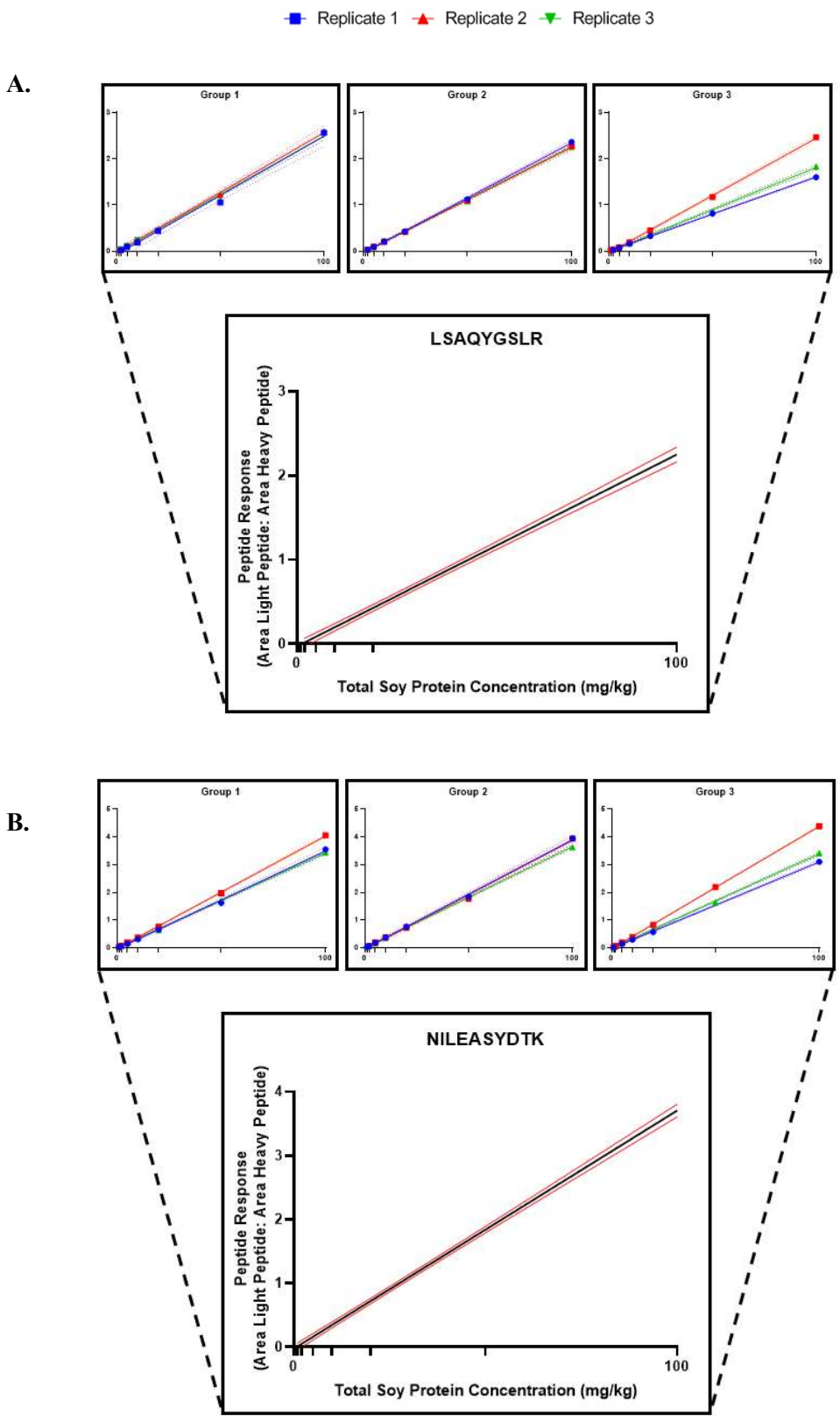
[A]Within group variation (n=3)
[B]Among group variation (n=3)
[C]Critical F value = $F_{0.05, 2, 6} = 5.143$
[D]Non significant as determined by the F test (within/among)
[E]Significant as determined by the F test (within/among)

The lowest total soy protein level was excluded because of missing peptide ratio responses. Additionally, the 1 mg/kg value is expected to vary because it is near the limit of detection for the method. An F test can be used to determine if two variances are significantly different between standard curve concentrations (*18*). The significance of the variation for within day (between replicates) was compared to the variance among groups (between days) using an F test with a critical p-value of 0.05. The F test for within group variation divided by among group variation was determined to be not significant. All mg/kg levels for all three peptides were found to have equivalent variation for peptide responses for within day and among groups. An exception was 10 mg/kg for the peptide

LSA, which found a significant difference between the variability between replicates and days. This was not concerning because of the non-significance of the six other total soy protein concentrations among replicates and between groups. It is assumed that the equal variance of the other concentrations will control for the significant variation at the 10 mg/kg level for this peptide. It is concluded by the F test that the variance between the peptide responses at each total soy protein concentration run within the same group and different groups is not significantly different. Therefore, peptide response from each concentration among replicate standard curves within a group and among groups do not vary significantly.

Variation of Linear Slopes

The next objective was to determine the variability of the slopes among all groups for each peptide. Three replicate standard curves, representing three quantitative peptides, were plotted for each replicate. The peptide response was measured as the ratio of the total peak areas of the light peptide to the heavy peptide. Each external standard dilution was injected in duplicate and the average peptide response was calculated. The average values were then used to plot a standard curve. Standard curves are plotted as follows: the x-axis corresponds to the external standard dilution (mg/kg total soy protein) and the y-axis corresponds to the peptide response (ratio). A final regression line was fit to a curve with all standard curve replicates (n=9 for each concentration except for 1 mg/kg). The peptide response is expected to increase in a concentration-dependent manner. Therefore, the standard curves were fit using an ordinary linear regression model. This final standard curve was used to interpolate the incurred samples to determine the protein recovery (Figure 3-3).


Replicate 1
Replicate 2
Replicate 3
A.
Group 1
Group 2
Group 3
LSAQYGSLR
Peptide Response
(Area Light Peptide: Area Heavy Peptide)
Total Soy Protein Concentration (mg/kg)
B.
Group 1
Group 2
Group 3
NILEASYDTK
Peptide Response
(Area Light Peptide: Area Heavy Peptide)
Total Soy Protein Concentration (mg/kg)

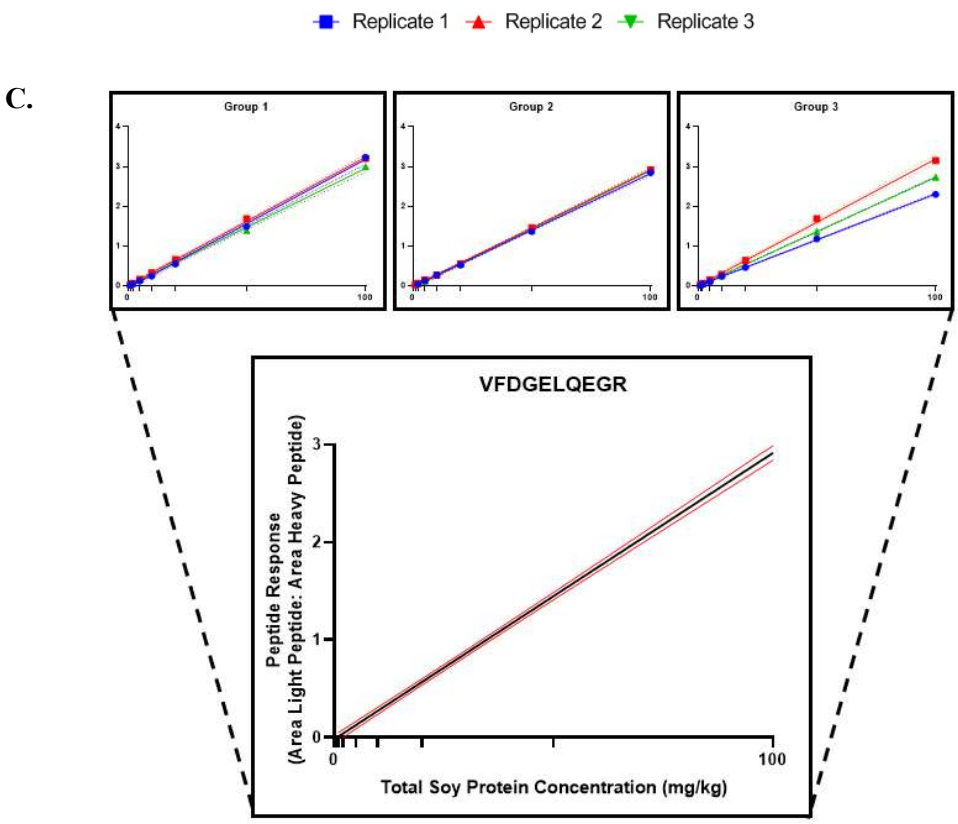


Figure 3-3. External standard curves prepared with the after reconstitution (AR) method of dilution. Each peptide response was graphed separately and is represented in each panel LSAQYGSLR (A), NILEASYDTK (B), and VFDGELQEGR (C). Each group consists of triplicate standard curves labeled as "Replicate 1", "Replicate 2", and "Replicate 3". The bottom graph in each panel shows the pooled standard curve (n=9 replicates). The black line shows the best fit average curve, and the red lines show the 95% confidence intervals.

The random effects of within group and among group on the linear regression model were determined for each peptide (Table 3-3). Within day and across day are considered random variables. The variance components are several orders of magnitude less than one, indicating a small impact of these random effects on the linear regression of standard curve replicates. For future experiments, the standard curve may not be required to be prepared in tandem with every sample because preparing samples on different days will not result in higher variability than samples prepared in tandem. The standard curve can be representative of the variation of a set of unknown samples if prepared on a different day. This is important for streamlining the method because of the time intensive sample preparation and MS acquisition time contributed by the standards.

Table 3-3. Variance estimations of the random replicate effects within group and across groups on the linear regression model.

	Variance Estimate			Standard Deviation		
Random Effect	LSA	NIL	VFD	LSA	NIL	VFD
Within	0.0025	0.0072	0[A]	0.051	0.085	0.062
Across	0.0041	0	0.322	0.064	0	0.0191
Residual Error	0.0155	0.02	0.719	0.124	0.142	0.105

[A]Standard deviation of the variance estimate
[B]Values reported as 0 were $< 1 \times 10^{-6}$

The linear regression model was first fit to the data without forcing the line through x=0 or y=0. The 95 % confidence interval of the y-intercept and x-intercept both included 0. Since the 95% confidence interval included 0, it was concluded these intercept values were not significantly different from 0. The final regression model was fit with an ordinary linear regression slope that was forced through x=0 and y=0. Linear regression slopes were then tested for any significant differences within groups. Several replicate group slopes were determined to be significantly different. This significant slope variation can be explained by a residual analysis. As can be seen in Figure 3-4, there is an

increase in the variation of the peptide response as the concentrations increases. All peptides had this variation from a linear regression fit.

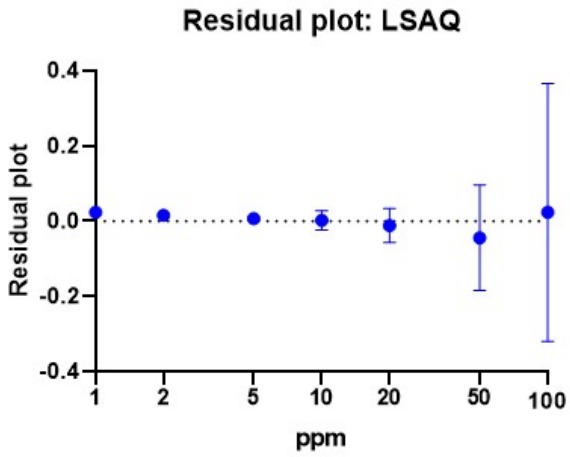


Figure 3-4. Residual plot of the linear regression at each external standard concentration (1, 2, 5, 10, 20, 50 and 100 mg/kg total soy protein) measured for the peptide LSA.

Although the slopes may be statistically significantly different, it is not necessarily significant for the purpose of the method. The significance between the slopes of the final quantification value for the incurred samples was then investigated. To test the effect of the different slopes on the quantification of unknown samples, three slope values were used to estimate soy protein in the incurred samples. The three slope values were obtained from the final standard curve of each peptide. The low and high range of slope values from the 95% confidence interval and the best-fit value from the pooled standard curve. The slope values tested for each peptide were as follows: LSA (0.0217, 0.0224, 0.0231). NIL (0.0364, 0.0369, 0.0375), and VFD (0.0287, 0.0291, 0.0295). The estimated quantitative values of each extract from the three slopes is shown in Figure 3-5.

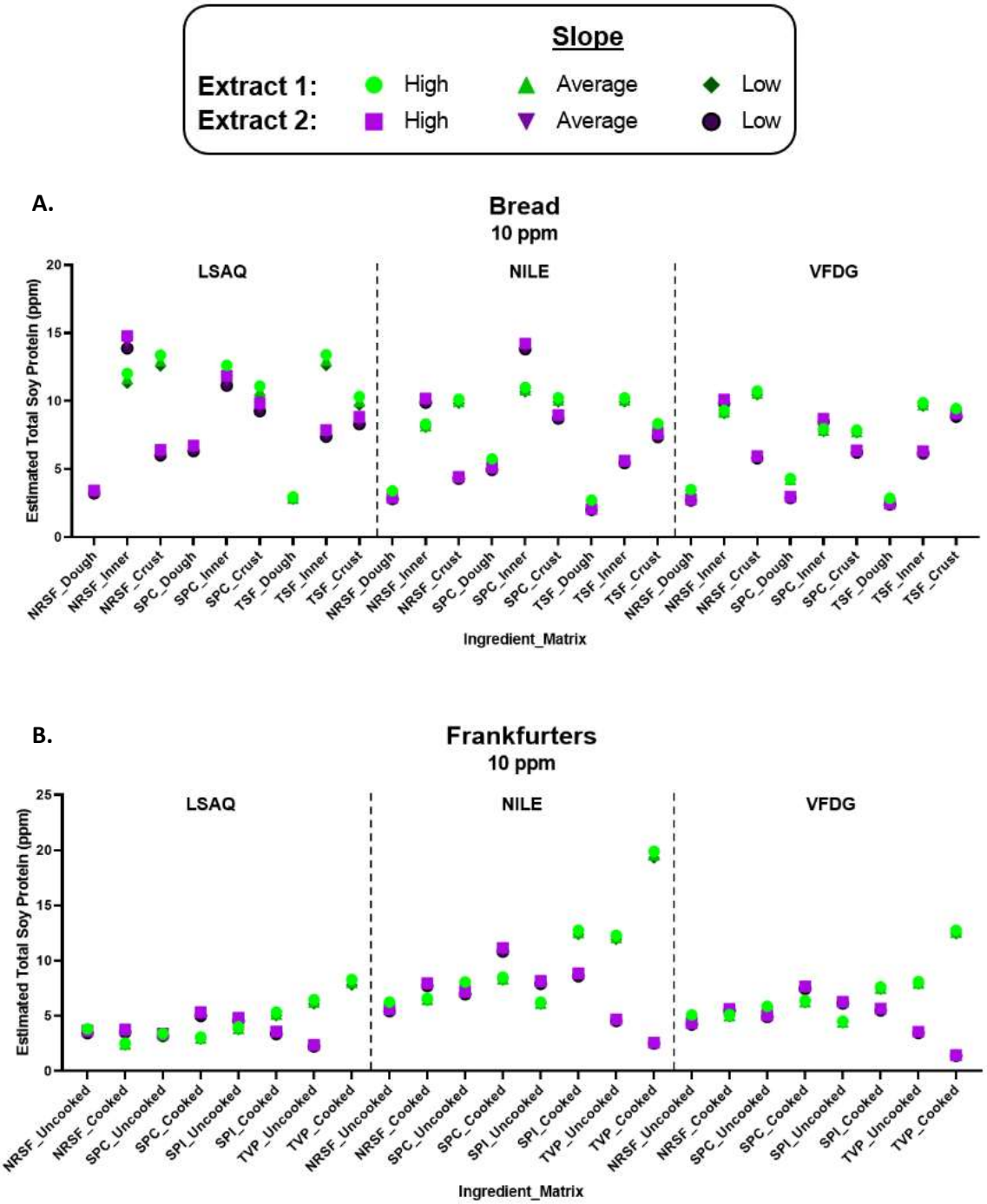


Figure 3-5. The total soy protein concentration of incurred samples was estimated using linear slope values of the pooled standard curve. Three slope values were used to estimate total soy protein: the best fit average and the high and low range of the 95 % confidence interval of the best fit line. Duplicate extracts were estimated and each is graphed in a separate color. All incurred samples have 10 mg/kg total soy protein.

The three estimated quantitative values appear almost superimposed with one another when quantifying a single extract. There is a higher variation among replicate extracts compared to the slope values for the estimated quantitation. Therefore, the variance between the slopes across days was determined to not affect the final quantification value. These predictive values were in a narrow enough range to conclude that the slopes are not significantly different for the purpose of the method. The protein recovery of the incurred samples was then calculated using quantification values from the average slope value.

C. Percent Recovery of Total Soy Protein in Model Foods

Cooked and uncooked matrices were evaluated to determine the effect of heat treatments on the recovery of soy protein. Total soy protein was spiked into the matrix based on the total uncooked weight. The total soy protein for uncooked and cooked bread matrices was then adjusted to account for water loss during proofing and baking (Table 3-4). The uncooked frankfurters have no water loss, and the total protein concentration was only adjusted for the cooked frankfurters (Table 3-5).

Table 3-4. Adjusted total soy protein concentrations of white bread matrices after heat treatment.

Total Soy Protein Concentration (ppm[A])	Cooked Matrix Sample Preparation	Pair of heat treated matrices	Soy-Derived Ingredient		
			NRSF[B]	TSF[C]	SPC[D]
1	WL[E]	Uncooked	1.08	1.09	1.11
		Cooked	1.20	1.17	1.20
	S[F]	Uncooked	1.09	1.08	1.10
		Cooked	1.18	1.21	1.19
10	WL	Uncooked	10.03	10.05	10.17
		Cooked	11.01	10.88	10.88
	S	Uncooked	10.02	10.12	10.12
		Cooked	10.95	10.99	10.87
100	WL	Uncooked	100.14	101.58	101.23
		Cooked	109.62	109.02	109.23
	S	Uncooked	100.68	101.38	101.44
		Cooked	110.98	109.21	109.64

[A]ppm = mg total soy protein/ kg matrix
[B] Non-roasted soy flour
[C]Toasted soy flour
[D]Soy Protein Concentrate (Arcon S)
[E]Whole Loaf
[F]Slices

Table 3-5. Adjusted total soy protein concentrations of white bread matrices after heat treatment.

Total Soy Protein Concentration (ppm[A])	Replicate	Pair of heat treated matrices	Soy-Derived Ingredient			
			NRSF[B]	SPC[C]	TVP[D]	SPI[E]
10	1	Uncooked	10	10	10	10
		Cooked	12.32	11.31	13.34	12.27
	2	Uncooked	10	10	10	10
		Cooked	11.83	12.22	13.01	12.36
50	1	Uncooked	N/A	N/A	50	50
		Cooked	N/A	N/A	67.81	60.34
	2	Uncooked	N/A	N/A	50	50
		Cooked	N/A	N/A	66.68	62.74

[A]ppm = mg total soy protein/ kg matrix
[B] Non-roasted soy flour
[C]Soy protein concentrate (Arcon S)
[D]Texturized vegetable protein
[E]Soy protein isolate (non-hydrolyzed)

Each sample was extracted and injected in duplicate (n=4). The average of the peptide ratios from each duplicate injection were calculated for one extract. Therefore, three peptide quantitative values were estimated for each extract, resulting in a total of six soy protein concentration estimates for each incurred matrix. In an attempt to reduce the complexity of the results, the estimated values were condensed into a single quantitative value. A single result is easier to interpret in a routine quality control analysis. ELISA is one of the most widespread protein assays for quality control purposes in the food industry. The main advantages are the ease of use during sample preparation and data analysis. ELISA kits have software that requires minimal user expertise to understand results. Therefore, it is important to try to streamline MS-based methods, so lab personnel

are not required to have extensive expertise for interpretation of the results. This MS method consists of quantification results expressed as total soy protein from three peptides. The results can be condensed into one final quantitative value using these three peptide responses. The final result was condensed either by taking the average or the maximum of all the quantitative results for each extract (Figure 3-6).

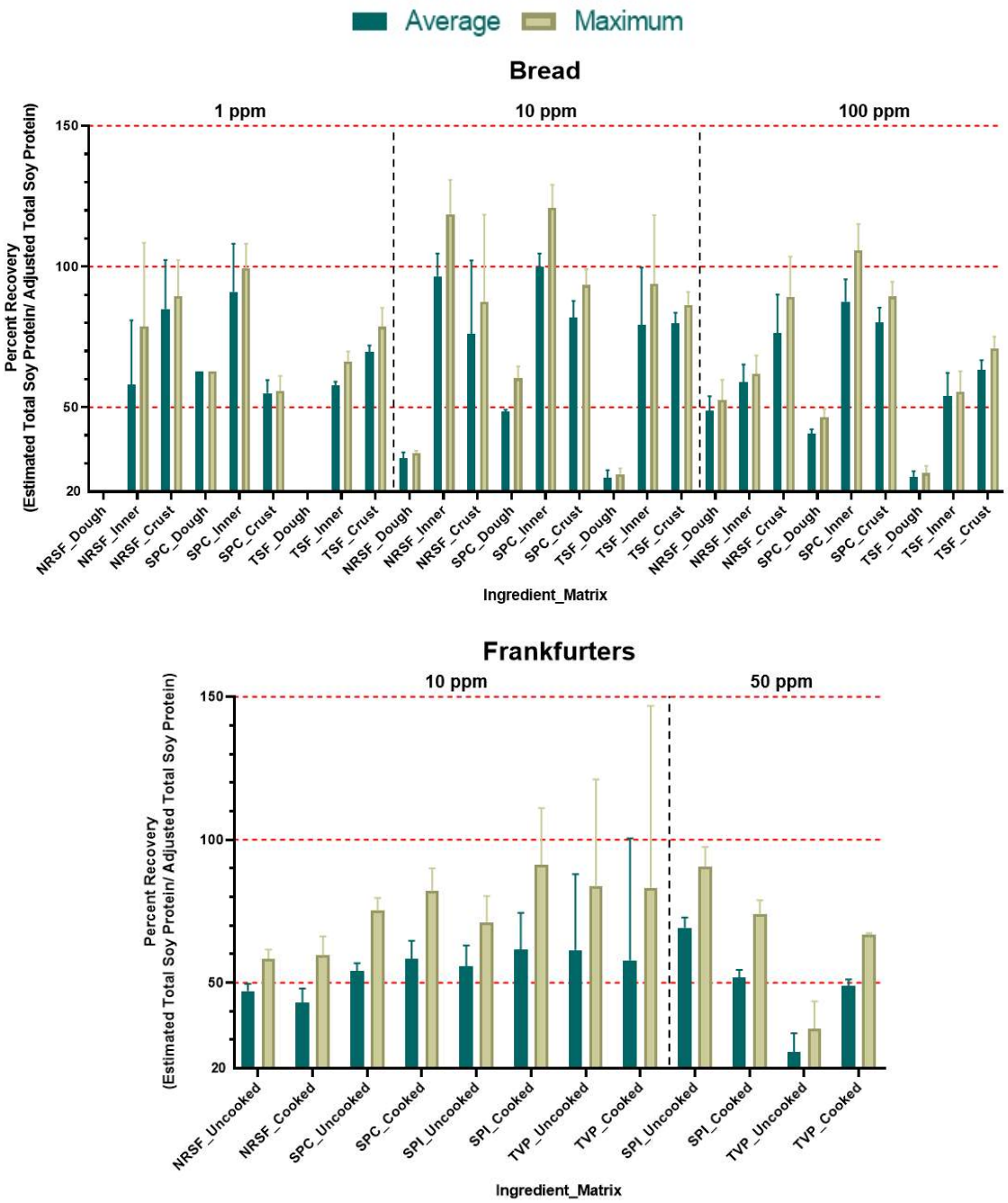


Figure 3-6. Percent recovery of the total soy protein concentration (mg/kg) in bread and frankfurter incurred matrices. The estimated total protein concentration was determined for each quantifying peptide using the best fit average slope. The average and maximum of all peptide estimations was then calculated for each extract. Each bar is the mean percent recovery of the duplicate extracts and error is represented as the standard error of the mean.

Three forms of bread were quantified: dough, inner, and crust. The dough matrices correspond to the replicate batches prepared in tandem with the sliced bread. Only a single injection for 1 mg/kg dough incurred with SPC was able to be quantified. Dough samples with higher total soy protein levels were able to be quantified at 10 and 100 mg/kg. At each level, the protein recovery of the dough, for all ingredients, was appreciably lower compared to the cooked matrices.

Overall, the cooked matrices had higher percent recoveries in bread at every incursion level compared to the uncooked. At 1 mg/kg, all inner and crust samples were able to be quantified for all injections. At 10 mg/kg, the inner portions of bread had slightly higher recoveries than the crust counterparts. A higher recovery of soy protein in the inner was expected and has been observed in previous studies (*19*). Interestingly, at 100 mg/kg the crust had higher protein recoveries for NRSF and TSF samples. Percent protein recoveries reported by the maximum and average had a relatively small difference. The cooked matrix fell between 50-120% recoveries and the uncooked matrices were between 25-35 % recoveries.

Two forms of frankfurter were quantified: uncooked and cooked. Similar to the bread matrices, higher percent protein recoveries were estimated for the cooked matrices. NRSF had the lowest total percent recoveries for the cooked and uncooked matrices. The largest variation was observed for the replicate extracts of TVP, as can be seen by the error in the 10 mg/kg cooked matrix. This variation was from dramatic difference in estimation between the replicate extracts. This may be explained by the dispersibility or solubility of TVP in the frankfurter matrix. The other soy ingredients, such as SPI or

SPC, are processed to increase the solubility. Therefore, there may be less variation among replicates for SPI and SPC compared to TVP.

D. Effect of Matrix and Heat on Protein Recovery

Uncooked and cooked matrices were evaluated to determine the effect of heating on the detection and quantification of target soy peptides. Heat can cause structural modifications to food proteins. These changes decrease protein extractability because of protein aggregation and can introduce variability to results depending upon the extraction method (*8*). Heat can also drive chemical modifications of food proteins. A well-known chemical reaction, known as the Maillard reaction, between proteins and reducing sugars is driven by heat. This reaction induces chemical changes such as the cross-linking of proteins (*7*). Because of these principles, the uncooked matrices were expected to have higher protein recoveries compared to the cooked matrices. Interestingly, the opposite was observed, and higher protein recoveries were calculated for both uncooked bread and frankfurter models within each total soy protein spike level. Therefore, the matrix was determined to be the most influential factor on protein recovery and will be further discussed.

Model Bread Matrix

The decreased protein recoveries in the uncooked compared to the cooked bread is most likely from the strength of the glutinous matrix (*20*). Gluten consists of two major classes of proteins, gliadin and glutenin, which contribute strength and elasticity, respectively, to the structure of wheat bread dough (*21*). These proteins form a three dimensional network after the addition of water and mixing. Covalent bonds between the proteins stabilize the polymer network with several types of intra- and intermolecular

crosslinking bonds between side chains (disulfide bonds) or amino acids (tyrosine bonds) (*22*). Other non-covalent bonds (e.g. hydrogen bonding) contribute to the structure of gluten and overall viscoelastic properties of dough (*21*). The decrease in total soy protein recovery of uncooked bread matrices may be from the strong elastic network of proteins that may remain intact during extraction, which prevents complete protein extraction.

The total protein extraction in gluten may be lower even in MS-compatible buffers. Gluten proteins are not readily solubilized in aqueous buffers. Increased solubility of gluten proteins has been observed in ethanol buffers containing reducing agents at higher extraction temperatures (*23*). Although MS-compatible buffers have harsher extraction techniques containing chaotropic agents (urea) and reducing agents (dithiothreitol), the gluten may not be completely solubilized. A comparison of total protein extracted in different MS-compatible buffers found lower total protein recoveries of gluten compared to other proteins (*24*). Soy protein was shown to be highly soluble in buffers (e.g. urea), from 88-95 % total protein extracted, compared to gluten under the same extraction conditions, from 8-39 % total protein extracted (*24*). A decrease in the total protein extraction may explain the overall decrease in the total soy protein recovery of the dough matrices. However, not all soy proteins may be affected the same because of peptide specific interactions with the matrix. The recovery of milk proteins in a model glutinous matrix using ELISA found a higher recovery of casein protein in the cooked matrix compared to the uncooked matrix (*25*). In contrast, other milk proteins tested, such as β-lactoglobulin, had higher recoveries in the uncooked glutinous matrix compared to the cooked (*25*). For the bread matrix, similar low protein recoveries were obtained for all quantifying peptides, representing the proteins beta-conglycinin and glycinin.

The digestion conditions for this method were optimized using extracts of NRSF as the reference material. Additionally, two additions of 1:100 (trypsin:protein) was determined to generate similar peptide responses compared to a single addition of 1:50. However, the low peptide responses in the dough matrices may suggest gluten is not completely digested with trypsin. Trypsin is an endoprotease present in the digestive tract which specifically cleaves peptide bonds after lysine and arginine from the carboxyl end, except after proline. Gluten is known to be resistant to proteases in the digestive tract, causing chronic adverse immune responses for people with celiac disease (*26*). Proline is an amino acid that does not contain a side group contributing to unique secondary structure configurations. Proteins with high concentrations of proline are hypothesized to be resistant to digestion because of the steric hinderance influenced by the unique folding (*27*). Proteins in gluten have documented to be resistant to trypsin digestion because of the high concentration of proline residues (*28*). In MS workflows, a cocktail of digestive enzymes including trypsin, chymotrypsin, and pepsin are commonly used to increase the number of peptides during digestion for higher coverage of the protein (*29*). Therefore, only a single proteolytic enzyme may not effectively digest gluten proteins especially for the detection of residual amounts of total soy protein (1 mg/kg).

The cooked matrix may have a weakened protein network which allows an easier release of proteins. Air is incorporated into the gluten matrix during proofing, and after cooking the protein network expands as it entraps air (*30*). The gluten network may be weakened by other components in bread such as the swelling of starch granules (*20*). The expansion of the protein network increases the surface area available for solubilization by the buffer and may allow accessibility of trypsin for more complete digestion. Therefore,

extraction and digestion efficiency are increased in cooked matrix by a weakened protein network, which allows an easier release of proteins, larger solvation space for the extraction buffer, and more accessibility to sites that may not be available in the uncooked matrix.

The viscoelastic form of the uncooked matrix seemed to contribute to the decreased extraction of soy protein in uncooked dough. The cooked bread samples were able to be effectively ground into uniform and small particle sizes, as determined visually. The bread particles were able to disperse uniformly in the extraction buffer after thorough mixing. The dough samples started as a more heterogeneous material. The uncooked samples were unable to be blended in a food processor due to the viscoelastic texture. Instead, dough subunits were “homogenized” with physical force into smaller pieces. These pieces were larger and more heterogeneous in size compared to the cooked matrix. As gluten heats, the protein elasticity increases and adsorbs to the walls of the equipment, such as test tubes. This prevents uniform distribution of the matrix in the buffer. This phenomenon was especially evident following extraction. After extraction, the warm dough matrix would coagulate at the bottom of the tube, preventing redistribution even after a 40-minute shaking step. Another physical form of the dough was tested by grinding the dough samples in a Freezer/Mill (SPEX 6850). This resulted in sample particles that were finer and more homogenous compared to the cooked sample particles. Preliminary analysis of the peptide recoveries between the two physical forms of dough concluded there was no improved detection of any target peptide. Therefore, although the Freezer/Mill physical form of the uncooked dough was smaller and more

homogenous, the rheological behavior of gluten still inhibits the extraction and detection of soy protein.

The low recoveries of soy protein in the uncooked dough matrix is likely from combined factors including the partial protein solubilization and incomplete digestion of gluten proteins. The gluten structure may remain intact during extraction and decreases the total availability of protein in solution. The physical structure of gluten, especially after heating, prevents the homogenous solubilization of dough pieces during extraction. The decreased particle size of the dough matrix did improve the extraction of total soy protein from the incurred matrices. The increased extraction temperatures cause the dough matrix to stick to equipment and prevents uniform distribution of sample in the extraction buffer. The cooked matrix was more effectively homogenized in buffer and the weakened gluten structure during cooking improve protein extraction and downstream detection. Future work should include other measures to weaken the gluten protein network before analysis.

Model Frankfurter Matrix

Sausages, hotdogs, bologna, and frankfurters are all commonly consumed meat products. Frankfurters are a type of meat product that originated from Germany and are characterized by the type of meat used or spices incorporated (*31*). The detection of soy proteins in adulterated meat products has been extensively studied (*9, 32, 33*). For MS-based methods, many detection methods have been developed focusing on soy protein peptide markers in meat products for food fraud purposes (*34-36*). There remains a need for the quantification of soy protein in meat products for the purpose of food allergen risk assessments.

The cooked matrices were expected to have lower total protein recoveries due to the heat denaturation of proteins. Previous work by Montowska et al., compared the effect of heat on the total protein extraction of slices of different types of meat and found a correlation between lower total protein extraction of meat samples with longer thermal processing due to protein aggregation (*33*). Interestingly, similar to the bread matrices, the cooked frankfurter matrices analyzed in the current study had slightly higher percent recoveries of total soy protein compared to the uncooked matrices. The physical limitations discussed for the lower percent protein recoveries in uncooked bread may also play a significant role in the extraction of the uncooked frankfurter. The cooked frankfurter was able to be ground using a food processor into finer, homogenous particles. These particles were then able to be distributed uniformly in the extraction buffer after mixing. The uncooked frankfurter was pre-weighed before cooking and directly extracted. The final physical form of the sample was a single large mass that did not evenly distribute in the buffer. It is probable that the buffer may not be effectively penetrating the sample matrix and solvating the protein as effectively compared to the ground cooked particles.

Frankfurters are emulsions between meat proteins, water, and fat, in combination with salt, seasonings and other processing agents (e.g. nitrite, phosphate) (*37*). The combination of salt, water, and shearing force all act to solubilize and swell meat proteins. The resulting product forms a three-dimensional protein network with a gel-like texture that entraps and stabilizes fat in the meat emulsion (*38*). The main stabilizing interaction between the proteins is disulfide bonds, although noncovalent bonds (e.g. hydrogen bonding) are also present (*39*). Crosslinking of the proteins is stabilized by

other chemical bonds, specifically ε-N-(γ-Glutamyl)lysine bonds in raw meat (*7*). The strength of the gel-like protein network may not be effectively disrupted during extraction for the detection of soy protein in the uncooked samples.

The high fat composition of the uncooked frankfurter may also interfere with the MS protein assay. A small liquid fraction of the frankfurter, containing water and fat, is expelled from the emulsion after cooking. For discussion purposes, this fraction is referred to as "purge". The total protein content of the purge could not be determined by the 660 nm assay. The high fat content in the sample extracts may have been incompatible with the assay reagents. Additionally, the downstream analysis of the sample by LC-MS/MS found no positive detections of any target peptide peaks for all purge samples. After cooking, the fat expelled in the purge fraction lowers the total fat content in the cooked frankfurter matrices. Therefore, while it may seem like there is a higher protein recovery in the cooked frankfurters, it may be a result of less interference of fat on the detection of soy peptides.

NRSF and SPC were both incurred at 10 mg/kg in bread and frankfurter samples. For both matrices, VFD and NIL were the most consistently detected peptides with all injections of incurred frankfurter matrices determined to have a positive detection. The cooked bread matrices had overall higher recoveries of total soy protein for each ingredient compared to the cooked frankfurters. The opposite trend was seen in the uncooked matrices. A direct comparison of NRSF and SPC is challenging due to the numerous physical and chemical characteristics of the matrices which interfere with complete extraction of the total soy protein. However, the comparison does provide evidence of the matrix to be the primary factor affecting protein recovery.

E. Ingredient effects

The effect of protein recovery based on the type of ingredient was less apparent compared to the effect of the matrix. The detection of target peptides in bread incurred at 1 mg/kg was comparable across the ingredients, with 100 % detection of cooked matrices for at least one peptide. TSF has the lowest solubility compared to all the ingredients according to the manufacturer. The heat treatment throughout ingredient processing was expected to lower the extractability of TSF. This explains the slightly lower protein recoveries of the TSF incurred matrices at 1, 10, and 100 mg/kg.

The protein recoveries of SPC was slightly higher at 100 and 10 mg/kg compared to TSF and NRSF. According to the manufacturer information, SPC is a functional soy protein ingredient that has been alcohol-washed and pH-adjusted to improve the solubility. This brand of SPC is used as an emulsifier in soup and meat products because of the solubility properties. The high solubility of SPC was evident because of the detection of soy peptides in dough at 1 mg/kg. As discussed previously, the other ingredients were not detected in all injections at this level because of the inability to effectively extract protein from the matrix.

TVP had lower counts of positive peptide detection compared to the other soy ingredients. Interestingly, one of the replicate extracts at 10 mg/kg total soy protein showed low peptide responses for both the uncooked and cooked matrix. The other replicate extract, from a separate frankfurter, showed higher peptide responses that were comparable to other 50 mg/kg incurred matrices. This indicates TVP may have lower dispersion compared to the other ingredients in the meat base. This may cause a heterogeneous distribution in the incurred product where some subsamples may contain

higher concentrations of TVP. This explains the higher recovery of one of the replicate extracts and the very low recovery of the second. The low peptide recoveries were confirmed as positive detections and were above the limit of quantification (peptide response from the 1 mg/kg external standard). It is possible these proteins may be from cross-contamination from other high protein samples prepared in tandem, but this is unlikely because of the absence of any target peptide peaks in the blank samples. TVP is an extruded soy protein product with a fibrous texture which mimics the mouthfeel of muscle (*37*). This ingredient may be partially insoluble, effecting the homogeneity in the starting material and thus affecting the extraction of protein for MS analysis. This may explain the large variation between replicate extracts of TVP incurred frankfurters.

V. SUMMARY

The factors affecting the accuracy of the MS-based protein assay was the type of soy ingredient, heat treatment, matrix composition, and total soy protein level. In some cases, the lower total protein concentrations had higher percent recoveries compared to the higher incursion levels. This may be a result of the limit of solvation of the buffer rather than the extraction efficiency of the ingredient. The bread matrices had lower recoveries of soy protein in uncooked matrices for all soy ingredients. The cooked matrices had percent recoveries between 50-150 % for all total soy protein levels. The frankfurter matrices had similar protein recoveries for cooked and uncooked matrices, with the largest variation among the TVP extracts. The relatively high sample recoveries is an example of how MS-based methods can be advantageous over ELISA methods. As discussed in Chapter 1, preliminary analysis of the protein recovery of these soy-derived ingredients using ELISA was dependent upon the processing of the ingredient. Direct

extracts of TSF showed total soy protein recoveries as low as 2 % in the Veratox Soy ELISA. Processing-induced changes are expected in the structure of the protein and immunological-based methods rely on the structural integrity of a protein epitope for optimal protein binding. The ability of this method to detect highly processed soy ingredients, among other matrix and ingredient factors, allows for a more accurate determination of the risk of the soy protein in complex foods. Further risk assessment analysis is needed for a more accurate determination.

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