Spring 4-21-2020

Development of a Sandwich ELISA Targeting Cashew Ana o 2 and Ana o 3

Morganne Schmidt
University of Nebraska - Lincoln, morganne.schmidt@huskers.unl.edu

DEVELOPMENT OF A SANDWICH ELISA

TARGETING CASHEW ANA O 2 AND ANA O 3

by

Morganne Schmidt

A THESIS

Presented to the Faculty of
The Graduate College at the University of Nebraska
In Partial Fulfillment of Requirements
For the Degree of Master of Science

Major: Food Science & Technology

Under the Supervision of Professor Joseph L. Baumert

Lincoln, Nebraska

April, 2020

DEVELOPMENT OF A SANDWICH ELISA

TARGETING CASHEW ANA O 2 AND ANA O 3

Morganne Schmidt, M.S.

University of Nebraska, 2020

Advisor: Joseph L. Baumert

Cashew nut is the second leading tree nut allergy in the US. Cross-contact of cashew nut poses potential food safety risks for individuals with cashew allergies. Highly-processed foods, such as HTST/UHT cashew milks may lead to problems in cashew protein detection by current allergen detection methods. Therefore, the aim of this study was to develop a robust sandwich ELISA for detection of highly processed cashew proteins. Commercial cashew ELISAs were evaluated for their robustness and sensitivity in detecting cashew milk cashew protein. After unreliable results were determined, cashew Ana o 2 (11S) and Ana o 3 (2S) were semi-purified using established methods. Cashew Ana o 2 was reduced (11S R/A) for improved extraction and both the 2S and 11S R/A were used for rabbit immunization. Cashew specific IgG antibodies were monitored by determining their titer values. A 1:1 pool of the rabbit sera (11S R/A:2S) was used as the capture reagent while sheep anti-roasted cashew sera was used as the detector reagent in the optimized sandwich ELISA (LOQ; 0.3 ppm cashew protein). Potential matrix interference and cross-reactivity were evaluated in 58 food matrices including plant milks, tree nuts, spices, baking ingredients, and seeds. No matrix interference was found with any tested plant milk, with matrix interference found from 9 select seeds, spices and tree nuts. Certain foods in the Anacardiaceae family (pink peppercorn, pistachio, mango seed) were found to be cross-reactive. The sensitivity of the developed ELISA was

evaluated further with cashew protein incurred in pre- and post-processed almond milk and cookies. The high percentage recovery of cashew protein in almond milk, above 10 ppm cashew protein, before and after processing indicates that the developed ELISA can reliably detect heat-processed cashew nut proteins in foods. With cookies, high percentage recovery was obtained with incurred baked cookie while incurred cookie dough showed overestimation. More validation work is needed to ensure that the developed ELISA will support allergen detection for various food matrices and processes.

ACKNOWLEDGEMENTS

Firstly, I would like to thank God for giving me the opportunity to learn, apply, and meet such generous, kind, and talented people during my time here at the University of Nebraska-Lincoln. Without Him I can do nothing.

Secondly, I am so grateful for the guidance and teaching from my advisor, Dr. Joseph Baumert. If it wasn't for your willingness to lead me, I wouldn't have learned so much about allergen detection, safe food, experimental procedure and so much more. Not only did you give me the opportunity to learn academically, but also professionally. From attending out of state conferences and pushing me to apply my skills into the food industry, I have become a brighter, more confident leader who is ready for the road ahead.

I am also very thankful for a scientist who I most admire, Shyamali Jayasena. From helping me understand basic math (again), to assisting in what I saw as complicated experiments, to being someone who was always willing to drop something to help or teach a new task, I am forever grateful. I would not be where I am today without you. You are the smartest person I have ever had the pleasure of meeting and I will miss your shouts every day.

Thank you to Justin Marsh, for your expertise and time in helping with the mass spectrometry portion of my project. Your ability to teach and thoroughly explain was essential to me understanding some of the most difficult aspects of mass spec analysis.

To my friends and lab mates, Kristina, Jenna, Tengfei, Bini, Vera, and Shimin, thank you so much for the constant guidance when I asked for it or for the pep talk to get the job done. Your extra help was always given with a smile and I cannot thank you enough.

Thank you to the entire FARRP group, especially Julie, Pat, and Lynn. All of you were essential to me completing my time here. You always were there to talk through a project or offer some assistance. Thank you so much for your generosity.

To the Food Science Department, especially my committee members, Dr. Melanie Downs, Dr. Randy Wehling, and Dr. Steve Taylor, thank you for your time, commitment, and guidance on my project and overall academic success.

Lastly, I'd like to thank some of the most important people in my life. Thank you to my forever friends, Nevena, Sam, and Danielle for your never-ending support and consistent reminder to keep going and get the job done. Without you all I would not be the person I am today. Thank you to my family, mom, dad, Taylor, Kendall, Payton, Bonnie, Tammy, grandma, all of you, for pushing me outside of my comfort zone throughout my life and telling me that I can be whoever I want to be. I'm so blessed to have you and I hope I can continually make you proud. Finally, thank you to my husband-to-be, Aaron. You supported every aspect of my crazy path, long-distance and all. I can't wait to experience the rest of life with you.

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER 2

CHAPTER 3

CHAPTER 4

LIST OF FIGURES

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 1:LITERATURE REVIEW

I. INTRODUCTION

More than half of the world population has an allergy to a food or environmental allergen; making allergies a top concern worldwide (Owen, et al., 2013). Allergies in the body have two different types of manifestations: Immunoglobulin E (IgE)-mediated and cell-mediated. IgE-mediated reactions are much more common and because of this, unless otherwise noted these will be the type referenced when allergies are discussed. Usually, IgE is secreted in small amounts and used as the body's defense mechanism against parasites. However, after the initiation of an allergic reaction, IgE is secreted in excess toward whatever antigen (protein) is inducing its release (Owen, et al., 2013). Cross-linking of two cell surface-bound IgE antibodies, or the linking of two separate antibody epitopes to a single antigen, leads to a downstream series of events that eventually releases inflammatory mediators associated with the immune system which trigger the allergic response symptoms that are commonly seen (trouble breathing, rash, itchy throat, etc). To prevent these life-threatening symptoms from occurring consistently, when a person suspects they have an allergy, the typical procedure is to go to an allergist and get diagnosed. This diagnosis goes through a stepwise procedure of understanding the possible allergies the person could have based on exposure to an allergen and the resulting history of past reactions and then testing the person for eventual diagnosis. This can be done through skin prick tests (SPT), blood tests, food challenges, and patch tests, all of which help identify if a person is allergic to the assumed food or environmental factor (Muraro & Arasi, 2018). Each of these methods have been

developed to aid in the diagnosis of the allergy and in some instances attempting to understand an individual's reactive dose and severity of the allergic response.

Outside of diagnostic methods for allergic individuals, steps are also taken to monitor the presence or absence of current allergens in the food system. These steps include validation of allergen control programs and cleaning procedures which try to prevent cross-contact or mislabeling of undeclared allergens. This is of particular use for food allergens where cross-contact or mislabeling between allergens can occur on incoming raw materials, shared production lines, and even finished prepackaged food products that reach grocery store shelves. Both quantitative and qualitative analytical methods are used to determine if a food allergen is present in ingredients or finished products or on shared food contact surfaces. The common quantitative, immunochemical assay used for allergen detection is the enzyme-linked immunosorbent assay (ELISA). This assay is used frequently for its sensitivity, reliability, and ease of use. The common qualitative assay is the lateral flow assay, used frequently due to its commercial availability and quick response time in detecting the allergenic protein of choice (Monaci & Visconti, 2010). Other methods used for allergen detection are liquid chromatography-tandem mass spectrometry (LC-MS) and polymerase chain reaction techniques (PCR). However, LC-MS techniques are very expensive and are still being developed for commercial use while PCR techniques are only able to detect DNA from the allergenic source of interest, thus proving themselves to have limitations in regards to allergenic protein detection (Schubert-Ullrich, et al., 2009). These facets together make the ELISA the most commercially available and sensitive allergen detection method used in the food industry.

The high prevalence of tree nut allergies in the world continues to be of concern as it affects around 2% of the human population (McWilliam, et al., 2015). Food safety regulations and allergen control procedures aim to understand the potential risks associated with tree nut allergen exposure and maintain control over unclear labeling on foods and the unintended presence of allergen residue in foods to protect these individuals. Of the tree nuts, cashew nut allergy is consistently a high-risk allergen as it ranks second only to walnut in the US in the prevalence of tree nut allergy and is in the top tree nut allergies worldwide (Sicherer, et al., 2010). To detect these cashew proteins, cashew ELISA methods have been optimized and developed for both whole cashew extracts and individual allergenic proteins (Gaskin & Taylor, 2011; Wei, et al., 2003; Zhao, et al., 2019). In addition to ELISA methods, other methods of detection such as lateral flow assays have been found to be beneficial in detecting the presence of cashew protein (Masiri, et al., 2016).

While work is being done to ensure that cross-reactivity is not a concern on current cashew ELISA methods, heat processing can also have an imact on the effectiveness of an ELISA. As mentioned by Monaci & Visconti (2010), heat processing can cause significant changes to the tertiary binding epitopes of antigens. These changes can then affect the accuracy of an ELISA method in detecting the allergen of choice. If the antibodies which recognize the pertinent protein are not able to detect a denatured or modified protein from a highly processed system, problems in detection and recovery of the protein for testing may occur (Monaci & Visconti, 2010). Understanding how an ELISA detects a heat-treated allergen sample is important for a robust detection method.

Upon testing of current commercial cashew ELISA methods as described in the following chapters of this thesis, it was found that UHT processed cashew milk protein was not detectable or was detectable with low reliability. Because of the increasing prevalence of highly processed cashew beverages (e.g. ultra-high temperature cashew milk) and highly processed cashew food products (e.g. high pressure pasteurized cashew cheese dip), reliable methods to detect cashew proteins are needed. Based on immunoblotting using current commercial cashew antibodies, it was decided that a cashew ELISA which targeted Ana o 2 (11S) and Ana o 3 (2S) cashew proteins could be developed because of the proteins' overall stability as seen by initial testing and reported by other researchers as well (Mattison, et al., 2016). The purpose of this project was thus to develop a more reliable, sensitive, and robust cashew ELISA method which would be capable of detecting cashew protein from a highly processed cashew matrix.

II. ALLERGIES

a. Mechanism & Symptoms

In simple terms, allergies occur when an individual reacts to some type of allergen(s)/antigen(s). An allergic reaction is then initiated in the body and the individual exhibits a physical response. There are four possible pathways for an allergic reaction to occur; however, the most common is through the immune mediator, IgE. When an allergen enters the body through the mouth, skin, or respiratory tract, that allergen stimulates Th2 cells (a subset of T helper cells) to stimulate B cells, which then secrete allergen-specific IgE antibodies. The IgE antibodies bind to two Fc receptors, which are on mast cells and blood basophils. The cross-linking of two surface-bound IgE antibodies by the offending allergen then initiates the release of histamines and other inflammatory mediators from mast cells and basophils which in turn cause the typical allergic reactions of muscular contraction, increased vascular permeability, and vasodilation (Owen, et al., 2013). This shows the specificity of IgE and the importance of the location of mast cells or basophils during an allergic reaction.

Two things need to occur for an allergic reaction to manifest. The first is that the person must be sensitized to the allergen. This means that the person must be exposed to the allergen and an unknown immune trigger in their body results in the production of IgE antibodies towards that antigen. During this sensitization, no allergic reaction occurs. Dendritic cells in the intestine take up the food proteins and internalize them by phagocytosis. Ubiquitin detects these allergenic proteins and begins to degrade them by breaking them down into peptides. Major histocompatibility complex class-II (MHC-II) then presents these degraded peptides to naïve CD4 and T-helper cells. These T-helper

cells, induced by a variety of cytokines, mainly IL-4, then interact with B cells which go through a process of class-switching the antibodies produced in the cells into allergen-specific IgE antibodies. Activation occurs when IgE binds to the surface of mast cells or basophils. The activated IgE now can induce a series of downstream events including the release of specific cytokines and histamine which can in turn elicit the typical allergic reaction symptoms upon secondary exposure. This then leads to the second requirement for an allergic reaction to occur, the ingestion of the food protein a subsequent time. Upon the subsequent ingestion, the reaction/elicitation phase occurs. This is where the sensitized antibodies bound to the surface of mast cells or basophils can cross-link with the food protein to stimulate the release of histamine and cytokines to initiate an immune response. This produces the typical symptoms of an allergy which include hives, rashes, itchiness, and/or anaphylactic shock (Kumar, et al., 2012).

The symptoms of an allergic reaction range from minor to very severe and can vary person to person. A potentially severe reaction is systemic anaphylaxis, which may start out with symptoms from any of the organ systems associated with allergies such as the respiratory system, moving to anaphylactic shock and finally asphyxiation. It is estimated that food allergies account for 30-50% of anaphylactic reactions; indicating its large impact (Cianferoni & Muraro, 2012). Anaphylactic shock is often fatal and can be controlled only if epinephrine is injected into the body very early in the course of an allergic reaction to counteract the effects of mast cell or basophil degranulation (Owen, et al., 2013). Other serious, yet sometimes minor reactions only affect localized areas of the body. For example, rashes on the skin, sneezing, or a runny nose can be attributed to

allergens that have come into direct contact with the surface of the skin, ingested, or inhaled due to environmental factors.

Overall, allergies can manifest themselves in multiple ways which can be different for each person. An allergic reaction, stemmed from an increase in mast cell production of mediators such as histamine or cytokines, causes symptoms ranging from simple sneezing to fatal anaphylactic shock. Identifying these allergens for an individual has proven to be difficult. The two main categories of allergens are food and environmental factors. Environmental factors can include airborne allergens such as dust mites, pollen, or animal dander. Both of these categories revolve around supposed reference thresholds and symptoms that are characteristic of that category. Considerable research has been done to characterize allergenic proteins, although identifying common allergens in the overall population is difficult, due to environmental factor changes and the individual changes of a person, which is why differing diagnostic methods must be used from person to person.

b. Types and Prevalence

Worldwide, efforts have been made to make the public aware of possible allergens. Possible allergens can be from food or certain environmental factors and each comes with its own set of problems in diagnosing, identifying, and understanding. Certain reference doses have been established for protein from allergenic sources to help identify what the general public might be able to handle in terms of allergies (Allen, et al., 2014; Taylor, et al., 2014). These reference doses are especially important for

considerations of when to use precautionary labeling due to their impact on consumers at home, in restaurants, and in stores.

In the US, there are the “Big 8” allergens of milk, eggs, fish, crustacean shellfish, tree nuts, peanuts, wheat, and soybean that are considered the priority/major allergenic sources that require source allergen labeling. In addition, there has been a recent request of information by the FDA (FDA-2018-N-3809) in response to a citizens petition to include sesame as part of the Big 8 list in response to new research on the prevalence and reported severity of sesame allergy (Adatia, et al., 2017). In Europe there are the top 14, which include the Big 8 and additional allergenic sources including: cereals containing gluten (instead of wheat specifically), celery, mustard, sesame, Sulphur dioxide and sulphites, lupin, and mollusks (in addition to crustacean shellfish). All of these foods can be ingested, mostly imparting an IgE-mediated response in affected individuals (Gendel, 2012). Recent studies have also been looking into data which suggests that food allergies are on the rise. Researchers such as Loh et al. (2018) have estimated as much as 10% of the western population to have some type of food allergy, based on food-challenges given (Loh & Tang, 2018).

Food allergies occur when the food consumed initiates an IgE-mediated response as previously discussed. People react differently to different allergens however, there are specifically identified proteins in the food which cause the response in a majority of allergic individuals. For example, in peanuts the proteins which initiate the allergic reaction could be Ara h 2, a major peanut allergen and/or Ara h 10, a more minor peanut allergen, depending on the person (Santos, et al., 2018). This introduces a complex idea

of what a reference threshold for a person can be for a food, especially since doses change person to person. Taylor et al. (2014), along with other researchers, were able to identify certain reference doses, low doses which will not impart a reaction in approximately 99% of the population, for approximately 11 different common allergens. For example, whole peanuts were tested and given a reference dose of 0.8 mg whole peanut (0.2 mg of peanut protein). Through the low-dose oral challenges, they were able to reasonably assume, with room for error, an amount that would be safe for the vast majority in the allergic population to consume (Taylor, et al., 2014). This can be extremely beneficial for the food industry and for voluntary labeling purposes on packages such as "May contain traces of…" or "Made in a facility that processes…" as the use of reference doses could aide in the risk assessment and management processes needed to help decide if that facility needs to make these types of claims or not. Allergic consumers would benefit from the adoption of reference doses as potentially more products may become available for them to safely consume.

In addition to food allergies, environmental allergies are also very common. These environmental allergies can be induced by animal sources, mold, pollen, or other items people may come into contact with, such as latex or enzymes used in detergents (Basketter, et al., 2015; Rusznak & Davies, 1998). Environmental allergies usually cause respiratory problems, such as asthma or breathing problems, as well as some skin reactions (Rusznak & Davies, 1998). Atopic dermatitis is a common skin problem that is now being associated with allergies, suggesting that individuals who have this skin problem may be more susceptible to allergies from dust mites and pet dander (Cid, et al., 2019). These environmental allergies pose threats for affected individuals on a daily basis

and although common, can sometimes have less of a diagnosis than food allergies (Ferastraoaru, et al., 2017).

The prevalence of food and environmental allergies has been shown to be increasing by different researchers, both in the US, EU, and western countries (Loh & Tang, 2018). Currently in the US it is estimated that 11% of adults have a food allergy while it is estimated that 8% of children have a food allergy (Gupta, et al., 2018; Gupta, et al., 2019). Similarly, in the EU food allergy prevalence continues with an estimated 6% of adults having a food allergy and 8% of children (Loh & Tang, 2018; Lyons, et al., 2019; Nwaru, et al., 2014). Prevalence however, changes based on location in the EU as well, depending on the country (Lyons, et al., 2019). Environmental allergies worldwide manifest as hay fever or rhinitis and their prevalence is also high, with 10-30% of the population diagnosed with hay fever (Pawankar, et al., 2014). While food and environmental allergens can be separate allergies for individuals, there can also be cross-over between the two. For example, individuals who are allergic to pollen may also be allergic to certain food proteins. These people may then be diagnosed with pollen food syndrome (PFS), occurring when a pollen-allergic individual also reacts to food proteins found in certain fruits and vegetables (Edwards & Halton, 2019). This phenomenon is possible due to pollen proteins having a high degree of homology to the proteins found in some fruits and vegetables such as apples and celery. It has been found that 2-5% of the population may have this syndrome and thus efforts to understand these allergic diseases are also high (Edwards & Halton, 2019).

With the high prevalence of allergies worldwide, certain allergies are of more of a concern than others in terms of age and type. Food allergies are of high concern due to the severity and frequency of reactions, mostly in part due to accidental ingestion in foodstuffs. Age of an allergic individual matters as younger individuals can outgrow certain allergies. For example, children who are allergic to milk and eggs are likely to outgrow their allergy (Kim, et al., 2020). In comparison, children who are allergic to peanut, shellfish and tree nuts are less likely to outgrow these allergies (Gupta, et al., 2013). Type of allergy also shows importance in determining the severity of allergies. Of the Big 8 allergens in the US, shellfish, peanuts and tree nuts account for some of the most common food allergies (Gupta, et al., 2019). Of those common allergies, tree nuts are a major concern due to the potency of the allergen (Bock, et al., 2001). Of those tree nut allergies, cashew along with walnut are the most prevalent in the US and other western countries (Mendes, et al., 2019). Gupta and others (2019) determined that an estimated 1.2% of the US population has a tree nut allergy, with 0.5% having a cashew allergy. A review by van der Valk et al. (2014) found that cashew nut allergy is increasing, thought to be due to increased cashew nut consumption (van der Valk, et al., 2014). A separate study by McWilliam and others (2015) estimated that over the past ten years, the prevalence of cashew allergen in children has increased by almost 2%, while the prevalence of food allergies in adults shows some evidence of increasing as well (McWilliam, et al., 2015). Due to the increasing prevalence, severity, and potency, cashews are considered a major allergen and need to be regulated for the safety of those cashew-allergic individuals.

III. CASHEW NUT

a. Consumption & Allergenic Proteins

Cashew nuts are widely consumed around the world. The International Nut and Dried Fruit Council's statistical report (2017-2018) claimed that cashews were within the top three most popular nuts consumed in the US and Europe, alongside almonds and walnuts (Council, 2017). In the US, cashew consumption is on the rise, accounting for approximately 17% of tree nuts consumed (Council, 2017). Cashew farming has also been on the rise, increasing 32% over the past ten years in comparison to the previous decade (Council, 2017). Even though cashews don't vary widely in origin based on the limited climate they can grow in, cashew varieties have been proven to have similar nutrition and allergenic protein content (Reitsma, et al., 2018).

Cashews are from the genus Anacardium and of the species, occidentale (Mori, 1987). According to the USDA, raw cashews are approximately 18% protein, 43% fat, and 30% carbohydrates (USDA, 2018). The three major cashew allergens are Ana o 1 (7S), Ana o 2 (11S), and Ana o 3 (2S) (Mendes, et al., 2019). The 7S seed storage protein has a molecular weight ~50 kDa (Reitsma, et al., 2016). This protein consists of vicilin-like, sucrose-binding proteins (Wang, et al., 2002). The 11S seed storage protein has a molecular weight ~53 kDa, is a part of the legumin family and has been shown to be immunogenic (Reitsma, et al., 2016; Wang, et al., 2003). The 2S cashew protein has a molecular weight ~12.6 kDa and is a part of the albumin family, also showing immunogenic capabilities (Teuber, et al., 2002). Under reducing conditions, the 11S will split up into an acidic 33 kDa subunit and a basic 20 kDa subunit while the 2S will split up into 6, 8, and 10 kDa large subunits with some, yet-to-be identified smaller subunits or

isoforms (Teuber, et al., 2002). The 11S protein makes up about 50% of the total soluble protein in a cashew while the 2S protein makes up about 11.5% and the 7S protein makes up about 5% (Roux, et al., 2003; Sathe, 1994; Zhao, et al., 2019).

b. Detection Methods

Because of the high prevalence of cashew consumption and the commonality of cashew allergy, it is imperative that current cashew detection methods meet the needs of the food industry and regulating bodies. To ensure safe food for cashew allergic consumers, multiple ELISA methods have been developed to increase sensitivity, robustness, and reliability based on differing protocols (Gaskin & Taylor, 2011; Wei, et al., 2003; Zhao, et al., 2019).

ELISA works by detecting residues of allergenic proteins after binding to IgG antibodies specific to that protein. There are four main types of ELISA methods used in protein detection: Competitive, Indirect, Sandwich, and Direct. The sandwich method is most widely used for cashew allergen detection due to its high sensitivity and reliability (Aydin, 2015). In a sandwich ELISA, the capture IgG antibody, raised specifically for the desired antigen(s), is bound to the solid phase. The sample extract containing the analyte of interest is then added to the plate and incubated. During this time, the antigen (if present) binds to the antibody and a complex is formed. The plate is then washed to get rid of any unbound analyte and other compounds in the extract. An enzyme-labeled secondary antibody is then added, which binds to the antigen-antibody complex already bound to the solid phase. A wash step follows and a final substrate specific to the enzyme is then added. When the enzyme reacts with the substrate, a color change is observed and this color change indicates the presence of the analyte. Finally, the color can be measured

using a spectrophotometer which then gives an absorbance reading which is compared to a calibrated standard curve. The concentration of the protein in the sample can then be calculated and used for protein quantification (Aydin, 2015).

The competitive ELISA is the other common method for protein detection (Aydin, 2015). In a competitive ELISA, the antigen used to sensitize the animal for antibody development is bound to the solid phase. Once bound, the plate is blocked to prevent any unwanted protein from binding to the plate. During blocking, a separate plate incubates, containing both the raised antisera and the analyte of choice (if present in the sample extract). Once incubated, the sample extract is added directly to the blocked plate so that any unbound antibody can now bind to the coated plate. This is allowed to incubate and an enzyme-labeled antibody is then added. The enzyme-substrate reaction results in a color changes which indicates that the substrate has bound to the enzyme-labeled antigens, not the analyte of choice as in a sandwich ELISA. Thus, the spectrophotometer absorbance values have an inversely proportional relationship to the concentration of the protein of interest. A higher concentration of the analyte of choice means a lower absorbance while a lower concentration of analyte means a higher absorbance (Sharma, et al., 2009).

The first cashew ELISA method to be developed was by Wei et al. (2003). They created a sandwich ELISA which focused on targeting the 11S (identified as 13S at the time) cashew protein, Ana o 2, using antibodies raised against cashew 11S in both rabbits and goats. This method was based on a standard curve of the cashew major protein (11S) and thus recorded a limit of detection of 0.02 ppm 11S protein. Approximately 50% of cashew is 11S protein so for comparison, this is a limit of detection of 0.04 ppm cashew

protein (Wei, et al., 2003). Gaskin & Taylor (2011) developed a sandwich cashew ELISA as well; however, they raised antibodies against a crude extract of whole cashew nut soluble proteins instead of a specific protein. Their method also proved to be successful with a limit of detection of 0.023 ppm cashew protein (Gaskin & Taylor, 2011). A recently developed sandwich cashew ELISA, developed by Zhao et al., (2019) focused on targeting the 2S cashew protein, Ana o 3, due to its high resistance to both pH and heat. This method also proved to be successful with a stated limit of detection of 0.04 – 0.06 ppm cashew protein (Zhao, et al., 2019).

Other ELISA methods for cashew have been developed such as multiple allergen recognition assays. These multi-allergen screening immunoassays are able to recognize more than one allergen at one time, rather than the usual ELISA, which can only detect allergens individually. A competitive indirect assay, developed by Ben-Rejeb et al., (2005) was able to detect peanut, cashew, almond, hazelnut, and brazil nuts in one system. This ELISA was specifically used for a chocolate matrix as these tree nuts are found in chocolate containing confections due to cross-contact of shared processing equipment. The limit of detection for their matrix was found to be 0.01 ppm of allergenic protein for each allergen and was shown to be very sensitive in the chocolate matrix in comparison to similar LODs of cashew (Ben-Rejeb, et al., 2005). Another more recent multi-allergen detection system is the fluorescent multiplex array (xMAP). This system uses a variety of monoclonal and polyclonal antibodies to detect multiple allergens in the same complex food. Black et al. (2019) recently developed an xMAP which could detect eleven major food allergens from peanut, cow's milk, shellfish, egg, cashew, soy and hazelnut. They showed a lower limit of detection of 0.01 ppb for some of the purified

allergens with an overall recovery on all purified allergens between 70-130%, indicating the possibility of this multi-allergen detection system being used in the food industry (Black, et al., 2019). In the future, multi-allergen immunoassays may become more popular as they are developed to make testing easier for food companies and to ensure quicker results.

Other methods to detect cashew residue include lateral flow devices (LFD), mass spectrometry, and PCR methods. A lateral flow device, while informative, only gives qualitative evidence on the presence or absence of cashew proteins by giving a negative or positive result. The device itself has a sample pad and a conjugate release pad. The sample pad allows the liquid sample with the analyte to be applied and drawn to the conjugate release pad. The sample pad contains specific reagents which allow the sample to flow through capillary action. The conjugate release pad then contains the antibodies which can bind to the liquid analyte. Once binding occurs, the liquid continues to flow into the detection zone. This is where a test line, a control line, and then finally an adsorbent pad are placed. The test line only shows if the antibodies have bound to the specific analyte and can vary in intensity depending on the type of LFD. The control line proves that the liquid has made it to the detection zone which in turn helps to prevent false positives. The adsorbent pad ensures that no backflow occurs in the system and that all the liquid is wicked away (Masiri, et al., 2016). New lateral flow devices have been developed to specifically be able to detect cashew protein in non-dairy based beverages as well. The advantage of this is that the matrix, which may interfere with a lateral flow assay, would have a negligible effect since the device is specifically meant for a cashew milk matrix. Masiri et. al (2016) recently designed both a sandwich-based and a direct

lateral flow assay with a limit of detection of 1 ppm in food to detect cashew milk protein. This method was termed “semi-quantitative” as it was able to give rough estimates of the protein content when paired with a calibrated electronic strip reader (Masiri, et al., 2016). This LFD method proved to be effective across multiple nut milks including soy, almond, cashew, and others and was deemed as a fast and simple way to detect the protein of interest.

Real-time PCR methods which focus on cashew detection in different matrices are also being developed. These PCR methods amplify the DNA of the desired analyte, which then gives an indication of which proteins are present in a given matrix/food. A method developed by Lopez-Calleja et al. (2015) was successful in identifying cashew DNA in over 200 different commercial foods and was found to be very sensitive with a limit of detection of 0.1 ppm of total cashew (López-Calleja, et al., 2015). The disadvantage to this method is that DNA does not always equal protein and thus even if the DNA is extractable, its presence does not always indicate the presence of proteins. Mass-spectrometry methods have also been developed to identify proteins in a variety of allergenic foods. These methods have looked at identifying major peptides in tree nuts, such as cashew, and also changes in the proteins following heat treatment. Work by Mattison et al., (2016) focused on understanding the changes in 5 major peptides of Ana o 1, Ana o 2, and Ana o 3 following different heat treatments. They reported that peptides from Ana o 1 and Ana o 2 change the most during heat treatment while peptides from Ana o 3 do not show any heat induced variations. However, due to the abundance of Ana o 2, its inclusion in future work is necessary for accurate cashew protein quantification

(Mattison, et al., 2016). This work led to understanding the stability of these allergenic cashew proteins.

c. Potential Cashew Cross-Reactivity

Cross-reactivity, the possibility for a person to be allergic to another food which has similar protein epitopes with cashew, has been suggested with multiple foods such as citrus fruits, peanut, and other tree nuts. This is thought to be due to either similar protein structure type or similar family type (van der Valk, et al., 2014). Cross-reactivity with cashew in the Anacardiaceae family has also been seen with pistachio, mango, pink peppercorn, and sumac. In a study by van der Valk et al., (2017) cross-reactivity to mango and cashew was shown by SPT or immunoblot. However, no positive food challenge occurred when a cashew-allergic, mango sensitized individual was given mango indicating that not all sensitization equals clinical reactivity (van der Valk, et al., 2017). Another study by Che et al., (2017) showed the heightened possibility for a sumac allergy in a cashew-allergic individual, but no inhibition blotting or testing on the food source was done to prove this (Che, et al., 2017). Another recent study by Bastiaan-Net et al., (2019) tested the clinical cross-reactivity for pistachio, pink peppercorn, mango and sumac, and while pistachio and pink peppercorn showed clinical cross-reactivity, mango and sumac only showed co-sensitization (Bastiaan-Net, et al., 2019).

Cross-reactivities of cashew have been found to both pistachio and pink peppercorn, both thought to be due to their shared botanical family, Anacardiaceae. The cross-reactivity clinically proven to occur in both pistachio and pink peppercorn suggests that the proteins in either one could trigger an allergic response in a cashew-allergic individual (van der Valk, et al., 2014). A study by Noorbakhsh et al. (2011) found that

pistachio had both serological (immunoblots with IgE) and clinical evidence (food challenge) of cross-reactivity with evidence through inhibition immunoblotting and inhibition ELISA (Noorbakhsh, et al., 2011). Another study by Wilson et al. (2008) showed similar results, suggesting clinical cross-reactivity between pistachio and cashew. These data also suggested that similarities between Ana o 1 and Pis v 3 may be the cause of this observed cross-reactivity (Willison, et al., 2008). A study by Fong et al. (2019) found cross-reactivity to pink peppercorn in cashew-allergic individuals. This was clinically proven in two cases where a cashew-allergic individual had an allergic reaction that was thought to be caused by pink peppercorns. This was further proven by inhibition blotting and was thought to be the pink peppercorn protein albumin showing cross-reactivity to the Ana o 3 (2S albumin) cashew protein (Fong, et al., 2019).

d. Heat Processing Effects

Food allergies cause some of the most life-threatening reactions as they are caused by products which many people do not find problematic. It is for this reason that many studies focus on food allergen identification and research and is also the reason they are the focus of this study. Much research has been conducted concerning the most common allergens (milk, eggs, fish, crustacean shellfish, tree nuts, peanuts, wheat, soybeans as defined by the US Congress and enforced by the FDA); however, foods undergo many different types of treatments that at times, these allergenic proteins can be different from their native proteins (Thompson, et al., 2006). Many food processing methods can begin to degrade proteins in a food matrix, and heat processing is especially impactful due to its ability to denature proteins and also result in processes such as deamidation reactions or racemization (Damodaran & Parkin, 2017). Overall, multiple

heat processing methods have been shown to either enhance or lessen the allergenicity of multiple proteins in different food matrices. These modifications to the allergenic protein may create problems when detection methods are being developed. It is important to understand the effects heat processing can have on a food system so that these changes are taken into account during protein detection method development.

Cashew proteins can change or be modified following certain heating procedures. Some findings suggest that certain cashew proteins can have either an increased or decreased solubility after heating. A study by Reitsma et al. (2018) found that different heat treatments of in-shell cashews did not have a large effect on the solubility of Ana o 1, Ana o 2, and Ana o 3; however, out of shell cashews had varying changes when subjected to heat treatments. (Reitsma, et al., 2018). Mattison et al. (2016) suggested that the solubility of Ana o 1 and Ana o 2 can decrease after roasting, while that of Ana o 3 increases. These changes in solubility after roasting alter the ability to detect IgE binding for Ana o 1 and Ana o 2, while slightly increasing the ability to detect IgE binding for Ana o 3 (Mattison, et al., 2016). Following multiple heat treatment tests, Venkatachalam et al. (2008) showed that boiling and pressure cooking may slightly decrease IgE binding of various cashew proteins while other treatments such as microwaving, frying, and roasting may lead to either a stable or increased IgE binding capacity (Venkatachalam, et al., 2008).

Ultra-high temperature treatment (UHT) and high-pressure processing (HPP) are two techniques which could have the ability to potentially modify cashew proteins. UHT treatment is used for pasteurization and shelf-stability purposes and usually requires a minimum time and temperature combination between 130-150°C for 2-5 seconds and can

be achieved either directly by steam injection or indirectly by heating the product through external tubular heat exchangers (Kwok & Niranjan, 1995). High-pressure processing includes pressures between 300-500 MPa for varying times, generally in the vicinity of five minutes. This, like UHT processing, destroys unwanted bacteria/pathogens and creates a more acceptable product (Dhakal, et al., 2014). Although no specific research has been carried out on cashew milk and the effects UHT and HPP have on cashew proteins, a study on almond milk suggests a decreased immunogenicity of almond proteins following HPP processing as determined by IgE-ELISA, which could translate over to cashew milk as well under similar conditions (Dhakal, et al., 2014). However, the decreased IgE binding after processing may also be due to decreased protein solubility which could be a limitation of this study. Since heating under these conditions changes proteins in unexpected ways, it is important to understand how these cashew milk matrices are detected in current cashew ELISAs, which is one of the main objectives of this study.

IV. PROTIEN PURIFICATION OF CASHEW

a. Ana o 1, 2 & 3

As mentioned previously, cashew contains Ana o 1 (7S), Ana o 2 (11S), and Ana o 3 (2S) proteins which have been shown to be allergenic (Mendes, et al., 2019). Multiple purification methods have been established to try to purify or isolate the allergenic proteins individually. This purification work is not only for characterization, but also for raising IgG antibodies directed against these cashew proteins and understanding the properties of each protein.

Ana o 1 proteins are 7S globulins which have a theoretical isoelectric point (pI) of 5.59 and have been sequenced using LC-MS/MS and PCR methods. Wang et al. (2002) looked at the amino acid sequence of Ana o 1 through a PCR method while Reitsma et al. (2016) characterized the Ana o 1 protein following MALDI-MS/MS analysis of purified Ana o 1. Purification of cashew 7S is theoretically very similar to that of other 7S seed storage protein purification. For example, Nagano et al. (1992) isolated the 7S globulin from soybeans using pH precipitation. The 7S proteins of peanut were purified using ammonium sulfate precipitation at 70-100% saturation followed by dialysis and ultrafiltration resulting in a 6-9% yield (Nagano, et al., 1992). Other purification methods for 7S seed storage proteins include the use of column chromatography (cation exchange or gel filtration) following ammonium sulfate fractionation instead of ultrafiltration for high purity; however, these methods usually result in a lower yield (Masuyama, et al., 2014). Several 7S purification techniques have also been also applied to cashew. Reitsma et al. (2016) was able to purify the 7S proteins from cashew using an optimized ammonium sulfate fractionation method. The researchers used ammonium sulfate

precipitation to yield a final fraction at 52.5% saturation where the resulting supernatant was subjected to ultrafiltration to isolate Ana o 1 with a 1% yield and 96.5% purity (Reitsma, et al., 2016). Due to the abundance of methods, the choice of which method to use for Ana o 1 purification depends on preferred technique, yield and materials available.

Ana o 2 proteins are 11S globulins which have a theoretical pI of 6.18. 11S cashew proteins have not been fully characterized by mass spectrometry; however, sequences exist due to methods developed by several research groups (Reitsma, et al., 2016; Robotham, et al., 2010). A common 11S purification method follows that of pH cyroprecipitation (Nagano, et al., 1992). For peanut 11S purification, both anion exchange chromatography (Koppelman, et al., 2003) and ammonium sulfate fractionation with dialysis have been used with success, with ammonium sulfate fractionation resulting in a yield of approximately 30% peanut 11S (Masuyama, et al., 2014). For cashew 11S purification, Reitsma (2016) used gel filtration to separate the 11S proteins in cashew using a pH 7.0 sodium phosphate buffer to elute the proteins. This resulted in cashew 11S with 92.6% purity and a 34% yield (Reitsma, et al., 2016). The most simple method for 11S purification would be cyroprecipitation, using the advantage of the high abundance of the protein in the raw material.

Ana o 3 proteins are 2S albumins which have a theoretical pI of 5.37. The 2S cashew sequence has been mostly characterized by MALDI-MS/MS, UPLC/PDA/ESI-MS, and epitope mapping (Reitsma, et al., 2016). In general, 2S albumins have been purified using ammonium sulfate precipitation, column chromatography, and pH precipitation. Peanut 2S has been purified using ammonium sulfate precipitation followed

by a variety of column chromatography techniques such as cation/anion exchange and/or hydrophobic chromatography (Sen, et al., 2002). Other methods for peanut 2S purification have used ammonium sulfate precipitation alone, optimizing the method to result in a 7-40% yield (Masuyama, et al., 2014). In cashews, 2S purification has also been carried out using ammonium sulfate precipitation followed by ultrafiltration and protease inhibitor addition, resulting in a 98.5% purity and a 3% yield (Reitsma, et al., 2016). Column chromatography methods have been implemented for cashew 2S purification as well. Mattison et al. (2016) used ammonium sulfate precipitation followed by ion-exchange chromatography to purify the cashew 2S proteins with high purity based on mass spectrometry analysis and SDS-PAGE protein analysis. A simple method for purifying cashew 2S uses the stability of 2S proteins at low pH for purification. Hummel et al. (2015) used a Glycine-HCl (pH 2.5) extraction buffer to purify the 2S proteins of multiple nuts including cashews. With a high yield suggested by evidence from mass spectrometry and SDS-PAGE data, this method promotes the most simple and efficient method of isolating cashew 2S for current researchers.

V. ELISA DEVELOPMENT

a. Antibody Production and Sera

IgG antibodies against cashew proteins can either be raised against an entire cashew extract (Gaskin & Taylor, 2011) or against specific cashew proteins (Zhao, et al., 2019). Antibody production was developed by Harlow and Lane (1988) and has been instrumental to the targeted capabilities of detecting very low amounts of protein (Harlow & Lane, 1988). During antibody production, an antigen is paired with an adjuvant for subcutaneous or intravenous injection to animals such as rabbits, horse, sheep, etc. An adjuvant helps stimulate the immune response and is necessary for good titers and antibody production (Harlow & Lane, 1988). Different adjuvants include Freund's, FIA, and TiterMax Gold which can be administered in tandem injections or separately depending on the developed protocol. It is suggested to always start with Freund's adjuvant due to its ability to stimulate a strong immune response with a low dosage of antigen (Harlow & Lane, 1988).

After the scheduled injections of the adjuvant/antigen pair are given to the animal for a set period of time, blood samples are drawn from the animal and sera is collected following centrifugation. Antibodies, commonly IgG antibodies, are contained in the sera and can be used for further analysis. These IgG antibodies are used for ELISA development are highly specific. Monoclonal or polyclonal antibodies can be produced for ELISA development methods in this manner. Polyclonal antibodies are antibodies which can detect a wide range of antigens as they are a mixture of different antigen-specific antibodies (Harlow & Lane, 1988). Polyclonal antibodies often detect multiple epitopes on the protein(s) included in the immunogen which can result in a robust system

for detection of the residue of interest. Monoclonal antibodies are produced via hybridoma production which is a technique which links an isolated antibody-secreting cell with a myeloma cell. Such linked cells can continue to replicate and secrete the same type of antibody continuously. In other words, a single monoclonal antibody detects a single epitope which results in a highly specific antibody pool for ELISA development (KÖHler & Milstein, 1975). One disadvantage of using monoclonal antibodies is that processing induced effects that alter the single epitope would render the detection method ineffective.

Determining which type of antibody to use is dependent on the specificity of the ELISA which is desired. For the following research, because heat-processed cashew matrices are being targeted for protein detection, Ana o 2 and Ana o 3 were isolated for immunization. Ana o 1 was not isolated because of its low stability in a processed matrix (Mattison, et al., 2016). Antibodies raised against specific cashew proteins can potentially have better protein recognition due to their ability to only detect one type of cashew protein. These semi-purified proteins are enriched with the desired protein of choice, still containing smaller amounts of other cashew proteins. This partial purity is thought to enhance the immune response by giving multiple opportunities for the animal to recognize one or more proteins as immunogenic (Zhao, et al., 2019). In addition, this enriched protein also gives the advantage of hyperimmunization effects such as class shifts to more IgG antibodies, affinity maturation from multiple injections, and clonal dominance for high affinity antibodies, while also being specific for the targeted antigen (Harlow & Lane, 1988). Because two major, heat-stable and prevalent cashew allergens

are used in this study, it is hypothesized that matrices which have gone through any type of heat processing can be better detected by the developed ELISA.

Once sera has been collected from the animals, antibodies can be tested for strong antigen-antibody binding capabilities. This is performed through determining which sera have the highest titers. Because antibodies do not usually show up until 7-10 days after the first immunization, bleeds are usually taken after the second and continuing booster injections where higher titer values occur around days 10-14. Booster injections are usually administered between 2-4 weeks after the first injection, resulting in consistent antibody production. The third and following booster injections are the most important for antibody production as these antibodies produced usually have the highest titers because of high affinity and maturity (Harlow & Lane, 1988).

b. Extraction Buffers

Optimized extraction buffers are key to ensuring the soluble protein from the matrix of choice is extracted for optimum antibody recognition. Multiple studies have been done on the best conditions for such cashew extractions with variations on time, temperature, reagents, and other additions. Overall, defatting, roasting, and a high ratio of extraction buffer to protein seem to have positive effects on the extractability of soluble nut proteins (L'Hocine & Pitre, 2016a). In addition, BSA, non-fat dried milk, and Tween 20 are also common ELISA extraction buffer additions which help separate the unwanted contaminants from the soluble proteins (Zhao, et al., 2019). These additives help bind unwanted polyphenols to result in better soluble protein yields by decreasing background absorbance.

Optimum time for extraction varies across the literature. Sathe et al. (2009) found that for cashews, an optimum time of 240 minutes is able to yield the highest soluble cashew protein; however, because of time constraints, 60 minutes also yields a significant amount of soluble protein in comparison to both 45 and 120 minutes (Sathe, et al., 2009). This work suggests that an increased extraction time does benefit cashew extraction. However, L'Hocine & Pitre (2016) showed that for a variety of tree nuts and peanuts, extraction time has no effect on the amount of soluble proteins extracted (L'Hocine & Pitre, 2016b). However, this study did not include cashews which may account for the discrepancy between studies even though its applicability was looked at across a variety of nuts.

It was found that cashew yields the most soluble protein in a 0.1 M NaOH buffer system with no large effects found with a change in ionic strength (Sathe, et al., 2009). Another study by Sathe (1994) focused solely on cashew protein solubilization and showed that 0.1 M NaOH was yet again the buffer system of choice for high protein extraction in addition to an optimum extraction buffer pH between pH 7-8 (Sathe, 1994). However, due to the high basicity of NaOH, buffered sodium borate (BSB) was suggested as a general extraction buffer for all nuts because of its more neutral pH and general applicability across systems (Sathe, et al., 2009).

Reducing agents such as DTT and sodium sulfite have also been evaluated for their potential to increase extraction efficiency. Studies have shown that both sodium sulfite and sodium bisulfite produce very similar results when used interchangeably as a reducing agent (Mattison, et al., 2014). Mattison et al. (2014) looked at the influence DTT, sodium sulfite, sodium bisulfite and other reducing agents have on cashew

proteins' ability to bind to antibodies. Overall, researchers saw a clear decrease in IgG and IgE binding to both rabbit antibodies and human sera after cashew was extracted with a reducing agent. According to IgG and IgE immunoblots evaluations, Ana o 1 and Ana o 2 had reduced binding after being extracted with a reduced extraction buffer while Ana o 3 appeared to have similar binding profiles either way (Mattison, et al., 2014). Overall, the use of reducing agents may have the ability to resolubilize cashew proteins into the buffer solution and thus enhance the soluble protein extraction, even though it may diminish IgG binding.

c. Optimizing ELISA Conditions

For an ELISA plate, a high-binding material such as polystyrene or polyvinyl is used. This ensures adequate binding can take place between the coating buffer and the solid phase. General incubation temperature and time varies throughout the assay however, the standard temperatures for incubation are room temperature (RT), 37°C or 4°C (Gaskin & Taylor, 2011). Most often, the coating buffer of choice is a carbonate/bicarbonate buffer of pH 9.6 which results in the best sensitivity for most ELISA assays. Zhao et al. (2019) chose this buffer for the recent development of a 2S targeted cashew ELISA. However, other coating buffers including phosphate buffered saline (PBS) and Tris buffered saline can be used although differing pHs can affect antibody binding (Deshpande, 1996). To bind the analyte in an ELISA, high-affinity antibodies are added to the coating buffer and used as the primary capture antibody. The concentration of antibodies needs to be optimized so that the entire surface of the well is coated for adequate binding to the antigen. This is mostly done through checkerboard titration which can effectively determine the amount of antigen and antibody dilution

which is best suited for analysis (Deshpande, 1996). Multiple methods have been established for antibody concentration optimization; however, many current ELISA methods use the checkerboard optimization technique in development (Zhao, et al., 2019).

The next step, blocking, is important to ensure non-specific binding does not occur with any unbound antibodies following antigen application. Proteins, nonproteins, and detergents/surfactants can be used as blocking agents. However, most often proteins are used. The most common proteins used for blocking includes BSA, nonfat dried milk (NFDM) or gelatin used in concentrations varying from 1-5% (Deshpande, 1996; Gaskin & Taylor, 2011; Zhao, et al., 2019). Washing the ELISA plate is also an important part of the ELISA procedure as it ensures that no unwanted residue is carried over to the next step. Wash buffers used are usually phosphate and Tris-HCl buffered saline with the addition of a detergent such as Tween 20. The detergent helps to remove unwanted nonspecific binding proteins (Engvall & Perlmann, 1971).

The detector antibody is important in a sandwich-type ELISA because the antibody needs to be able to bind to a different epitope on the antigen than the capture antibody. It is important to again optimize the concentration of the detector antibody through checkerboard titration or a similar method (Kato, et al., 1977). This secondary antibody can either be enzyme-labeled or can be without a label, with another enzyme-labeled antibody added on top. Usual enzymes which are used in ELISAs are alkaline phosphatase (AP) and horseradish peroxidase (HRP). Both are effective at amplifying the ELISA signal.

VI. SUMMARY

Allergies are a common occurrence in the US and worldwide. Food allergies can be a dangerous and life-threatening condition if not adhering to a near 100% avoidance diet. To help allergic individuals, methods have been developed to ensure food companies and the like are making efforts to accurately and reliably prevent cross-contact and inadequate cleaning. A significant tree nut allergy is from cashew, being the second most common tree nut allergy in the US behind walnut. It is important that current cashew detection methods are meeting the needs of the food industry in detecting potential cashew allergens across raw materials, production lines, and in finished food products destined for grocery stores or for foodservice establishments. This promotes food safety, consumer trust, and potential life-saving knowledge for those affected. To detect these cashew allergens, ELISAs are used for sensitivity, robustness, and reliability. Currently, there is a lack of sensitivity in cashew ELISAs when testing highly processed cashew products such as cashew milk beverages which have undergone UHT/HTST processing. This inability for accurate detection of cashew allergens in these matrices leads to inaccurate labeling, possible unknown cross-contact, and the potential for life-threatening reactions.

Of the three major allergens identified in cashew (Ana o 1, Ana o 2, and Ana o 3), Ana o 2 (11S) and Ana o 3 (2S) proteins are known to be more stable and abundant in heat-processed matrices. Because of this, ELISA methods which use antibodies raised against these proteins can help ensure accurate protein detection in highly processed matrices. To achieve this objective, the 11S and 2S cashew proteins were purified using pH precipitation and low pH extraction techniques as described in this thesis. These

isolated proteins were used in rabbits to raise antibodies against cashew 11S and 2S proteins. To better protect cashew-allergic individuals and to promote further testing with other highly processed nut matrices, a more robust and sensitive cashew ELISA was developed targeting the cashew 11S and 2S proteins. This ELISA will further ensure that cashew residues are detected more reliably in the food industry, through the more robust cashew protein detection method.

VII. **REFERENCES**

Adatia, A., Clarke, A. E., Yanishevsky, Y., & Ben-Shoshan, M. (2017). Sesame allergy: current perspectives. *J Asthma Allergy, 10*, 141-151.

Allen, K. J., Remington, B. C., Baumert, J. L., Crevel, R. W. R., Houben, G. F., Brooke-Taylor, S., Kruizinga, A. G., & Taylor, S. L. (2014). Allergen reference doses for precautionary labeling (VITAL 2.0): Clinical implications. *Journal of Allergy and Clinical Immunology, 133*(1), 156-164.

Aydin, S. (2015). A short history, principles, and types of ELISA, and our laboratory experience with peptide/protein analyses using ELISA. *Peptides, 72*, 4-15.

Basketter, D. A., Kruszewski Fh Fau - Mathieu, S., Mathieu S Fau - Kirchner, D. B., Kirchner Db Fau - Panepinto, A., Panepinto A Fau - Fieldsend, M., Fieldsend M Fau - Siegert, V., Siegert V Fau - Barnes, F., Barnes F Fau - Bookstaff, R., Bookstaff R Fau - Simonsen, M., Simonsen M Fau - Concoby, B., & Concoby, B. (2015). Managing the Risk of Occupational Allergy in the Enzyme Detergent Industry. *Journal of occupational and environmental hygiene, 12*(7), 431-437.

Bastiaan-Net, S., Reitsma, M., Cordewener, J. H. G., van der Valk, J. P. M., America, T., Dubois, A. E. J., Gerth van Wijk, R., Savelkoul, H. F. J., de Jong, N. W., & Wichers, H. J. (2019). IgE cross-reactivity of cashew nut allergens. *Int Arch Allergy Immunol, 178*(1), 19-32.

Ben-Rejeb, S., Abbott, M., Davies, D., Cleroux, C., & Delahaut, P. (2005). Multi-allergen screening immunoassay for the detection of protein markers of peanut and four tree nuts in chocolate. *Food Addit Contam, 22*(8), 709-715.

Black, K. R., Filep, S., Smith, B. R. E., Lee, J., Murphy, B., Block, D., & Chapman, M. D. (2019). Simultaneous quantification of major food allergens using fluorescent multiplex array. *Journal of Allergy and Clinical Immunology, 143*(2), AB240.

Bock, S. A., Munoz-Furlong, A., & Sampson, H. A. (2001). Fatalities due to anaphylactic reactions to foods. *J Allergy Clin Immunol, 107*(1), 191-193.

Che, C. T., Douglas, L., & Liem, J. (2017). Case reports of peanut-fenugreek and cashew-sumac cross-reactivity. *J Allergy Clin Immunol Pract, 5*(2), 510-511.

Cianferoni, A., & Muraro, A. (2012). Food-induced anaphylaxis. *Immunology and allergy clinics of North America, 32*(1), 165-195.

Cid, B. J., Perez-Mateluna, G., Iturriaga, C., Zambrano, M. J., Vives, M. I., Valenzuela, P. M., & Borzutzky, A. A.-O. h. o. o. X. (2019). Is there an association between indoor allergens and the severity of atopic dermatitis? *International Journal of Dermatology, 58*(4), 433-439.

Council, I. N. a. D. F. (2017). Nuts and Dried Fruits. In *Statistical Yearbook 2017/2018*): International Nuts and Dried Fruits Council.

Damodaran, S., & Parkin, K. L. (2017). *Fennema's Food Chemistry.* (Fifth Edition. ed.): CRC Press.

Deshpande, S. S. (1996). *Enzyme Immunoassays From Concept to Product Development*: Chapman & Hall.

Dhakal, S., Liu, C., Zhang, Y., Roux, K. H., Sathe, S. K., & Balasubramaniam, V. M. (2014). Effect of high pressure processing on the immunoreactivity of almond milk. *Food Research International, 62*, 215-222.

Edwards, S. E., & Halton, F. F. (2019). Pollen food syndrome. *Paediatrics and Child Health, 29*(3), 137-141.

Engvall, E., & Perlmann, P. (1971). Enzyme-linked immunosorbent assay (ELISA) quantitative assay of immunoglobulin G. *Immunochemistry, 8*(9), 871-874.

Ferastraoaru, D., Shtessel, M., Lobell, E., Hudes, G., Rosenstreich, D., & de Vos, G. (2017). Diagnosing environmental allergies: Comparison of skin-prick, intradermal, and serum specific immunoglobulin E testing. *Allergy Rhinol (Providence), 8*(2), 53-62.

Fong, A. T., Du Toit, G., Versteeg, S. A., & van Ree, R. (2019). Pink peppercorn: A cross-reactive risk for cashew- and pistachio-allergic patients. *J Allergy Clin Immunol Pract, 7*(2), 724-725.e721.

Gaskin, F. E., & Taylor, S. L. (2011). Sandwich enzyme-linked immunosorbent assay (ELISA) for detection of cashew nut in foods. *J Food Sci, 76*(9), T218-226.

Gendel, S. M. (2012). Comparison of international food allergen labeling regulations. *Regul Toxicol Pharmacol, 63*(2), 279-285.

Gupta, R. S., Lau, C. H., Sita, E. E., Smith, B., & Greenhawt, M. J. (2013). Factors associated with reported food allergy tolerance among US children. *Annals of Allergy, Asthma & Immunology, 111*(3), 194-198.e194.

Gupta, R. S., Warren, C. M., Smith, B. M., Blumenstock, J. A., Jiang, J., Davis, M. M., & Nadeau, K. C. (2018). The public health impact of parent-reported childhood food allergies in the United States. *Pediatrics, 142*(6), e20181235.

Gupta, R. S., Warren, C. M., Smith, B. M., Jiang, J., Blumenstock, J. A., Davis, M. M., Schleimer, R. P., & Nadeau, K. C. (2019). Prevalence and severity of food allergies among US adults. *JAMA network open, 2*(1), 1-14.

Harlow, E., & Lane, D. (1988). *Antibodies: A Laboratory Manual*: Cold Spring Harbor Laboratory.

Hummel, M., Wigger, T., Höper, T., Westkamp, I., & Brockmeyer, J. (2015). Simple, rapid, and selective isolation of 2S albumins from allergenic seeds and nuts. *Journal of Agricultural and Food Chemistry, 63*(26), 6035-6040.

Kato, K., Hamaguchi, Y., Okawa, S., Ishikawa, E., & Kobayashi, K. (1977). Use of rabbit antiboty IgG bound onto plain and aminoalkylsilyl glass surface for the enzyme-linked sandwich immunoassay. *J Biochem, 82*(1), 261-266.

Kim, M., Lee, J. Y., Yang, H., Won, H. J., Kim, K., Kim, J., & Ahn, K. (2020). The natural course of immediate-type cow's milk and egg allergies in children. *International Archives of Allergy and Immunology, 181*(2), 103-110.

KÖHler, G., & Milstein, C. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature, 256*(5517), 495-497.

Koppelman, S. J., Knol, E. F., Vlooswijk, R. A., Wensing, M., Knulst, A. C., Hefle, S. L., Gruppen, H., & Piersma, S. (2003). Peanut allergen Ara h 3: isolation from peanuts and biochemical characterization. *Allergy, 58*(11), 1144-1151.

Kumar, S., Verma, A. K., Das, M., & Dwivedi, P. D. (2012). Molecular mechanisms of IgE mediated food allergy. *International Immunopharmacology, 13*(4), 432-439.

Kwok, K.-C., & Niranjan, K. (1995). Review: Effect of thermal processing on soymilk. *International Journal of Food Science and Technology, 30*, 263-295.

L'Hocine, L., & Pitre, M. (2016a). Quantitative and qualitative optimization of allergen extraction from peanut and selected tree nuts. Part 1. Screening of optimal extraction conditions using a D-optimal experimental design. *Food Chemistry, 194*, 780-786.

L'Hocine, L., & Pitre, M. (2016b). Quantitative and qualitative optimization of allergen extraction from peanut and selected tree nuts. Part 2. Optimization of buffer and ionic strength using a full factorial experimental design. *Food Chemistry, 194*, 820-827.

Loh, W., & Tang, M. L. K. (2018). The Epidemiology of Food Allergy in the Global Context. *International journal of environmental research and public health, 15*(9), 2043.

López-Calleja, I. M., de la Cruz, S., González, I., García, T., & Martín, R. (2015). Development of real-time PCR assays to detect cashew (Anacardium occidentale) and macadamia (Macadamia intergrifolia) residues in market analysis of processed food products. *LWT - Food Science and Technology, 62*(1, Part 1), 233-241.

Lyons, S. A., Burney, P. G. J., Ballmer-Weber, B. K., Fernandez-Rivas, M., Barreales, L., Clausen, M., Dubakiene, R., Fernandez-Perez, C., Fritsche, P., Jedrzejczak-Czechowicz, M., Kowalski, M. L., Kralimarkova, T., Kummeling, I., Mustakov, T. B., Lebens, A. F. M., van Os-Medendorp, H., Papadopoulos, N. G., Popov, T. A., Sakellariou, A., Welsing, P. M. J., Potts, J., Mills, E. N. C., van Ree, R., Knulst, A. C., & Le, T. M. (2019). Food allergy in adults: substantial variation in prevalence and causative foods across europe. *The journal of allergy and clinical immunology. In practice., 7*(6), 1920-1928.

Masiri, J., Benoit, L., Meshgi, M., Day, J., Nadala, C., & Samadpour, M. (2016). A novel immunoassay test system for detection of modified allergen residues present in almond-, cashew-, coconut-, hazelnut-, and soy-based nondairy beverages. *Journal of Food Protection, 79*(9), 1572-1582.

Masuyama, K., Yamamoto, K., Ito, K., Kitagawa, E., & Yamaki, K. (2014). Simplified methods for purification of peanut allergenic proteins: Ara h 1, Ara h 2, and Ara h 3. *Food Science and Technology Research, 20*(4), 875-881.

Mattison, Bren-Mattison, Y., Vant-Hull, B., Vargas, A. M., Wasserman, R. L., & Grimm, C. C. (2016). Heat-induced alterations in cashew allergen solubility and IgE binding. *Toxicology Reports, 3*, 244-251.

Mattison, C. P., Desormeaux, W. A., Wasserman, R. L., Yoshioka-Tarver, M., Condon, B., & Grimm, C. C. (2014). Decreased immunoglobulin E (IgE) binding to cashew allergens following sodium sulfite treatment and heating. *Journal of Agricultural and Food Chemistry, 62*(28), 6746-6755.

McWilliam, V., Koplin, J., Lodge, C., Tang, M., Dharmage, S., & Allen, K. (2015). The prevalence of tree nut allergy: a systematic review. *Curr Allergy Asthma Rep, 15*(9), 54.

Mendes, C., Costa, J., Vicente, A. A., Oliveira, M. B., & Mafra, I. (2019). Cashew nut allergy: clinical relevance and allergen characterisation. *Clin Rev Allergy Immunol, 57*(1), 1-22.

Monaci, L., & Visconti, A. (2010). Immunochemical and DNA-based methods in food allergen analysis and quality assurance perspectives. *Trends in Food Science & Technology, 21*(6), 272-283.

Mori, J. D. M. S. A. (1987). Memoirs of the New York Botanical Garden. In T. N. Y. B. Garden (Ed.), *The Cashew and Its Relatives (Anacardium: Anacardiaceae)*, vol. 42 (pp. 1-76). New York.

Muraro, A., & Arasi, S. (2018). Biomarkers in food allergy. *Curr Allergy Asthma Rep, 18*(11), 64.

Nagano, T., Hirotsuka, M., Mori, H., Kohyama, K., & Nishinari, K. (1992). Dynamic viscoelastic study on the gelation of 7 S globulin from soybeans. *Journal of Agricultural and Food Chemistry, 40*(6), 941-944.

Noorbakhsh, R., Mortazavi, S. A., Sankian, M., Shahidi, F., Tehrani, M., Azad, F. J., Behmanesh, F., & Varasteh, A. (2011). Pistachio allergy-prevalence and in vitro cross-reactivity with other nuts. *Allergol Int, 60*(4), 425-432.

Nwaru, B. I., Hickstein L Fau - Panesar, S. S., Panesar Ss Fau - Roberts, G., Roberts G Fau - Muraro, A., Muraro A Fau - Sheikh, A., & Sheikh, A. (2014). Prevalence of common food allergies in Europe: a systematic review and meta-analysis. *Allergy, 69*(8), 992-1007.

Owen, J., Stranford, S., & Punt, J. (2013). *Kuby Immunology, 7th edition*: W. H. Freeman.

Pawankar, R., Walkter Canonica, G., Holgate, S., & Lockey, R. (2014). White Book on Allergy 2011-2012 Executive Summary. In W. H. Organization (Ed.)).

Reitsma, M., Bastiaan-Net, S., Sforza, S., van der Valk, J. P. M., van Gerth van Wijk, R., Savelkoul, H. F. J., de Jong, N. W., & Wichers, H. J. (2016). Purification and characterization of Anacardium occidentale (cashew) allergens Ana o 1, Ana o 2, and Ana o 3. *Journal of Agricultural and Food Chemistry, 64*(5), 1191-1201.

Reitsma, M., Bastiaan-Net, S., Sijbrandij, L., de Weert, E., Sforza, S., Gerth van Wijk, R., Savelkoul, H. F. J., de Jong, N. W., & Wichers, H. J. (2018). Origin and processing methods slightly affect allergenic characteristics of cashew nuts (Anacardium occidentale). *Journal of Food Science, 83*(4), 1153-1164.

Robotham, J. M., Xia, L., Willison, L. N., Teuber, S. S., Sathe, S. K., & Roux, K. H. (2010). Characterization of a cashew allergen, 11S globulin (Ana o 2), conformational epitope. *Mol Immunol, 47*(9), 1830-1838.

Roux, K. H., Teuber, S. S., & Sathe, S. K. (2003). Tree nut allergens. *International Archives of Allergy and Immunology, 131*(4), 234-244.

Rusznak, C., & Davies, R. J. (1998). ABC of allergies. Diagnosing allergy. *Bmj, 316*(7132), 686-689.

Santos, A. F., Couto-Francisco, N., Becares, N., Kwok, M., Bahnson, H. T., & Lack, G. (2018). A novel human mast cell activation test for peanut allergy. *Journal of Allergy and Clinical Immunology, 142*(2), 689-691.

Sathe, Venkatachalam, M., Sharma, G. M., Kshirsagar, H. H., Teuber, S. S., & Roux, K. H. (2009). Solubilization and electrophoretic characterization of select edible nut seed proteins. *Journal of Agricultural and Food Chemistry, 57*(17), 7846-7856.

Sathe, S. K. (1994). Solubilization and electrophoretic characterization of cashew nut (Anacardium occidentale) proteins. *Food Chemistry, 51*(3), 319-324.

Schubert-Ullrich, P., Rudolf, J., Ansari, P., Galler, B., Fuhrer, M., Molinelli, A., & Baumgartner, S. (2009). Commercialized rapid immunoanalytical tests for determination of allergenic food proteins: an overview. *Anal Bioanal Chem, 395*(1), 69-81.

Sen, M., Kopper, R., Pons, L., Abraham, E. C., Burks, A. W., & Bannon, G. A. (2002). Protein structure plays a critical role in peanut allergen stability and may determine immunodominant IgE-binding epitopes. *J Immunol, 169*(2), 882-887.

Sharma, G. M., Fau, R. K., & Sathe, S. K. (2009). A sensitive and robust competitive enzyme-linked immunosorbent assay for Brazil nut (Bertholletia excelsa L.) detection. *Journal of Agricultural and Food Chemistry, 57*(2), 769-776.

Sicherer, S. H., Munoz-Furlong, A., Godbold, J. H., & Sampson, H. A. (2010). US prevalence of self-reported peanut, tree nut, and sesame allergy: 11-year follow-up. *J Allergy Clin Immunol, 125*(6), 1322-1326.

Taylor, S. L., Baumert, J. L., Kruizinga, A. G., Remington, B. C., Crevel, R. W. R., Brooke-Taylor, S., Allen, K. J., & Houben, G. (2014). Establishment of reference doses for residues of allergenic foods: report of the vital expert panel. *Food and Chemical Toxicology, 63*, 9-17.

Teuber, S. S., Sathe, S. K., Peterson, W. R., & Roux, K. H. (2002). Characterization of the soluble allergenic proteins of cashew nut (Anacardium occidentale L.). *Journal of Agricultural and Food Chemistry, 50*(22), 6543-6549.

Thompson, T., Kane, R. R., & Hager, M. H. (2006). Food allergen labeling and consumer protection act of 2004 in effect. *Journal of the American Dietetic Association, 106*(11), 1742-1744.

USDA. (2018). Nuts, cashew nuts, raw. In U. S. D. o. A.-A. R. Service (Ed.), *USDA National Nutrient Database for Standard Reference*).

van der Valk, J. P., Dubois, A. E., Gerth van Wijk, R., Wichers, H. J., & de Jong, N. W. (2014). Systematic review on cashew nut allergy. *Allergy, 69*(6), 692-698.

van der Valk, J. P. M., Bouche, R. e., Gerth van Wijk, R., de Groot, H., Wichers, H. J., Dubois, A. E. J., de Jong, N. W. J. C., & Allergy, T. (2017). Low percentage of clinically relevant pistachio nut and mango co-sensitisation in cashew nut sensitised children. *Journal of Clinical and Translational Allergy, 7*(1), 8.

Venkatachalam, M., Monaghan, E. K., Kshirsagar, H. H., Robotham, J. M., O'Donnell, S. E., Gerber, M. S., Roux, K. H., & Sathe, S. K. (2008). Effects of processing on Immunoreactivity of cashew nut (Anacardium occidentale L.) seed flour proteins. *Journal of Agricultural and Food Chemistry, 56*(19), 8998-9005.

Wang, F., Robotham, J., S Teuber, S., Sathe, S., & H Roux, K. (2003). Ana o 2, a major cashew (Anacardium occidentale L.) nut allergen of the legumin family. *International Archives of Allergy and Applied Immunology, 132*(1), 27-39.

Wang, F., Robotham, J. M., Teuber, S. S., Tawde, P., Sathe, S. K., & Roux, K. H. (2002). Ana o 1, a cashew (Anacardium occidental) allergen of the vicilin seed storage protein family. *Journal of Allergy and Clinical Immunology, 110*(1), 160-166.

Wei, Y., Sathe, S. K., Teuber, S. S., & Roux, K. H. (2003). A sensitive sandwich ELISA for the detection of trace amounts of cashew (Anacardium occidentale L.) nut in foods. *Journal of Agricultural and Food Chemistry, 51*(11), 3215-3221.

Willison, L. N., Tawde, P., Robotham, J. M., Penney, R. M. t., Teuber, S. S., Sathe, S. K., & Roux, K. H. (2008). Pistachio vicilin, Pis v 3, is immunoglobulin E-reactive and cross-reacts with the homologous cashew allergen, Ana o 1. *Clin Exp Allergy, 38*(7), 1229-1238.

Zhao, Y., Sun, X., Marquis, C. P., & Lee, N. A. (2019). Development of a sensitive sandwich ELISA specific to 2S albumin (Ana o 3) as a stable protein marker for cashew nut residue detection in pre-packaged food products. *Food Control, 96*, 432-440.

CHAPTER 2: COMPARISON OF THREE COMMERCIAL CASHSEW ELISA KITS & AN IN-HOUSE CASHEW ELISA ON THE DETECTION OF CASHEW FROM SIX DIFFERENT CASHEW MILK MATRICES

I. INTRODUCTION

The prevalence of US food allergies in children is estimated to be 8% while in adults, the prevalence of food allergies ranges from 10.8% to 19% (Gupta, et al., 2011; Gupta, et al., 2019). To protect these food-allergic individuals, methods have been developed to detect these allergens in ingredients, finished products or on contact surfaces of shared production equipment. One of the most commonly used analytical platforms for detection of residues from allergenic sources is the enzyme-linked immunosorbent assay (ELISA) which uses antibodies raised against specific target proteins to detect the protein(s) residue of interest (Monaci, et al., 2010). These ELISAs are specific to the antigen of choice and provide both qualitative as well as quantitative tools that are critical in food allergen detection. Of the major food allergens, tree nuts are among the highest concern as they and peanut account for almost 90% of all fatalities due to anaphylactic shock (Bock, et al., 2001). Because cashew is the second most common tree nut allergy in the US, its accurate detection is of the utmost importance for consumer safety (McWilliam, et al., 2015; Sicherer, et al., 2010).

With the high prevalence and severity of cashew allergy and clear need for reliable detection for qualification of allergen control and cleaning programs, cashew ELISA methods have been developed with high robustness and sensitivity (Gaskin, et al., 2011; Wei, et al., 2003; Zhao, et al., 2019). However, new research on other tree nuts has suggested that several processing techniques, such as high-temperature short time

(HTST), ultra-high temperature (UHT) and high pressure processing (HPP) can modify protein epitopes resulting in lower antibody binding (Monaci, et al., 2010). To understand the effect of UHT processing on detection of an almond-containing product, Dhakal et al. (2014) tested almond milk for its reactivity using antibodies directed against almond protein residue. The almond milk showed lower immunoreactivity overall and suggested that other UHT treated tree nut matrices may show similar behavior with currently available immunoassays (Dhakal, et al., 2014).

Because of the need for robust and reliable methods for cashew detection, the objective of this study was to determine if current cashew ELISAs can detect cashew proteins from a highly processed cashew matrix. Thus, six different commercially available cashew milks were tested using three commercial cashew ELISAs and one proprietary in-house ELISA for their reliability, sensitivity, and accuracy in detecting cashew proteins from cashew milk matrices.

II. MATERIALS AND METHODS

a. Cashew Milk Soluble Protein Characterization

Six commercial cashew milk brands were identified from the current cashew milk beverage market. The six different brands of cashew milk used in the current study were Silk; Pacific Foods; Cashew Dream; Forager Project; Elmhurst; and SoDelicious. Each sample was shaken thoroughly and aliquots were stored at both 4°C and -20°C until used for further analysis.

i. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Cashew Milk Proteins

All six commercial cashew milk samples were analyzed using SDS-PAGE for protein profile comparison. Additionally, an extract of raw (blanched) whole cashews (R.U. Nuts Co, Lincoln, NE) was included as a positive control with a known quantity of cashew protein. Before testing, all of the cashew milk samples were brought to room temperature (RT) and shaken thoroughly to ensure a homogenous sample was used. Raw (blanched) whole cashews were hand-sorted and homogenized using a freezer mill (SPEX 6850). To prepare samples for extraction, 1.0 g of each cashew milk sample was extracted in 2.5 mL of phosphate buffered saline (PBS) [0.01 M PBS containing 0.85% NaCl, pH 7.4] at 60ºC in a shaking water bath (200 rpm) for 25 min. For raw (blanched) whole cashew nuts, 1.0 g of ground, homogenized cashew nut was extracted in 20 mL of PBS. The resulting supernatant from the centrifugation at 12000 xg for 10 min was used for SDS-PAGE. Each sample extract was prepared 5:1 (v/v) using 6X Laemmli sample buffer with or without dithiothreitol (DTT) to evaluate the protein profiles under reducing and non-reducing conditions, respectively. The samples were heated for 10 min at 95ºC

and loaded (12 µL/lane) onto 4-20% Ready Gel precast TRIS-glycine gels. Gels were run for ~40 min at a constant voltage of 200 V. Following gel electrophoresis, gels were fixed (10% (v/v) acetic acid, 50% (v/v) methanol in water) for 40 min. The gels were then rinsed x3 times (5 min each), in distilled water. The gels were then stained O/N in Coomassie Brilliant Blue G-250 staining solution (Thermo Fisher Scientific, Waltham, MA). The following day, gels were destained using distilled water and the images were captured using a Kodak Gel Logic 440 Imaging System (Eastman Kodak Company) and Kodak Gel Logic ID v. 3.6.5 software (Kodak Scientific Imaging Systems, New Haven, CT).

ii. Protein Estimation using the 2-D Quant Protein Assay (GE Healthcare Life Sciences, Piscataway, NJ)

For a robust comparison of protein extractability under different conditions, each of the cashew milks was extracted in five different extraction buffers at two different temperatures. Raw, ground cashew nut was also extracted in each buffer for comparison. For the cashew milk samples, 1.0 g of each sample was extracted in 2.5 mL of the respective extraction buffers. For raw (blanched), ground cashew, 0.5 g of cashew was extracted in 10 mL of the respective extraction buffers. The extraction buffers used were:

- 0.01 M PBS
- 0.01 M PBS with 0.1 M Sodium Sulfite
- 0.01 M PBS with 0.1 M Sodium Sulfite & 1% SDS
- 0.01 M PBS with 0.1 M Sodium Bisulfite
- 0.01 M PBS with 0.1 M Sodium Bisulfite & 1% SDS

All PBS extraction buffers were adjusted to pH 7.4 prior to the addition of the other reagents. Extractions were carried out at both 60°C for 25 min and 100°C for 10 min in a shaking water bath (200 rpm). The extracts were then cooled to RT followed by centrifugation (Beckman Coulter Microfuge 16 Centrifuge, Beckman Coulter Life Sciences, Indianapolis, IN) at 12500 xg for 10 min at RT. The supernatants were then stored at -20°C until used for further analysis.

For 2-D Quant protein analysis, sample extracts were analyzed according to the manufacturer's instructions (2-D Quant Kit, GE Healthcare Life Sciences, Piscataway, NJ).

b. Commercial & In-House ELISA Testing

All six commercial cashew milk samples and a whole cashew extract were tested using three commercial cashew ELISA kits and an in-house cashew ELISA developed by the Food Allergy Research and Resource Program (FARRP) at the University of Nebraska-Lincoln. The three commercial kits used were the R-Biopharm RIDASCREEN FAST Cashew ELISA Kit (Germany); BioFront Monotrace Cashew ELISA Kit (Tallahassee, FL); and the 3M Cashew Protein ELISA Kit (St. Paul, MN). All samples were tested by ELISA using the protocols supplied by each kit manufacturer. The in-house cashew ELISA was performed per procedural guidelines (Gaskin, et al., 2011). Direct, 10-fold, 50-fold, 100-fold, and 500-fold dilutions of each cashew milk sample were tested.

For the in-house ELISA, the following procedure was followed: A 96-well polyvinyl microtiter plate (NUNC-Immuno™ MaxiSorp™ 96-MicroWell™ plates, Nagle

Nunc Intl., Rochester, NY, USA) was coated with 100 µL/well of 2.25 µg/mL sheep anti-roasted cashew antisera prepared in coating buffer (0.015 M Na_2CO_3, 0.035 M $NaHCO_3$, pH 9.6) and incubated overnight at 4°C. Following the overnight incubation, the plate was washed with wash buffer (0.05% Tween 20 in 0.01 M PBS, pH 7.4) four times, and blocked with blocking buffer (0.1% gelatin in 0.01 M PBS, pH 7.4) for 1 h at 37°C. The plate was washed four times and the protein standards (prepared by using a 10000 ppm roasted cashew extract diluted three-fold from 100 ppm total cashew to 0.097 ppm total cashew) and samples were added (100 µL/well) and incubated for 1 h at 37°C. The plate was then washed four times, followed by the addition of rabbit anti-roasted cashew antibody (100 µL/well;1.5 µg/µL) in conjugate buffer (0.1% BSA in 0.01 M PBS, pH 7.4) and incubated for 1 h at 37°C. The plate was then washed four times and incubated with 1:5000 v/v alkaline phosphatase-labeled goat anti-rabbit IgG in conjugate buffer (100 µL/well) for 1 h at 37°C. The plate was washed four times and developed using p-nitrophenyl phosphate substrate (p-NPP SigmaFast™ Tablets, Sigma Chemical Co., St. Louis, MO) dissolved in 0.2M Trizma buffer (100 µL/well). The reaction was stopped by adding 1 M NaOH and the absorbance read at 405 nm using a plate reader (ELx808 Ultraplate, BioTek Instruments, Inc., Winooski, VT). The standard curve was generated using a Sigmoidal Curve, 2 variable equation with quantitative results taken from the linear portion of the curve.

c. Protein Recognition Using Animal Antisera from Commercial & In-House ELISA Antibodies (IgG Immunoblotting)

Western blots were performed for all cashew milks using the antibodies provided with each ELISA kit. The objective of the IgG immunoblot analysis was to determine

which cashew proteins from the cashew milk samples were being recognized by the conjugated antibodies used in each ELISA kit. Since the coating antibodies are bound to the microwells provided with the commercial kits, we are unable to evaluate which proteins were recognized by these antibodies specifically. We were able to evaluate both the coating and detection antibodies used in the in-house ELISA.

IgG immunoblotting procedures (Towbin, et al., 1979) were followed. Samples were prepared as previously mentioned for SDS-PAGE. Following gel electrophoresis, the proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Immunoblin-P PVDF membrane, 0.45 μm, Millipore Corporation, Billerical, MA). Following the transfer, the membrane was washed twice with wash buffer (0.01 M PBS with 0.05% Tween 20) and then blocked with blocking buffer (0.01 M PBS, 0.02% BSA, 0.05% Tween 20) for 2 h at RT. The membrane was then washed twice in washing buffer and incubated with the primary diluted antibody of choice in blocking buffer for 1 h at RT. The membrane was washed four times for 5 min each with wash buffer and then, if necessary, such as for the in-house cashew ELISA, incubated with the secondary antibody for 1 h at RT and washed again. The antibodies and their respective dilutions (diluted in blocking buffer) used were:

- BioFront Monotrace Cashew ELISA conjugated antibody: diluted 1:20
- R-Biopharm RIDASCREEN FAST Cashew ELISA Kit conjugated antibody: diluted 1:20
- 3M Cashew Protein ELISA Kit conjugated antibody: diluted 1:20
- In-house (Coating Antibody) antibody: diluted 1:15000
- In-house (Detection Antibody) antibody: diluted 1:10000

The recognized bands were developed by incubating the membrane with the SuperSignal™ West Dura Extended Duration Substrate (Thermo Fisher Scientific, Waltham, MA) and visualized using the Kodak imager.

III. RESULTS AND DISCUSSION

a. SDS-PAGE Profiles & 2-D Quant Protein Estimation of Cashew Milks

A brief description of all six cashew milk samples used in the current study is provided in Table 2.1. SDS-PAGE was used to compare the protein profiles of each of the commercial cashew milk samples under reducing and non-reducing conditions (Figure 2.1).

Table 2.1. Commercial cashew milk brand information and reported protein content (g/240 mL).

Cashew Milk Brand	Product Line	Ingredient List	Protein Content (g/240 mL)*
Forager Project	Organic Cashew Milk	cashewmilk (filtered water, cashews*), coconut cream* tapioca starch*, gluten-free oats*, sea salt	1
Danone North America (Whitewave)	Silk Cashew Milk	cashewmilk (filtered water, cashews), contains 2% or less of: almond butter, vitamin and mineral blend (including calcium carbonate, vitamin e acetate, vitamin a palmitate, vitamin d2), sea salt, natural flavor, sunflower lecithin, locust bean gum, gellan gum, ascorbic acid	<1
Danone North America (Whitewave)	SoDelicious Cashew Milk	cashew milk (water, cashews), canola oil, natural flavor, tricalcium phosphate, guar gum, sea salt, magnesium phosphate, carob bean gum, gellan gum, l-selenomethionine (selenium), vitamin a acetate, vitamin d-2, zinc oxide, vitamin b-12	1
Campbell's	Pacific Foods Cashew Non-Dairy Beverage	water, cashew butter (fair trade certified (tm) cashews*, sunflower oil*)*, contains less than 1% of: gellan gum, guar gum*, sea salt, sodium citrate, tricalcium phosphate, xanthan gum	1
Hain Celestial	Dream Original Cashew Drink	filtered water, organic cashew butter, organic tapioca starch, calcium carbonate, sea salt, gellan gum, natural flavors, sunflower lecithin, dipotassium phosphate, gum acacia, xanthan gum, vitamin e (d-alpha tocopheryl acetate), vitamin a palmitate, vitamin d2, vitamin b12	1
Elmhurst Milked	Cashew Milk	filtered water, cashews, cane sugar, salt, natural flavors	4

*Protein Content is reported in terms of g/240 mL serving size based on reported quantities on the nutrition fact panel for each product. Items in RED may contribute additional protein in the cashew milk beyond the cashew ingredient.

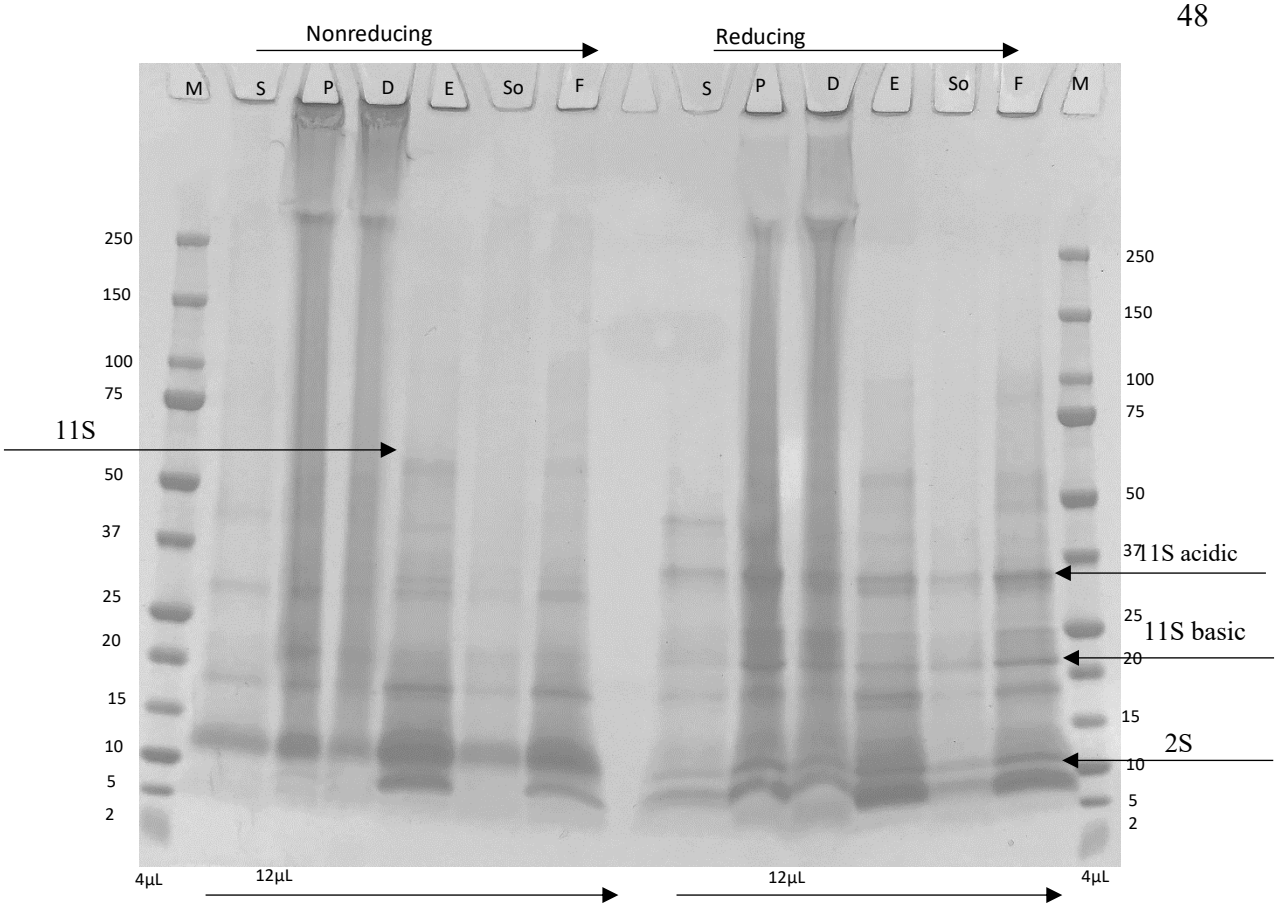


Figure 2.1. Coomassie Brilliant Blue G-250 stained SDS-PAGE profile of the six cashew milks under both non-reducing and reducing conditions. The letters indicated in each lane correspond to the following samples; Lane M – Molecular Weight Marker (in kDa); Lane S – Silk; Lane P – Pacific Foods; Lane D – Dream; Lane E – Elmhurst Milked; Lane So – SoDelicious; Lane F – Forager Project.

The 2-D Quant protein assay compared the total soluble protein in each of the six cashew milk samples (Table 2.3). While most of the cashew milks had varying sources of protein from ingredients or other nuts, Elmhurst contained only cashew as a source of protein and can be depicted as the simplest cashew milk (Table 2.1 & Figure 2.1, Lane E). Figure 2.1 indicates the presence of two major allergenic cashew proteins under both non-reducing and reducing conditions. Under non-reducing conditions, the 11S cashew proteins (Ana o 2) are present as a 53 kDa band in its native form. Under reducing conditions, the cashew

11S acidic and 11S basic sub units have molecular weights of 33 kDa and 22 kDa, respectively (Figure 2.1) (Wang, et al., 2003). The cashew 2S proteins (Ana o 3) fall between 8-12 kDa under reducing conditions (Figure 2.1) (Teuber, et al., 2002). The Cashew 7S protein (Ana o 1; 53 kDa) cannot be visualized on SDS-PAGE (Figure 2.1) which is in agreement with current literature due to its low stability to heat processing and the inability to resolubilize the cashew 7S protein (Mattison, et al., 2016). Overall the cashew 2S proteins are consistently present across all cashew milks while the cashew 11S protein seems to be faint or even absent in some of the cashew milks under non-reducing conditions (Figure 2.1). The cashew 11S could be faint under non-reducing conditions due to a lessened ability of the 11S to solubilize without reducing conditions (Abtahi, et al., 1997). The acidic subunit of the cashew 11S protein is present in all of the processed cashew milk samples indicating that it could perhaps provide a good target for detection of highly processed cashew residues.

2-D quant protein estimation of the cashew milk and cashew nut samples varied based on the type of extraction buffer used (Table 2.3). Depending on extraction conditions and the presence/absence of additives, the amount of cashew protein extracted differed (Table 2.2).

Table 2.2. 2-D Quant protein estimation (ppm) of cashew nuts using different extraction buffers.

Protein Estimation of Whole Raw Cashew Nuts (ppm)					
	Extraction Buffer				
Extraction Temperature	**PBS**	**PBS with Sodium Sulfite**	**PBS with Sodium Bisulfite**	**PBS with Sodium Sulfite & SDS**	**PBS with Sodium Bisulfite & SDS**
60 °C	1300	919	1021	4241	1077
100 °C	2155	4017	767	5336	ALQ

*2D Quant Assay (GE Healthcare, Chicago, IL) was used for protein estimation. ALQ; Above the Limit of Quantification (50 µg protein). PBS; 0.01 M PBS, pH 7.4.

Prior to testing the six cashew milks, ground cashew nut was extracted in each of the 5 extraction buffers under 2 temperatures (60°C and 100°C) to understand how the extraction buffers perform in the solubilization of cashew proteins. The reducing agent, sodium sulfite, has been shown to effectively reduce the cysteine bonds similar to 2-mercaptoethanol (2-ME), a common reducing agent, but sodium sulfite is more environmentally friendly (Ito, et al., 2016). For this reason both sodium sulfite and sodium bisulfite were chosen as reducing agents for our initial extraction experiments. The addition of sodium sulfite or sodium bisulfite at 60°C did not have an effect on extracting soluble cashew protein from cashews. However, the presence of sodium sulfite when extracted at 100°C did have an appreciable effect on increasing the yield of extracted cashew protein. This was dependent on the type of reducing agent added, as the addition of sodium bisulfite did not have the same increase in protein yield when extracted at 100°C (Table 2.2). This indicates a difference on the ability to use the two reagents interchangeably for reduction purposes, contrary to research done by Abtahi and others on soy (Abtahi, et al., 1997). However, the molecular formula weight shows a

difference in percentage of SO_2 (% SO_2) for the two reagents, with Sodium Bisulfite (Molecular weight; 104 g/mol) being lower at 63.4% compared to 76.9% for Sodium Sulfite (Molecular weight; 126 g/mol). Since the % SO_2 contributes to the reduction power, increasing the amount of Sodium Bisulfite in the future to match that of Sodium Sulfite may have a positive effect on extraction. Following the addition of 1% SDS to the sodium sulfite extraction buffer, an appreciable increase was found in the soluble proteins extracted from cashew nuts at both extraction temperatures (Table 2.2). This was not the case for the addition of 1% SDS to the sodium bisulfite extraction buffer, as an appreciable increase in the soluble proteins extracted was only found in the extraction done at 100ºC. Again, at 60°C, the addition of sodium bisulfite did not have a large increase in the extraction of soluble cashew protein even with the addition of the denaturing agent (Table 2.2). Based on these results, the extraction buffers selected for comparing cashew milk protein extractability were the extraction buffers containing either sodium bisulfite or sodium sulfite with 1% SDS at both 60°C and 100°C in PBS. Although the soluble protein content of the PBS extraction in comparison to the PBS with sodium bisulfite and SDS extraction showed no appreciable increase, the reducing buffer was still chosen because this comparison was conducted on somewhat minimally processed ground cashew. From other work, we know that the solubility will decrease in further processed products and therefore we anticipated that PBS would not provide a good extraction buffer for the cashew milks that undergo extensive heat treatment (Abtahi, et al., 1997).

All cashew milks, except for Pacific Foods, showed an increase in the amount of protein extracted when any type of additive was used in PBS (Table 2.3).

Table 2.3. Comparison of proteins (ppm) extracted using different buffers from cashew milk and cashew nut extracts.

	Extraction Buffer/ Temp and Time				
Brand of Cashew Milk	**PBS/ 60 °C 25 min**	**PBS with sodium bisulfite & SDS/ 60 °C 25 min**	**PBS with sodium sulfite & SDS/ 60 °C 25 min**	**PBS with sodium bisulfite & SDS/ 100 °C 10 min**	**PBS with sodium sulfite & SDS/ 100 °C 10 min**
Silk	BLQ	759	697	777	729
SoDelicious	BLQ	375	586	631	615
Cashew Dream	766	1084	1129	1030	1164
Elmhurst Milked	590	2836	5264	3096	5014
Pacific Foods	1134	1116	1690	1732	1610
Forager Project	424	1996	2310	2382	2150
Cashew Nut	1300	1077	4241	ALQ	5336

*BLQ; Below the Limit of Quantification (10 µg protein). ALQ; Above the Limit of Quantification (50 µg protein). PBS; 0.01 M PBS, pH 7.4

Cashew nuts also benefitted from the addition of a reducing agent and detergent. Proteins extracted from the cashew milk brands “Silk,” “Pacific,” and “Dream” had very little variation between the use of different reducing agents and temperatures of extraction (Table 2.3). Proteins extracted from the cashew milk brands “SoDelicious” and “Forager Project” showed similar extraction patterns except for slightly lower protein extracted when sodium bisulfite was used at 60ºC. Proteins were best extracted from the cashew milk brand “Elmhurst” with PBS containing sodium sulfite and SDS at either 60ºC or 100°C (Table 2.3). Contrary to the results seen in Table 2.2, the protein extracted from cashew milks seemed to be fairly consistent no matter which reducing agent was used. This agrees with data shown by Abtahi (1997) in arguing that both sodium sulfite and sodium bisulfite extract soy proteins similarly (Abtahi, et al., 1997). However, these results could be due to the SDS being present in all of the extraction buffers which may

have an effect on the extraction of protein no matter what reducing agent is included in the extraction buffer. As shown before, the proteins from whole cashew nuts were best extracted using PBS containing either sodium sulfite or sodium bisulfite and SDS at 100ºC (Table 2.3).

From the buffers evaluated for their efficacy in extracting cashew proteins from heat processed cashew milk samples, the most efficient was PBS containing sodium sulfite and SDS, extracted at 60ºC (Table 2.3). Even though an extraction temperature of 100ºC extracted more protein from four of the cashew milks and from whole raw cashew nuts, a lower extraction temperature is preferred. A 60ºC extraction temperature is preferred over a 100ºC temperature because higher extraction temperatures may denature proteins, causing proteins to potentially aggregate (Mattison, et al., 2016). In comparing the use of sodium sulfite and sodium bisulfite, sodium sulfite appeared to extract more proteins at the preferred lower extraction temperature of 60°C, especially in cashew nuts (Table 2.3). This observation was surprising as a number of studies had reported no significant difference between the two reducing agents although the protein, amount extracted, and % SO_2 may have had an influence on this (Abtahi, et al., 1997; Liu, et al., 2007).

Due to the well-known inability to effectively use high concentrations of SDS in an ELISA format (McCabe, et al., 1988), the optimum extraction buffer was further evaluated as descripted in Chapter 4.

b. Detection and Quantification of Cashew Proteins Using the Commercial and In-House Cashew Immunoassays

Three commercial cashew ELISA kits (R-Biopharm, BioFront, and 3M) and one in-house cashew ELISA were used to quantify cashew proteins from six cashew milk brands (Silk, Dream, SoDelicous, Elmhurst Milked, Pacific Foods, Forager Project). The goal of running each ELISA was to determine if each kit could detect the presence of cashew protein from the selected cashew milk products.

The 3M kit, following the testing of multiple dilutions (1:10, 1:100, 1:1000) of the sample extracts, appeared to have somewhat consistent cashew protein (ppm) values; however, nonspecific binding and/or recovery issues were observed for all cashew milks (Table 2.4). As dilutions increased for each sample extract, the protein values reported by the kit were shown to be variable (data not shown). The inconsistent protein values could be due to some type of sample interference as no dilution of the cashew milks could define a consistent and reliable protein concentration to record. Another possible explanation could be an innate problem with the ELISA concerning repeatability; however, more analysis would need to be conducted with different production lots of the 3M ELISA kits to further evaluate this.

Table 2.4 Approximate cashew protein (ppm) concentrations detected from each ELISA kit. The two assays highlighted in gray did not yield reliable reporting values.

Cashew Milk Brand	3M	In-House FARRP	BioFront	R-Biopharm	Expected ppm values*
Silk	1980	25844	258	BLQ	4166
SoDelicious	2095	24208	74	BLQ	4166
Cashew Dream	1390	25643	1004	11	4166
Elmhurst Milked	3954	34104	39	BLQ	16666
Pacific Foods	4206	30873	938	9	4166
Forager Project	4475	29040	17	BLQ	4166

*Assuming all protein from the commercial nutrition fact panel is from cashew

The in-house and 3M assay did not show consistent results as the absorbance values did not show a decrease with increasing dilutions as would be expected when cashew residue is present (Table 2.4.). As a sample extract was diluted further, the corresponding absorbances should decrease to indicate that the amount of protein is being diluted. These assays did not show this typical linear decrease; instead we observed an increase in absorbance as the dilutions increased. Because of this, it is assumed that nonspecific binding was occurring with the cashew milks in this assay. Consequently, the in-house assay overestimated the amount of protein in all of the cashew milk samples (Table 2.4). Both assays reported varying amounts of protein in each cashew milk and are not quantitatively reliable.

The BioFront kit observed very consistent protein recovery amounts (ppm cashew protein) for each cashew milk sample across dilutions. However, the assay greatly underestimated the amount of protein for all cashew milk samples except for Cashew Dream and Pacific Foods, which only showed slight underestimation (Table 2.4). It is not understood why these two cashew milks had a higher cashew protein recovery. The

underestimation of the other four cashew milks is most likely due to a low recovery of protein during extraction for this assay or due to the antibodies not recognizing all the target epitopes due to the loss of epitopes by high temperature processing. As an example, the simplest matrix, the Elmhurst Milked cashew milk (Table 2.1), has an expected ppm value of over 16000 ppm (4g of protein per 240 mL serving based on the nutrition fact panel). While a high cashew protein value is expected, the BioFront kit reported only 39 ppm cashew protein indicating the potential for loss of epitopes from the cashew milk processing (Table 2.4).

The R-Biopharm kit detected cashew proteins in only two (Cashew Dream and Pacific Foods) of the six cashew milk samples tested (Table 2.4). The inability to detect cashew protein from the other four samples with this kit suggests that the antibodies used in the R-Biopharm kit may not be recognizing the cashew proteins in the cashew milk samples that were subjected to heat processing (Table 2.4). Overall, the R-Biopharm ELISA showed the lowest sensitivity for the cashew milk samples, and similar to other ELISA kits, was not reliable in cashew milk protein detection and/or quantification.

Overall, no ELISA was accurate or reliable in quantifying cashew proteins from the cashew milk samples. Detection by each kit was either overestimating or underestimating the amount of cashew protein that would be expected in the cashew milk samples. Similar problems have been found with other nut milks, with low protein detected by a variety of commercial ELISA kits. Slotwinski et al., (2018) tested almond milk with the Veratox for Almond Allergen assay and although they did detect almond protein, a large overestimation of the amount of detected almond protein was observed

(Slotwinski, et al., 2018). This observation aligns with our observations with overestimation of cashew protein using the in-house cashew assay. In a separate study by Dhakal et. al, (2014), almond milk was subjected to high pressure processing (HPP) and the immunoreactivity was determined by ELISA (Dhakal, et al., 2014). In comparison to no processing, HPP was able to decrease the immunogenicity of the almond milk, showing protein underestimation by ELISA (Dhakal, et al., 2014). This supports the notion that highly processed plant milk samples are not readily detected by ELISA similar to our findings on lower detection of cashew milk protein by ELISA.

In comparing all four assays, the BioFront kit gave the most reliable cashew protein values across all cashew milks; however, this assay also significantly underestimated the amount of protein in each cashew milk (Table 2.4). The in-house assay had a large amount of interference, possibly from non-specific binding, and therefore, is not reliable in testing UHT/HTST treated cashew milks due to its high overestimation of the amount of cashew protein in cashew milks. The 3M kit, providing no consistent results across dilutions, showed the possibility for non-specific binding based upon the variability that was observed across all cashew milks, thereby leading to an overestimation or underestimation of the amount of cashew protein for some samples. The R-Biopharm cashew ELISA consistently had a very low (or no) detection cashew milk protein as the recovery was either very low or below the limit of quantification. Based on the results from these four assays, low recovery or non-specific binding did not allow for an accurate detection of cashew protein from HTST/UHT treated cashew milks. Although all four assays were able to detect the presence of cashew from a qualitative standpoint from some or all of the samples, the quantitative results were not reliable and

do not accurately represent the amount of cashew protein in high-temperature treated cashew milks, thus demonstrating the need for the development of a more robust ELISA method for detection of cashew milk residue.

c. IgG Immunoblotting

IgG immunoblotting was carried out on the cashew milk samples using the antibodies supplied with the commercial ELISA kits (conjugated antibodies) and the in-house ELISA (coating and detection antibodies). The results from IgG immunoblotting indicated the specific cashew proteins are being recognized by each of these antibodies thereby supporting some of the quantitative results seen with the ELISAs. Based on information provided by the BioFront kit insert, it uses a cocktail of monoclonal antibodies. The immunoblot using the monoclonal antibodies supplied with the BioFront ELISA kit showed strong recognition of protein band(s) around 33 kDa and a somewhat weaker binding at 20 kDa under reducing conditions (Figure 2.2). The 53 kDa cashew 11S protein under non-reducing conditions was not being recognized as strongly by the antibodies from the BioFront kit which may be due to the proteins presenting as aggregates which are not separated by SDS-PAGE (Figure 2.2). These results may illustrate the consistency of the BioFront ELISA with the cashew milk samples since the antibodies recognize similar proteins across all six cashew milk samples under reducing conditions. Although the recovery of cashew milk protein with the BioFront ELISA was lower than expected, it was the most consistent across different dilutions which supports its reliability. Overall, the antibodies from the BioFront kit are recognizing the cashew 11S protein, with possible aggregation due to high heat processing under non-reducing conditions and stronger recognition under reducing conditions.

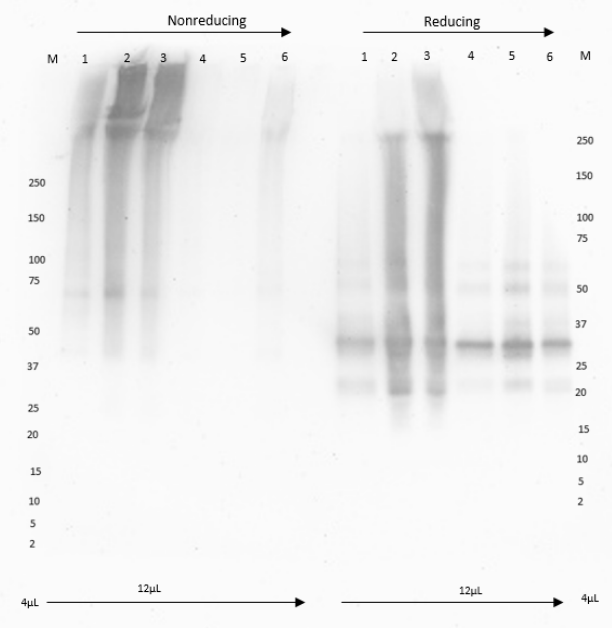


Figure 2.2. IgG immunoblot analysis using BioFront cashew ELISA conjugated antibody (antibody diluted X20). Lane letter or number corresponds to each of the following from left to right: Lane M – Molecular Weight Marker (kDa); Lane 1 – Silk; Lane 2 – Pacific Foods; Lane 3 – Dream; Lane 4 – Elmhurst Milked; Lane 5 – SoDelicious; Lane 6 – Forager Project.

The IgG immunoblot using the R-Biopharm ELISA antibodies resulted in almost no recognition of the proteins in the cashew milk samples under both reducing and non-reducing conditions (Figure 2.3). There was some recognition of proteins with a molecular weight of ~33 kDa under reducing conditions; however, there did not seem to be any recognition of the basic subunit for the cashew 11S protein at ~20 kDa (Figure 2.3). This correlates to the low yield seen with the R-Biopharm ELISA. Lanes 2 and 3 (Pacific Foods and Dream) do show slightly stronger recognition near 30 kDa under reducing conditions, correlating with the low but positive results seen for these two cashew milks in the quantitative ELISA results.

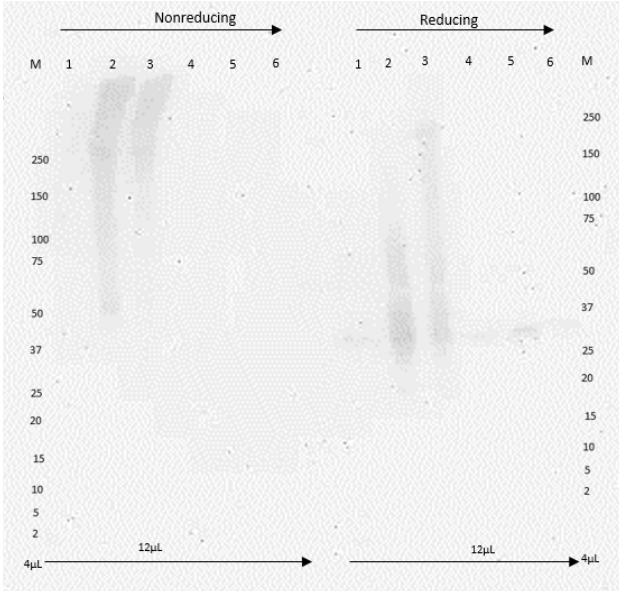


Figure 2.3. IgG immunoblot analysis using R-Biopharm cashew ELISA conjugated antibody (antibody diluted X20). Lane letter or number corresponds to each of the following from left to right: Lane M – Molecular Weight Marker (kDa); Lane 1 – Silk; Lane 2 – Pacific Foods; Lane 3 – Dream; Lane 4 – Elmhurst Milked; Lane 5 – SoDelicious; Lane 6 – Forager Project.

The IgG immunoblot analysis using the antibodies from the 3M ELISA have faint binding under non-reducing conditions (Figure 2.4). Some possible binding occurred at ~20 kDa under non-reducing conditions, corresponding to the cashew 11S basic subunit (Figure 2.4). This faint binding indicates that the antibodies may not be strongly recognizing this protein, especially in comparison to the other cashew antibodies tested. In addition, the apparent binding to smears may indicate a background/matrix interference which agrees with the unreliable results from the 3M and in-house ELISA.

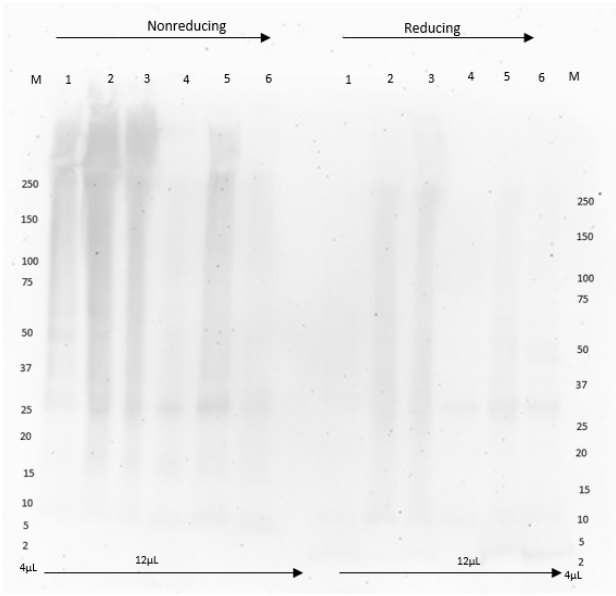


Figure 2.4. IgG immunoblot analysis using 3M cashew ELISA conjugated antibody (antibody diluted X20). Lane letter or number corresponds to each of the following from left to right: Lane M – Molecular Weight Marker (kDa); Lane 1 – Silk; Lane 2 – Pacific Foods; Lane 3 – Dream; Lane 4 – Elmhurst Milked; Lane 5 – SoDelicious; Lane 6 – Forager Project.

The in-house sheep anti-cashew antibody (coating antibody) recognized several of the cashew proteins from the cashew milk samples (Figure 2.5). This is because the in-house assay uses polyclonal antibodies which recognize multiple cashew allergen proteins (Gaskin, et al., 2011). Some of the cashew milks such as those in lanes 4, 5 and 6 (Elmhurst, SoDelicious, Forager Project) do show clear recognition of the cashew 7S, 11S, and 2S proteins under reducing conditions (Figure 2.5). This again supports the notion that there is better recognition under reducing conditions by these antibodies compared to non-reducing conditions. In addition, clear binding at 25-30 kDa seems to be present in the cashew milks under non-reducing conditions which indicates a higher presence of the cashew 2S proteins in the cashew milks (Figure 2.5).

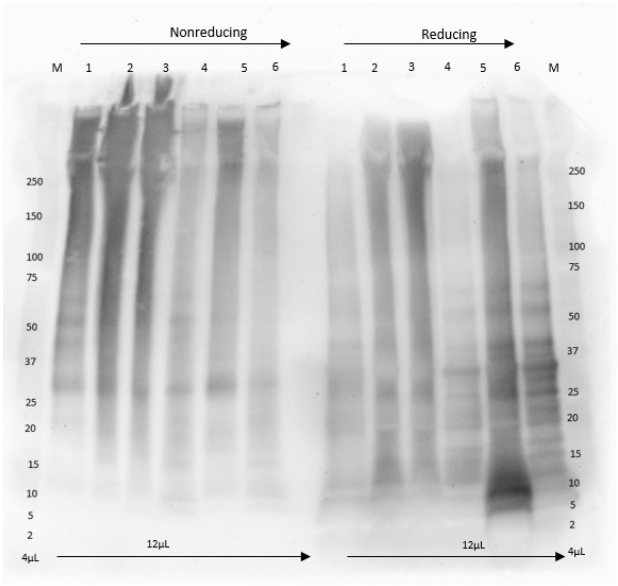


Figure 2.5. IgG immunoblot analysis using sheep anti-cashew antibody (antibody diluted X15000). Lane letter or number corresponds to each of the following from left to right: Lane M – Molecular Weight Marker (kDa); Lane 1 – Silk; Lane 2 – Pacific Foods; Lane 3 – Dream; Lane 4 – Elmhurst Milked; Lane 5 – SoDelicious; Lane 6 – Forager Project.

The in-house rabbit anti-cashew antibody (secondary antibody) shows relatively clear binding to the cashew 11S protein under reducing conditions (Figure 2.6). This can be seen with all six cashew milk samples with binding at 30 kDa and 20 kDa corresponding to the cashew 11S acidic and 11S basic subunits, respectively. In addition, possible recognition of the cashew 7S protein (~50 kDa) can be seen with all cashew milks under both reducing and non-reducing conditions. This indicates that the rabbit anti-cashew antibody may be better at detecting the cashew proteins in cashew milk in comparison to the sheep anti-cashew antibody. However, since the ELISA is a sandwich ELISA, binding is dependent on both antibodies for accurate protein recognition. In addition, the apparent binding to smears may indicate a background/matrix interference which agrees with the unreliable results from the in-house and 3M ELISA.

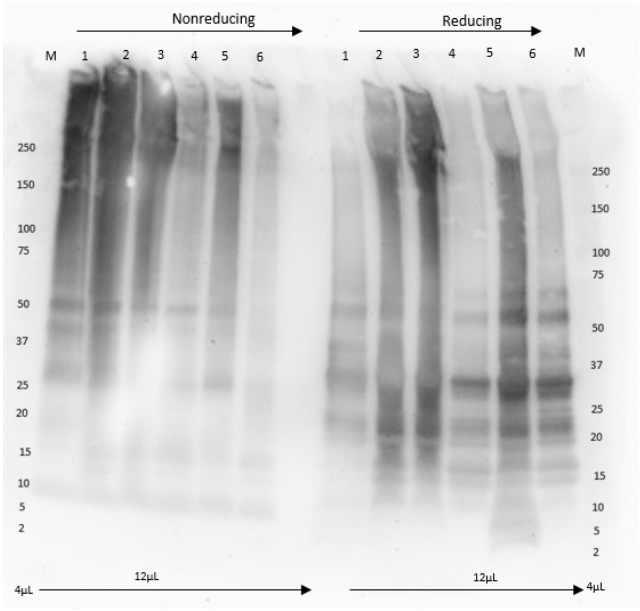


Figure 2.6. IgG immunoblot analysis using rabbit anti-cashew antibody (antibody diluted X10000) Lane letter or number corresponds to each of the following from left to right: Lane M – Molecular Weight Marker (kDa); Lane 1 – Silk; Lane 2 – Pacific Foods; Lane 3 – Dream; Lane 4 – Elmhurst Milked; Lane 5 – SoDelicious; Lane 6 – Forager Project.

Overall, all of the antibodies from the ELISAs identified the cashew 11S proteins which supports the data that the 11S is the most abundant protein (Wang, et al., 2003). Under reducing conditions, the cashew milk proteins are more easily extracted and recognized by IgG antibodies than under non-reducing conditions. It is for these reasons that the work described in the remainder of this thesis focused on the use of the reduced form of the proteins as targets for an improved ELISA for detection of processed cashew residue.

IV. CONCLUSIONS

SDS-PAGE of the cashew milks show multiple protein bands in each cashew milk, indicating that multiple cashew allergens are present. IgG immunoblot analysis demonstrated both specific (BioFront) and sporadic, non-specific (3M, in-house) recognition of these allergenic protein bands. 2-D quant protein estimation indicated varying amounts of soluble cashew protein from each cashew milk sample which was dependent on the extraction buffer as well as extraction conditions (temperature). This indicates that the protein solubility is dependent on both the extraction buffer and extraction temperature. The highest yield was observed with PBS containing sodium bisulfite and SDS, when extracted at 100°C. However, due to the preferred extraction temperature of 60°C, the extraction buffer with PBS containing sodium sulfite and SDS, when extracted at 60°C was chosen. Overall, cashew milks were not accurately and reliably detected by the current cashew ELISA methods. While the BioFront ELISA was able to detect cashew proteins consistently, it tended to underestimate the cashew protein in the cashew milk samples. The 3M and in-house assays showed matrix interference or nonspecific binding which led to overestimation and inconsistencies of the amount of cashew proteins present in all six cashew milk samples. The R-Biopharm assay detected cashew proteins just above the lower limit of quantification in some instances but failed to detect cashew protein in some samples. IgG immunoblot analysis using the antibodies provided by each of the four ELISAs showed recognition or binding to the cashew 11S acidic and basic units with enhanced recognition being observed under reducing conditions. In conclusion, these results indicate that current cashew ELISAs are unable to accurately and reliably detect cashew protein from HTST/UHT treated cashew milk

samples. Therefore, a more robust ELISA capable of reliably quantifying cashew from processed samples including cashew milks was developed, targeting the reduced 11S and native 2S cashew proteins, as described further in this thesis.

V. REFERENCES

Abtahi, S., & Aminlari, M. (1997). Effect of sodium sulfite, sodium bisulfite, cysteine, and pH on protein solubility and sodium dodecyl sulfate−polyacrylamide gel electrophoresis of soybean milk base. *Journal of Agricultural and Food Chemistry, 45*(12), 4768-4772.

Bock, S. A., Munoz-Furlong, A., & Sampson, H. A. (2001). Fatalities due to anaphylactic reactions to foods. *J Allergy Clin Immunol, 107*(1), 191-193.

Dhakal, S., Liu, C., Zhang, Y., Roux, K. H., Sathe, S. K., & Balasubramaniam, V. M. (2014). Effect of high pressure processing on the immunoreactivity of almond milk. *Food Research International, 62*, 215-222.

Gaskin, F. E., & Taylor, S. L. (2011). Sandwich enzyme-linked immunosorbent assay (ELISA) for detection of cashew nut in foods. *J Food Sci, 76*(9), T218-226.

Gupta, R. S., Springston, E. F., Warrier, M. R., Smith, B. M., Kumar, R., Pongracic, J., & Holl, J. L. (2011). The prevalence, severity, and distribution of childhood food allergy in the United States. *Pediatrics, 128*(1), 9-17.

Gupta, R. S., Warren, C. M., Smith, B. M., Jiang, J., Blumenstock, J. A., Davis, M. M., Schleimer, R. P., & Nadeau, K. C. (2019). Prevalence and severity of food allergies among US adults. *JAMA network open, 2*(1), 1-14.

Ito, K., Yamamoto, T., Oyama, Y., Tsuruma, R., Saito, E., Saito, Y., Ozu, T., Honjoh, T., Adachi, R., Sakai, S., Akiyama, H., & Shoji, M. (2016). Food allergen analysis for processed food using a novel extraction method to eliminate harmful reagents for both ELISA and lateral-flow tests. *Anal Bioanal Chem, 408*(22), 1618-2650 (Electronic).

Liu, C., Wang, H., Cui, Z., He, X., Wang, X., Zeng, X., & Ma, H. (2007). Optimization of extraction and isolation for 11S and 7S globulins of soybean seed storage protein. *Food Chemistry, 102*(4), 1310-1316.

Mattison, Bren-Mattison, Y., Vant-Hull, B., Vargas, A. M., Wasserman, R. L., & Grimm, C. C. (2016). Heat-induced alterations in cashew allergen solubility and IgE binding. *Toxicology Reports, 3*, 244-251.

McCabe, J. P., Fletcher, S. M., & Jones, M. N. (1988). The effects of detergent on the enzyme-linked immunosorbent assay (ELISA) of blood group substances. *Journal of Immunological Methods, 108*(1), 129-135.

McWilliam, V., Koplin, J., Lodge, C., Tang, M., Dharmage, S., & Allen, K. (2015). The prevalence of tree nut allergy: a systematic review. *Curr Allergy Asthma Rep, 15*(9), 54.

Monaci, L., & Visconti, A. (2010). Immunochemical and DNA-based methods in food allergen analysis and quality assurance perspectives. *Trends in Food Science & Technology, 21*(6), 272-283.

Sicherer, S. H., Munoz-Furlong, A., Godbold, J. H., & Sampson, H. A. (2010). US prevalence of self-reported peanut, tree nut, and sesame allergy: 11-year follow-up. *J Allergy Clin Immunol, 125*(6), 1322-1326.

Slotwinski, E., Almy, D., Viator, R., Abouzied, M., Klein, F., & Rice, J. (2018). Development and validation of a quantitative ELISA for the detection of almond residues in foods. *J AOAC Int, 101*(1), 118-123.

Teuber, S. S., Sathe, S. K., Peterson, W. R., & Roux, K. H. (2002). Characterization of the soluble allergenic proteins of cashew nut (Anacardium occidentale L.). *Journal of Agricultural and Food Chemistry, 50*(22), 6543-6549.

Towbin, H., Staehelin, T., & Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A, 76*(9), 4350-4354.

Wang, F., Robotham, J., S Teuber, S., Sathe, S., & H Roux, K. (2003). Ana o 2, a major cashew (Anacardium occidentale L.) nut allergen of the legumin family. *International Archives of Allergy and Applied Immunology, 132*(1), 27-39.

Wei, Y., Sathe, S. K., Teuber, S. S., & Roux, K. H. (2003). A sensitive sandwich ELISA for the detection of trace amounts of cashew (Anacardium occidentale L.) nut in foods. *Journal of Agricultural and Food Chemistry, 51*(11), 3215-3221.

Zhao, Y., Sun, X., Marquis, C. P., & Lee, N. A. (2019). Development of a sensitive sandwich ELISA specific to 2S albumin (Ana o 3) as a stable protein marker for cashew nut residue detection in pre-packaged food products. *Food Control, 96*, 432-440.

CHAPTER 3: CASHEW ANA O 2 & ANA O 3 PURIFICATION FOR IMMUNOGEN PREPARATION

I. INTRODUCTION

Cashew Ana o 2 and Ana o 3 have been identified as potential ELISA targets due to their abundance, resilience and heat stability (Mattison, et al., 2016). Ana o 2 is an 11S globulin which comprises about 50% of the soluble cashew protein (Robotham, et al., 2010). Ana o 3 is a 2S albumin which has been identified as being both heat and pH resilient and highly immunogenic (Hummel, et al., 2015). While Ana o 1 is another major seed storage protein in cashew with potential immunogenicity, it has been reported to be less stable to high heat and other processing techniques (Mattison, et al., 2016; Wang, et al., 2002). Multiple ELISAs have used antibodies directed against either whole cashew proteins (i.e. multiple proteins from cashew) or cashew 2S proteins for reliable cashew detection, but so far no ELISA methods have utilized antibodies that target the cashew 11S and 2S proteins collectively (Gaskin, et al., 2011; Zhao, et al., 2019). The reduced form of the cashew 11S proteins has also been found to be more soluble during extraction and thus, may be a better target than the native form (Abtahi, et al., 1997). Based on past research, targeting these two cashew proteins, namely intact cashew 2S and the reduced form of the cashew 11S proteins, should help increase the detection of highly processed forms of cashew protein residue in a cashew ELISA.

Proteins can be purified from a crude mixture of proteins using a number of different techniques, including column chromatography or wet chemistry methods such as pH precipitation. Gel filtration and anion exchange chromatography are two commonly used column chromatography techniques (Mattison, et al., 2019; Zhao, et al.,

2019). A disadvantage of gel filtration is that it only yields small amounts of purified protein, extending the time it takes to collect a sufficient quantity for raising antibodies and ELISA development (Mukherjee, 2019). Methods such as pH precipitation utilize the isoelectric point of the individual proteins to either precipitate or solubilize depending on what is desired (Boye, et al., 2010). This method has been used extensively for the purification of a number of seed storage proteins including from tree nuts and soy (Khan, et al., 2006; Nagano, et al., 1992; Sze-Tao, et al., 2000). The application of such a method for cashew could be beneficial to obtain a higher yield and efficiency in comparison to gel filtration.

Based on the techniques mentioned above, pH precipitation was used for cashew 11S purification while both anion exchange chromatography and a low pH extraction were evaluated for cashew 2S purification as described further in this chapter. The purified proteins were then used to produce polyclonal antibodies in rabbits and subsequently utilized for the development of a sandwich ELISA as described in Chapter 4.

II. MATERIALS AND METHODS

a. Cashew Nut Preparation

Whole raw organic cashew nuts were purchased from Terrasoul Superfoods (Fort Worth, Texas). The cashew nuts were kept in their original vacuum-sealed packaging and stored at 4 °C until further processing. To prepare cashew nuts for purification, whole raw cashew nuts were hand-sorted (to ensure only whole cashew nut pieces were included), split lengthwise to produce cashew nut splits, and washed ten times in distilled water to remove any potential residue that many have been present due to cross-contact with other nuts from surface contact. Cashew nuts were patted dry and air-dried overnight in a fume hood for 12 h and stored at 4 °C until further processing.

Cashew nuts were roasted for downstream analysis. For roasting, the already washed and dried cashew nuts were brought to RT and placed on a parchment-lined aluminum tray and roasted at 132°C for 15 min. The cashew nuts were cooled to RT and stored at -20°C until further processing. The roasted cashew nuts were homogenized by flash freezing with liquid nitrogen using a freezer mill (SPEX 6850). The ground cashew nuts were brought to RT and de-fatted in excess n-Hexane [1:5 (w/v)]. The defatted cashew flour was stored at -20°C until further analysis.

The total protein content of both the ground cashew nuts and the de-fatted cashew flour was determined using the Dumas nitrogen method using a LECO thermogravimetric system (LECO Corporation, St. Louis, MO).

b. Ana o 3 Purification

i. Column Chromatography

Cashew 2S protein was purified from roasted, defatted cashew flour using a method described by Zhao et al. (2019) with minor modifications. Briefly, 4 g of defatted cashew flour was extracted in 0.1 M Tris-HCl- containing 0.9% NaCl, pH 8.4 at RT for 60 min with constant magnetic stirring. The resulting supernatant following centrifugation at 13000 xg for 30 min at RT was filtered using a 0.45 um bottle top filter and degassed. A HiScale 26/20 column (106 mL column volume) (GE Healthcare Life Sciences, Chicago, IL) packed with Source 30Q anion exchange resin (GE Healthcare Life Sciences) was used for anion exchange chromatography. Forty mL of the prepared sample was loaded onto the anion exchange column and eluted using a linear gradient of 0-1 M NaCl in 20 mM Tris-HCl, pH 8.4. In total, 90 fractions were collected, each containing 14 mL/fraction. This resulted in only a few fractions containing the desired 2S cashew protein. Therefore, a second run was performed using a step-wise gradient of 0-0.12 M NaCl in 20 mM Tris-HCl, pH 8.4, holding the salt concentration at 0.12 M NaCl for 50 fraction volumes. The salt gradient was then continued from 0.12-1 M NaCl in 20 mM Tris-HCl, pH 8.4 to get rid of any leftover protein on the column. In total, 90 fractions were collected, each containing 14 mL/fraction. The resulting fractions were analyzed separately, with the fractions containing cashew 2S protein concentrated using 3 kDa Centriprep centrifugal filters (15mL) (Merck Millipore Ltd., Co. Cork, Ireland).

SDS-PAGE was carried out as described previously in Chapter 2.II.a for every fifth fraction collected from the anion exchange column from both runs, under reducing conditions. In addition, a silver stain of selected tubes (tubes 8-22) from the first run was

carried out according to the instructions provided by the manufacturer (BIO-RAD silver stain plus). Following gel electrophoresis, the gel was fixed in a fixative enhancer solution provided in the staining kit for 2 h at RT. The gel was washed five times for 5 min each in distilled water and stained using the provided staining solution for 20 min or until the protein bands were developed. The reaction was stopped with 5% acetic acid and the protein bands were visualized using a Kodak Gel Logic 440 imaging system (Eastman Kodak Company, New Haven, CT).

ii. Low pH Extraction

Cashew 2S protein from the prepared roasted, defatted cashew flour was purified using a low pH extraction method with minor modifications (Hummel, et al., 2015). Briefly, 60 g of defatted cashew flour was extracted in 600 mL of 100 mM Glycine-HCl, pH 2.5 at RT for 2 h with constant magnetic stirring. The resulting slurry was centrifuged at 9000 xg for 30 min and the protein in the supernatant was visualized by SDS-PAGE and confirmed by mass spectrometry to be enriched with the cashew 2S protein. The cashew 2S protein was dialyzed against 0.01 M PBS, pH 7.4 for the pH to be compatible for immunization to rabbits.

c. Ana o 2 Purification

Cashew 11S protein was purified using a method described by Nagano et al. (1992) followed by a method published by Hummel et al. (2015). Briefly, 100 g of defatted cashew flour was mixed with 1500 mL of pH adjusted (pH 7.5) distilled water and extracted at RT for 1 h with constant magnetic mixing. The resulting slurry was centrifuged at 9000 xg for 30 min at RT followed by the addition of dry sodium bisulfite (0.98 g of sodium bisulfite/L). The pH of the mixture was adjusted to pH 6.4 and kept at

4°C overnight (O/N). Following the incubation, the mixture was centrifuged at 6500 xg for 20 min at 4°C. The resulting supernatant was decanted and the precipitate was washed with distilled water and centrifuged again at 6500 xg for 20 min at 4°C. The supernatant was decanted and the wash step was repeated. The resulting precipitate (~8.0 g) was extracted in 10-fold Glycine-HCl (w/v) (pH 2.5) at RT for 2 h with constant magnetic mixing. The pH of the resulting supernatant following centrifugation at 9000 xg for 30 min at RT was adjusted to pH 5.0 and kept O/N at 4°C. The mixture was centrifuged at 6500 xg for 20 min at 4°C. The supernatant was decanted and the resulting precipitate was dissolved in 40 mL of 0.01 M PBS (pH 7.4) to yield cashew 11S protein fraction.

The cashew 11S protein was reduced and alkylated using DTT and iodoacetamide (IAA), respectively. Briefly, the 11S protein was reduced with 10 mM DTT at 60°C in a shaking water bath (200 rpm) for 20 min. The solution was cooled to RT followed by the addition of 50 mM IAA. The solution was placed in the dark for 90 min at RT with constant shaking and the final reduced and alkalized cashew 11S protein (11S R/A) was dialyzed against 0.01 M PBS at 4°C for 24 h with several changes of buffer. The protein concentration of the R/A cashew 11S protein was determined using the 2-D Quant protein assay (GE Healthcare) and was characterized by SDS-PAGE under both non-reducing and reducing conditions as described previously (Chapter 2.II.i) and mass spectrometry (Chapter 3.II.iv).

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

Whole cashew extract and two partially purified cashew proteins (cashew 11S R/A & 2S) intended for rabbit immunization, were characterized by LC-MS/MS analysis.

Preparation, workflow, and analysis of the samples for MS analysis was directed by Justin Marsh, PhD (FARRP, University of Nebraska-Lincoln). The protein purifications for the two partially purified cashew proteins are described above. The whole cashew extract used roasted defatted cashew flour, prepared as described in Chapter 3.II.a. The roasted defatted cashew flour was extracted in 6 M Urea, 20 mM DTT, 50 mM Tris-HCl, pH 8.6, at 50 mg/mL, by heating in a shaking water bath (200 rpm) at 60°C for 25 min. The extract was centrifuged at 12500 xg for 10 min and the supernatant was transferred to a fresh tube and was termed the whole cashew extract.

Following 2D Quant Assay (GE Healthcare) of the extract and partially purified proteins, 20 µg of protein was reduced, alkylated and digested with trypsin, according to the In-Solution Tryptic Digestion Kit protocol (Thermo Scientific, Waltham, MA). The resultant peptides were subjected to a Pierce™ C18 spin column (Thermo Scientific) clean-up, according to instructions provided by the manufacturer. The samples were reconstituted in 18 µL of 5% (v/v) acetonitrile,0.1% (v/v) formic acid and 2 µL of 200 fmol/µL of glycogen phosphorylase standard (Waters, Milford, MA). Prepared samples were analyzed using LC-MS/MS.

One-dimensional (1D) microscale liquid chromatography separation of tryptic peptides (2 µl injection) was performed, in duplicate (2 technical replicates), with an UltiMate 3000 RSL® liquid chromatography (UPLC) system (Thermo Scientific), equipped with a Javelin™ Direct-Connection Column Filter, 2.1 mm (Thermo Scientific), a Hypersil Gold aQ C18 1.9 µm, 20 x 2.1 mm pre-column (Thermo Scientific) and a Hypersil Gold C18 1.9 µm, 100 x 1 mm analytical reversed phase column (Thermo Scientific). Mobile phase A consisted of water containing 0.1% (v/v) formic acid, whilst

mobile phase B was 100% (v/v) acetonitrile containing 0.1% (v/v) formic acid. The sample was injected on-column and peptides were eluted from the analytical column and separated using a gradient of 2-40% mobile phase B over 60 min at a flow rate of 60 µL/min. The analytical column temperature was maintained at 35°C.

Mass spectrometric analysis utilized a Q Exactive Plus™ Hybrid Quadrupole-Orbitrap™ MS (Thermo Scientific™) in the data-dependent mode with survey scans acquired at a resolution of 70,000 at m/z 400, whereas the target value for the fragment ion spectra was set to resolution of 17500 at m/z 400. Up to the top 10 most abundant isotope patterns with charge 2 to 4 from the survey scan were selected with an isolation window of 1.5 Thomsons and fragmented by higher energy collisional dissociation with normalized collision energies of 27. The maximum ion injection times for the survey scan and the MS/MS scans were 100 and 60 ms, respectively, and the ion target value for scan modes were set to 1E6 and 2E5, respectively. Repeat sequencing of peptides was kept to a minimum by dynamic exclusion of the sequenced peptides for 10 s.

The results were analyzed using Peaks 8.5 with peptides compared to the currently available *Anacardiaceae* database in UniProt and the Glycogen Phosphorylase sequence (UniProt: P00489). All samples were normalized against the 40 fmol glycogen phosphorylase spike. A false discovery rate of 1%, a mass error tolerance of 5 ppm and a retention time shift tolerance of 6 min was set. Proteins were only reported which had greater than 2 unique peptides. Using these settings, 6 proteins were robustly detected as shown in Table 3.1. Protein concentrations were determined based on the normalized peak area of the top three unique tryptic peptides.

Table 3.1. Characteristics of allergens and associated peptide targets selected (Peaks). Proteins were only reported which had greater than 2 unique peptides. Selected peptides compared to the currently available *Anacardiaceae* database in UniProt.

UniProt Acc.	Allergen Name	Protein Family	#Peptides	#Unique Peptides
Q8GZP6	Ana o 2	11S	24	24
Q8L5L6	Ana o 1	7S	18	18
A0A1Z1G953	-	Ribulose bisphosphate carboxylase large chain	17	4
B2KN55	Pis v 11s	11S	5	5
Q8H2B8	Ana o 3	2S	7	7
I3RXT2	-	Glyceraldehyde-3-phosphate dehydrogenase	7	7
P00489	-	Glycogen phosphorylase (SPIKE)	41	41

*Of note is the detection of B2KN55. This is a pistachio protein, but presumably a second 11S isoform, similar to this accession exists in cashew.

e. Polyclonal IgG Antibody Production

Three rabbits each were immunized with partially purified cashew 11S R/A and 2S proteins for polyclonal IgG antibody production at Covance Research Products Inc. (Denver, PA). The immunization protocol was based off the method developed by Harlow & Lane with modifications (Harlow, et al., 1988). The rabbits were immunized subcutaneously with an initial dose of 200 µg cashew protein/rabbit mixed 1:1 with Freund's Complete Adjuvant (FCA). Monthly booster doses of 100 µg/rabbit were administered where the proteins are mixed with either Freund's Incomplete Adjuvant (FIA) or Titermax Gold at a 1:1 ratio. Test bleeds were collected every 2 weeks to monitor antibody production for each rabbit.

Rabbit antibody titers were monitored continuously. A titer value of 10,000 was considered as a sufficient titer, when antibodies can be effectively used for ELISA development as determined by past antibody products conducted in collaboration with Lampire Biological Laboratories (Pipersville, PA). Antibody titers were monitored using an indirect ELISA format using established protocols (Harlow & Lane, 1988). A 96-well polyvinyl microtiter plate (NUNC-Immuno™ MaxiSorp™ 96-MicroWell™ plates, Nagle

Nunc Intl., Rockester, NY, USA) was coated with 100 µL/well of 1 µg/mL cashew 11S R/A or 2S immunogen prepared in coating buffer (0.015 M Na_2CO_3, 0.035 M $NaHCO_3$, pH 9.6) and incubated O/N at 4°C. Following incubation, the plate was washed with wash buffer (0.05% Tween 20 in 0.025 M PBS, pH 7.4) four times and blocked with blocking buffer (0.1% gelatin in 0.025 M PBS, pH 7.4) for 1 h at 37°C. The plate was then washed four times and 10-fold dilutions of rabbit anti-cashew 11S R/A or 2S antisera in conjugate buffer (0.5% BSA, 0.2% Tween-20 in 0.025 M PBS, pH 7.4) were added, 100 µL/well, and incubated for 2 h at 37°C. The plate was washed four times and incubated with 1:5000 v/v alkaline phosphatase-labeled goat anti-rabbit IgG in conjugate buffer (100 µL/well) for 1 h at 37°C. The plate was washed four times and developed for 30 min using p-nitrophenyl phosphate substrate (p-NPP SigmaFast™ Tablets, Sigma Chemical Co., St. Louis, MO) dissolved in 0.2 M Trizma buffer (100 µL/well). The reaction was stopped by adding 1 M NaOH (100 µL/well) and the absorbance read at 405 nm using a plate reader (ELx808 Ultraplate, BioTek Instruments, Inc., Winooski, VT). Sigmoidal titration curves were generated using GraphPad Prism v8.0 (GraphPad Prism® Software, Inc., San Diego, CA). The allergen-specific IgG antibody titer was defined as the log reciprocal of the mid-linear portion of the resulting titration curve when 1 µg/mL cashew 11S R/A or 2S immunogen was coated onto the microtiter plate.

III. RESULTS AND DISCUSSION

Based on the DUMAS analysis, roasted cashew flour had a protein content of 19.42 ± 0.19 g protein per 100 g while roasted and defatted cashew flour had a protein

content of 36.25 ± 1.24 g protein per 100 g. All further protein purifications were performed using the roasted defatted cashew flour.

a. Purification of Cashew 2S Proteins Using Anion Exchange Chromatography

The first anion exchange run eluted purified 2S proteins at a 12% salt concentration (Figure 3.1). However, more of the cashew Ana o 3 (2S) protein eluted following the increase of the salt gradient from 12-50% along with other cashew proteins. Based on the work carried out by Zhao et al (2019), the cashew 2S protein was expected to elute at a concentration of ~ 12 mM NaCl (Zhao, et al., 2019). The current experiment yielded similar results, with the cashew 2S eluting at a NaCl concentration of 10-15 mM. The fractions containing the 2S protein ranged from tubes 9-22 (Figure 3.1).

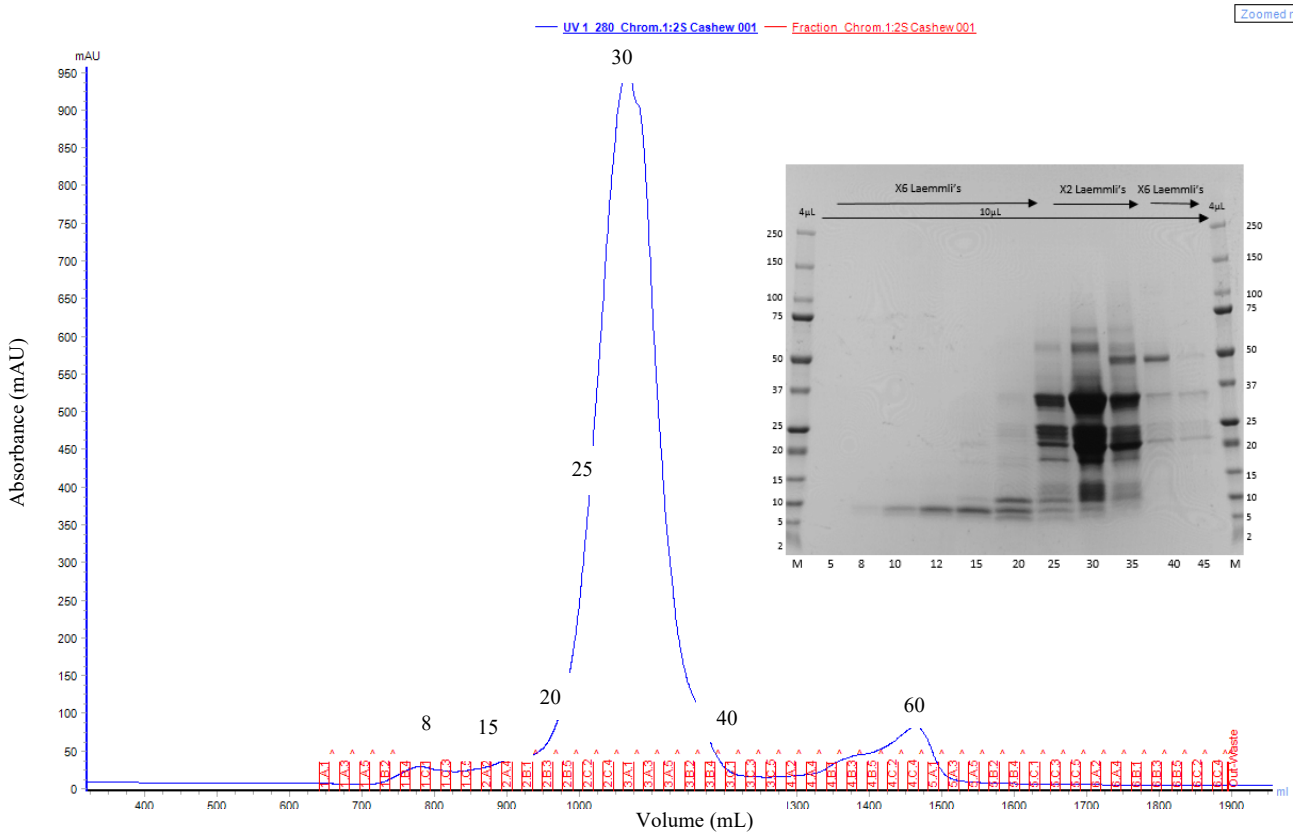


Figure 3.1 Elution profile of cashew protein extract off of the anion exchange column (Source 30Q). The column was equilibrated with 20 mM Tris-HCl, pH 7.4. Fractions corresponding to tubes 8, 15, 20, 25, 30, 40, and 60 are labeled. Inset: Coomassie Brilliant Blue G-250 stained SDS-PAGE profile of select anion exchange fractions under reducing conditions. M: Molecular Weight Marker (kDa). The numbers indicated below each lane correspond to the tube numbers of the fractions eluding off the anion exchange column. Laemmli's buffer (2X) and Laemmli's buffer (6X) were used for sample preparation based on the protein amount in each fraction.

Thus, using this method, cashew 2S protein was partially purified from roasted, defatted cashew flour similar to the results obtained by Zhao et al (2019), suggesting that this method can be used for the purification of cashew 2S protein. The chromatogram in Figure 3.1, indicates that additional peaks, such as the large peak corresponding to fractions 25-45, contains mostly cashew 11S proteins. A thorough separation between the cashew 11S and 2S proteins is needed for raising antibodies specific to these proteins.

Our goal was to develop a robust ELISA using a mixture of antibodies directed against both the cashew 11S and 2S proteins rather than develop highly specific ELISAs for each cashew protein. Therefore, a concentrated 2S immunogen was sufficient to develop a high titer 2S antisera. A minor amount of 11S in the immunogen did not affect our ability to develop the needed 2S antisera.

Selected fractions (tubes 4-45) were analyzed using SDS-PAGE where the protein profiles were visualized using both Coomassie G-250 staining and silver staining. When comparing the Coomassie stain (Inset of Figure 3.1) to the silver stain (Figure 3.2), fractions 8 & 10 showed similar profiles for cashew 2S protein (~8-12 kDa).

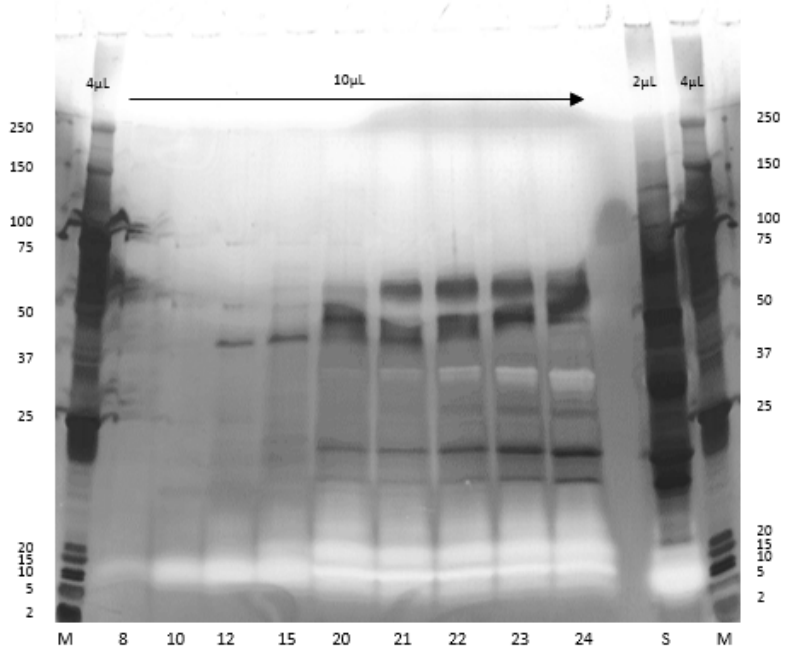


Figure 3.2 Silver stain of select fractions off of the anion exchange column following SDS-PAGE under reducing conditions. M – Molecular Weight Marker (in kDa). The numbers indicated below each lane correspond to the tube numbers of the fractions eluding off the anion exchange column. Laemmli's buffer (2X) and Laemmli's buffer (6X) were used for sample preparation based on the protein amount in each fraction.

Although the 2S bands appear bleached on the silver stain (Figure 3.2), the bleaching occurs in the molecular weight regions corresponding to the protein bands in the Coomassie stain (Inset of Figure 3.1) where there is a large quantity of protein in each band, thus the bleaching is likely due to the high concentration of protein in these areas. However, with the Coomassie staining (Figure 3.1), some of the other protein bands corresponding to tubes 12, 15, and 20 are less visible compared to that of the silver stain (Figure 3.2). This is not surprising as silver staining is known to be significantly more sensitive when compared to Coomassie staining of proteins (Neuhoff, et al., 1988). Based on the column and gel profiles, fractions corresponding to tubes 8-20 were pooled together.

Following the initial anion exchange run, a step-wise salt gradient was used which plateaued at 12mM NaCl to increase the yield of cashew 2S proteins in a second anion exchange run. Figure 3.3 gives both the elution profile and the SDS-PAGE profile of the second anion exchange run with a continuous 2S elution using a narrow salt gradient.

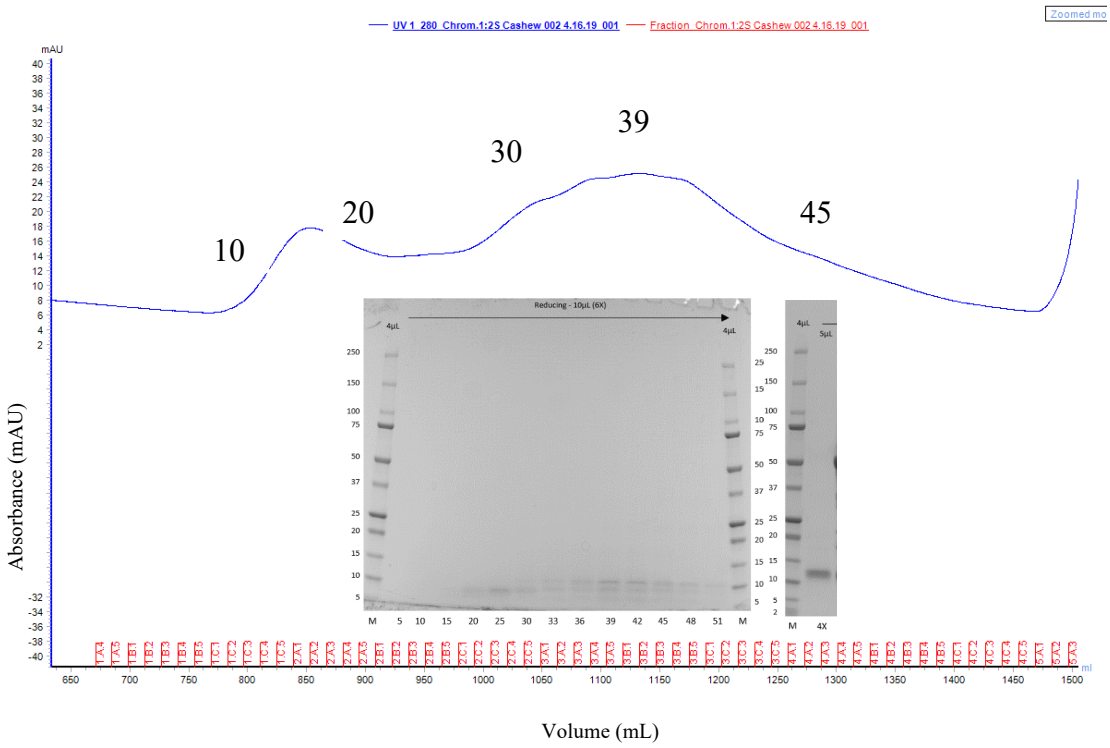


Figure 3.3 Elution profile of cashew protein extract off of the anion exchange column (Source 30Q). The column was equilibrated with 20 mM Tris-HCl, pH 7.4. Fractions containing cashew 2S (tubes 1-60) were pooled. Inset: Coomassie Brilliant Blue G-250 stained SDS-PAGE of select anion exchange fractions & 4X concentrated 2S Fractions (1-60) under reducing conditions. The labels indicated in each lane correspond to the following samples; M – Molecular Weight Marker (in kDa); 4X – 4X concentrated fractions 1-60. The numbers indicated below each lane correspond to the tube numbers of the fractions eluding off of the column (Source 30Q). Laemmli's buffer (2X) and Laemmli's buffer (6X) were used for sample preparation based on the protein amount in each fraction.

The cashew 2S protein eluting in fractions corresponding to tubes 1-60 based on the SDS-PAGE gel (Inset of Figure 3.3) were pooled and concentrated. The 4X concentrated cashew 2S sample in Figure 3.3 (Inset) does appear to be highly purified cashew 2S

proteins. The protein concentration of this sample was 0.16 mg/mL as determined by the 2-D quant protein assay (Table 3.2).

Table 3.2 2-D Quant protein estimation (mg/mL) of the concentrated cashew Ana o 3 proteins off the anion exchange chromatography.

Protein Estimation of Ana o 3 (2S) Concentrated Fractions (tubes 1-60)			
	2-D Quant (mg/mL)	**Total Volume (mL)**	**Total Protein (mg)**
Concentrated Cashew 2S	0.16	75	12

While this purification protocol provided highly purified cashew 2S protein, the yield of total cashew 2S protein (12 mg) was determined to not be sufficient to generate the quantity of purified 2S protein needed for immunization of rabbits for antibody production as well as for other downstream applications (Table 3.2). Consequently, a more efficient method that yielded higher protein quantities was considered for cashew 2S purification.

b. Purification of Cashew 2S Proteins Using Low pH Extraction

Figure 3.4A illustrates the protein profile of the isolated cashew 2S protein using the low pH, glycine-HCl extraction method (Hummel, et al., 2015). A significant amount of cashew 2S protein is present in the supernatant while the precipitate contains the majority of the other cashew proteins. The low pH extraction method worked exceedingly well at selectively extracting the cashew 2S protein as indicated by the SDS-PAGE protein profile (Figure 3.4A). Cashew 2S proteins have a molecular weight of ~8-12 kDa (Zhao, et al., 2019). However, there does appear to be some breakdown products or other proteins which may not be the 2S proteins, visible both below and above 20 kDa in the 2S supernatant (Figure 3.4A). These breakdown products may be stable proteins

which can withstand extreme conditions or dimers or trimers of the 2S proteins. These bands were not evident in the work carried out by Hummel et al. (2015) but are not of major concern for the purpose of producing antibodies against these 2S proteins since these bands are less intense (indicating perhaps a lower quantity of protein) than that of the 2S protein bands (Figure 3.4A) (Hummel, et al., 2015). These faint bands were also present in the cashew milk samples, further indicating both their stability with 2S cashew proteins and their possible resilience to pH and heat treatment (Figure 2.1). Following dialysis against 0.01 M PBS, pH 7.4 the protein profile of the purified cashew 2S proteins remained unchanged (Figure 3.4B). The protein concentrations of the cashew 2S immunogen used for rabbit immunizations are provided in Table 3.3.

Table 3.3. 2-D Quant protein estimation (mg/mL) of the dialyzed cashew Ana o 3 proteins after low pH extraction.

Protein Estimation of Ana o 3 (2S)			
	2-D Quant (mg/mL)	**Total Volume (mL)**	**Total Protein (mg)**
Dialyzed Cashew 2S	0.5	100	75

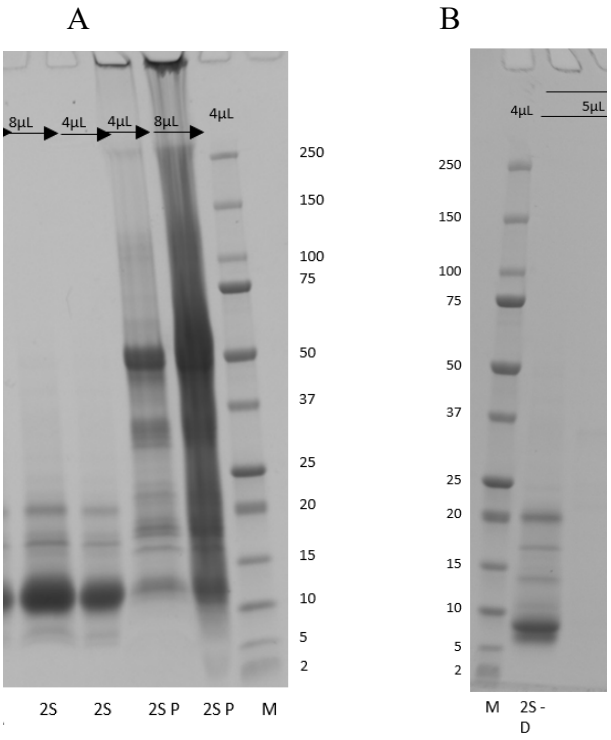


Figure 3.4 Coomassie Brilliant Blue G-250 stained SDS-PAGE (non-reducing) profile of both the supernatant (2S) and precipitate (2SP) of cashew proteins following glycine-HCl extraction (A). Coomassie Brilliant Blue G-250 stained SDS-PAGE (non-reducing) profile of dialyzed 2S cashew proteins (2S G-P) following glycine-HCl extraction (B). The letters indicated in each lane correspond to the following: M – Molecular Weight Marker (in kDa); 2S – 2S Supernatant; 2S P – 2S Precipitate; 2S - D – Dialyzed 2S Supernatant. Sample volume (µL) loaded onto the gel are listed above each lane.

c. Purification of Cashew 11S Proteins Using pH Precipitation Followed by Low pH Extraction

For cashew 11S purification, a pH precipitation method (Nagano, et al., 1992) followed by low pH extraction (Hummel, et al., 2015) and pH adjustment was performed. In the pH precipitation (pH 7.5) process, the cashew 11S protein was expected to precipitate out without any other commingled cashew proteins. However, the cashew 11S precipitated together with the cashew 2S protein and only the cashew 7S separated out into the supernatant (Figure 3.5). The method established by Nagano et al. (1992) was initially described for the separation of soy 7S and 11S proteins (Nagano, et al., 1992). Soy has a lower percentage of 2S proteins (maximum estimate of 8%) than cashew (11.5%) and thus, this could be a potential reason for the cashew 2S proteins to copurify together with the 11S proteins (Kinsella, 1979; Nagano, et al., 1992; Zhao, et al., 2019).

In order to remove some of the lower molecular weight proteins from the partially purified cashew 11S fraction, the cashew 2S purification (pH 2.5) method described by Hummel et al. (2015) was used in sequence with the pH precipitation (pH 7.5) method described by Nagano et al. (1992) to retain the 2S cashew proteins in the supernatant while keeping the 11S cashew proteins in the precipitate (Hummel, et al., 2015; Nagano, et al., 1992). Upon further analysis using SDS-PAGE, it was observed that the cashew 2S proteins remained in the supernatant as seen previously in the low pH extraction (Figure 3.4B). However, the majority of the 11S cashew proteins also remained in the supernatant instead of precipitating out (Figure 3.6). Since the majority of the cashew 11S proteins were present in the supernatant together with the other cashew proteins (Figure 3.6), it was decided that a pH adjustment (pH 5) could be used to separate the cashew proteins

based on their individual isoelectric points (11S: pI ~6.18; 2S: pI ~5.37 obtained using the UniProt database). To separate the cashew 11S proteins from the 2S proteins, the pH of the supernatant was changed over a range of pH 4.5 to pH 7. Consequently, at pH 5 the majority of the 2S cashew proteins remained in solution, while most of the 11S cashew proteins precipitated out (Figure 3.7).

The cashew 11S proteins (the precipitate) following pH adjustment (pH 5) was dissolved in 0.01 M PBS, pH 7.4. The resulting cashew 11S proteins in solution were reduced and alkylated and then dialyzed against 0.01 M PBS, pH 7.4 to remove some of the lower molecular weight proteins (Figure 3.7). Since previous work indicated that a reducing extraction buffer enhances protein extraction (Chapter 2.III.i), targeting the reduced form of the cashew 11S may allow for an increased antibody detection when used in tandem with a reducing extraction buffer for a future developed ELISA. The reducing extraction buffer can help solubilize these cashew 11S proteins which may lead to better antibody recognition with the antibodies raised against a reduced form of cashew 11S (Abtahi, et al., 1997). The protein concentrations of the cashew 11S native and 11S reduced/alkylated immunogen used for rabbit immunizations are provided in Table 3.4.

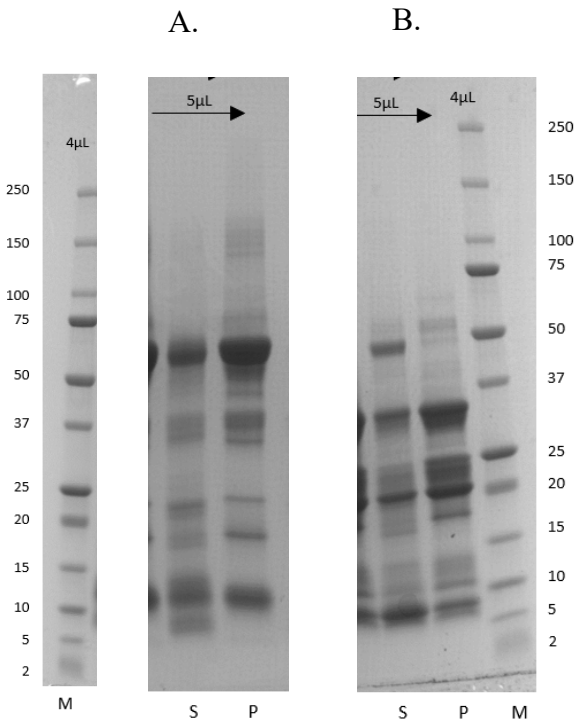


Figure 3.5 Non-reducing (A) and reducing (B) Coomassie Brilliant Blue G-250 stained SDS-PAGE profiles of both the supernatant (S) and precipitate (P) of roasted and defatted cashew extracts following pH precipitation. The letters indicated in each lane correspond to the following samples; M – Molecular Weight Marker (kDa); S – Supernatant; P – Precipitate.

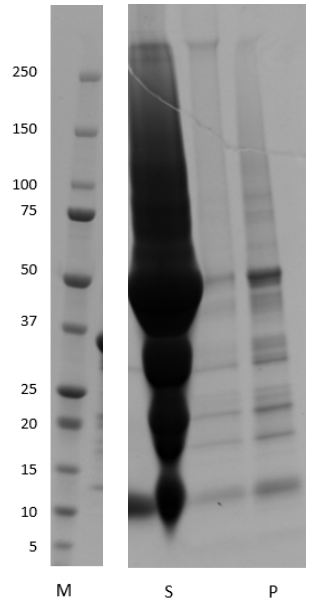


Figure 3.6 Non-reducing Coomassie Brilliant Blue G-250 stained SDS-PAGE profile of both the supernatant (S) and precipitate (P) of cashew following glycine-HCl extraction. The letters indicated in each lane correspond to the following samples; M – Molecular Weight Marker (kDa); S – Supernatant; P – Precipitate.

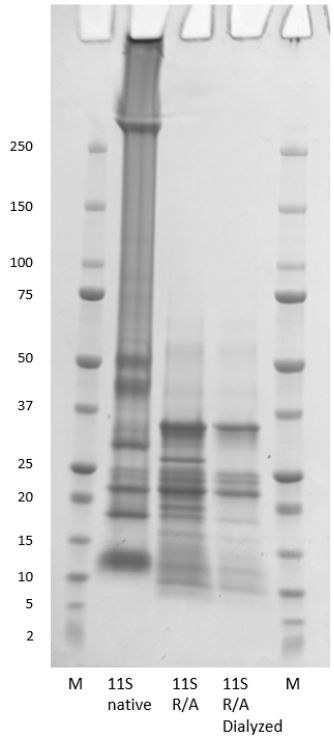


Figure 3.7 Non-reducing Coomassie Brilliant Blue G-250 stained SDS-PAGE profile of native (11S native), reduced & alkylated (11S R/A), and reduced and alkylated 11S following dialysis (11S R/A Dialyzed) cashew proteins following adjustment at pH 5.0. M – Molecular Weight Marker (kDa).

Protein Estimation of Cashew 11S Proteins (mg/mL)	
Sample	**2-D Quant**
11S native	30
11S R/A – Dialyzed	0.50

Table 3.4 2-D Quant protein estimation (mg/mL) of cashew 11S native (11S native) and reduced and alkylated 11S following dialysis (11S R/A Dialyzed) proteins.

d. Use of Mass Spectrometry for Further Characterization Cashew Ana o 2 & Ana o 3 Proteins

The UniProt *Anacardiaceae* database is incomplete and thus, a limitation to any MS analysis. This database holds only 20 accessions, limiting our analysis of the protein sequences such as minor seed storage proteins or lower molecular weight proteins (i.e. lipid transfer proteins), which could explain why the obtained results are slightly different than current literature sources. However, based on the available UniProt database, both the partially purified cashew 11S R/A and 2S proteins were of high purity. The peptides identified for the cashew extract, 11S R/A and 2S proteins are listed in (Table 3.5). As illustrated in Figures 3.8A/B, the cashew 11S R/A protein contained approximately 66.7 ± 0.8% of the 11S protein (Q8GZP6); 24.9 ± 0.8% of the pistachio like 11S protein (B2KN55) and 8 ± 0.02% of the 2S protein (Q8H2B8). The purified cashew 2S proteins has a purity of almost 100%, with very low levels of additional proteins (Figure 3.8B). This is in agreement with the SDS-PAGE protein profiles of the purified cashew proteins (Figures 3.4b & 3.7). Thus, the MS analysis confirms that the two purification methods used for the purification of these proteins resulted in samples with the desired cashew 11S or 2S proteins while decreasing the presence of the unwanted cashew proteins. Based on Figure 3.8A, the roasted, whole cashew extract contains approximately 50.6 ± 3.9% of the cashew 11S protein (Q8GZP6); 17.9 ± 1.7% of the pistachio like 11S protein (B2KN55); 28.0 ± 1.7%of the 2S protein (Q8H2B8) and 3.2 ± 0.4% of the 7S protein (Q8L5L6). This is in agreement with current literature as Sathe (1994) reported that the 11S makes up about 50% of the total soluble cashew protein in cashew while Zhao et al. (2019) reported that the 2S makes up about 11.5% (Sathe, 1994; Zhao, et al., 2019). Roux

et al. (2003) reported that the 7S makes up about 5% of the total soluble cashew protein in cashew, again in good agreement.

Of interest is that the pistachio-like 11S protein (B2KN55), a novel cashew 11S isoform that is moderately abundant in our cashew extract, and secondly, has very good evidence of hydroxyprolination (see Table 3.6). The only other food allergen with known hydroxyprolination at this point in time is the peanut 2S albumin, Ara h 2, which has shown evidence of high immunogenicity, in particular the Hyp region (Bernard, et al., 2015). This protein is obviously a candidate for future research and investigation.

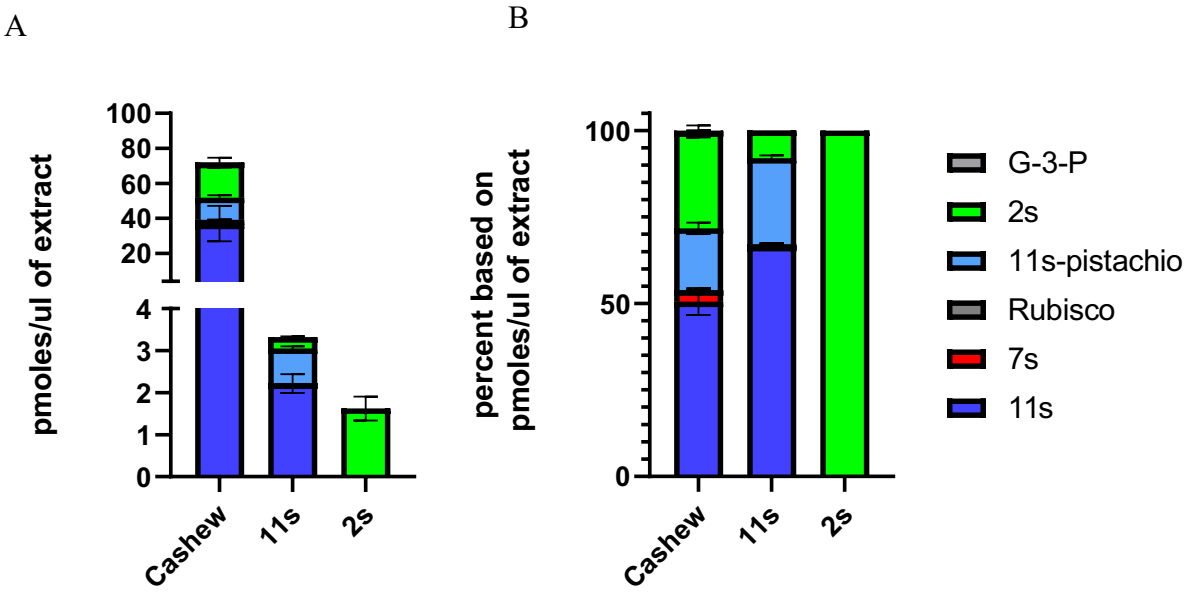


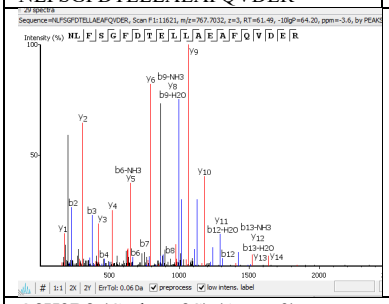
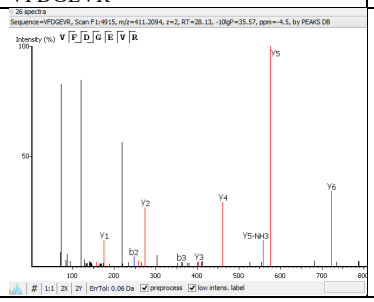
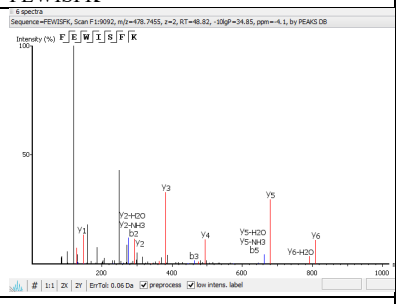
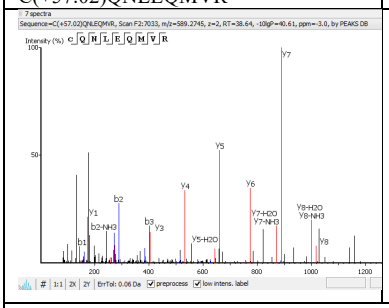
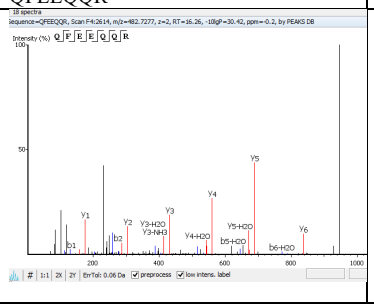
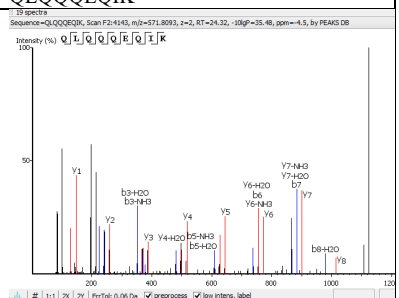
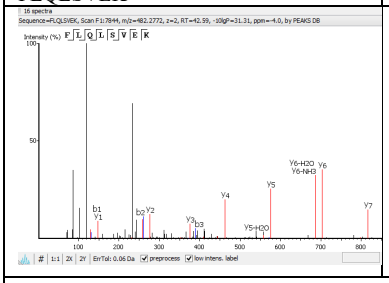
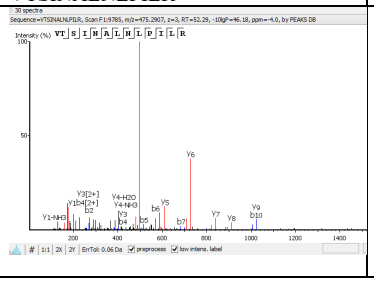
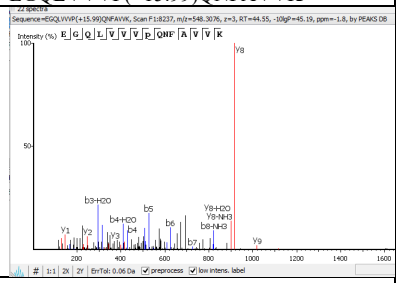
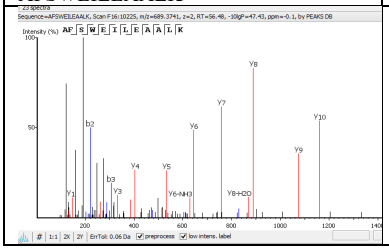
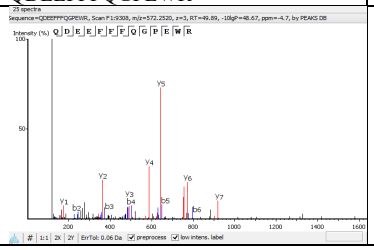
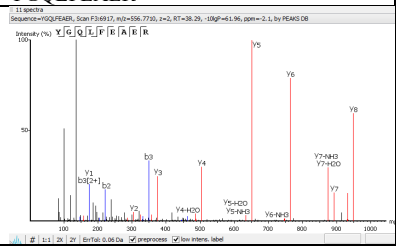
Figure 3.8. Quantification of allergen peptide targets in roasted cashew flour (Ro Cashew) using the LC-MS method. MS data are expressed in duplicate (± SEM) in (A) picomoles/ul or (B) percentage.

Table 3.5. Peptides used for Ana o 2 (11S) and Ana o 3 (2S) quantitation. For each major allergen, three peptides were selected for use in quantitation based on their presence in the most abundant identified allergen isoform and their abundance. Charge state, retention time (RT), and *m/z* of the precursor ion for each peptide are indicated.

Accession # (Genomic Isoform)	Peptide Sequence	*m/z*; charge state	RT
Q8GZP6 (Cashew 11S)	NLFSGFDTELLAEAFQVDER	767.7032 (3+)	61.49
	VFDGEVR	411.2094 (+2)	28.13
	FEWISFK	478.7455 (+2)	48.82
Q8H2B8 (Cashew 2S)	C(+57.02)QNLEQMVR	589.2761 (+2)	39.05
	QFEEQQR	482.7277 (+2)	16.26
	QLQQQEQIK	571.8093 (+2)	24.32
B2KN55 (Pistachio 11S – presumably another cashew isoform)	FLQLSVEK	485.2772 (+2)	42.59
	VTSINALNLPILR	475.2907 (+3)	52.29
	EGQLVVVP(+15.99)QNFAVVK	548.3076 (+3)	44.55
Q8L5L6 (Cashew 7S)	AFSWEILEAALK	689.3741 (+2)	56.48
	QDEEFFFQGPEWR	572.2520 (+3)	49.89
	YGQLFEAER	556.7710 (+2)	38.29

RT: Retention time; m/z: mass over charge ratio

Table 3.6. LC-MS/MS fragmentation pattern (Peaks) of top three peptides used for quantitation for 11S - Ana o 2 sequence Q8GZP6; 2S - Ana o 3 sequence Q8H2B8; 11S - pistachio like 11S protein B2KN55 and 7S - Ana o 1 Q8L5L6.

Q8GZP6 (Cashew 11S) (Ana o 2)		
NLFSGFDTELLAEAFQVDER	VFDGEVR	FEWISFK
		
Q8H2B8 (Cashew 2S) (Ana o 3)		
C(+57.02)QNLEQMVR	QFEEQQR	QLQQQEQIK
		
B2KN55: 11S- pistachio like 11S protein		
FLQLSVEK	VTSINALNLPILR	EGQLVVVP(+15.99)QNFAVVK
		
Q8L5L6: Cashew 7S (Ana o 1)		
AFSWEILEAALK	QDEEFFFQGPEWR	YGQLFEAER
		

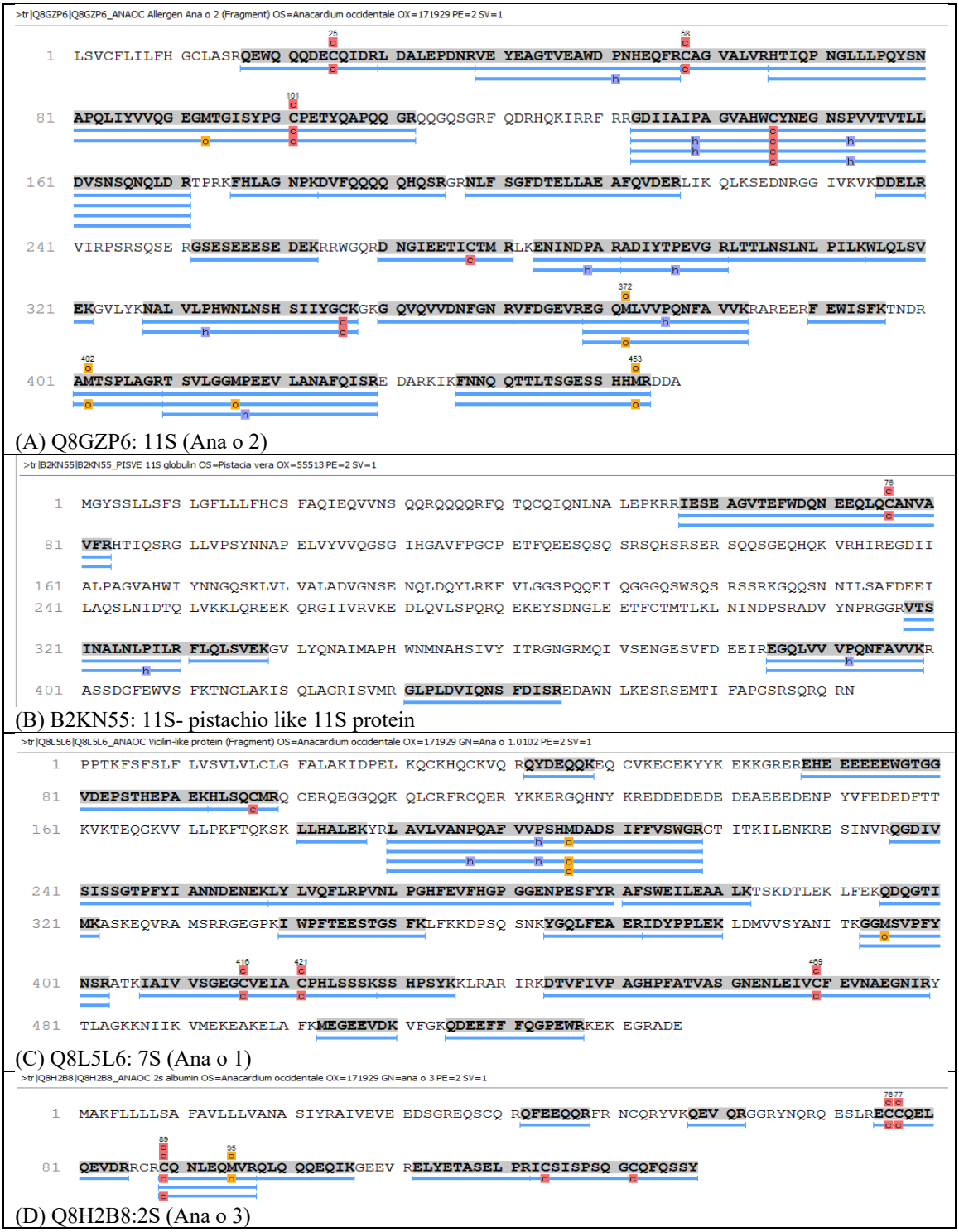


Figure 3.9. Results of LC-MS/MS-peptide coverage (shown in blue) for robustly identified proteins (A) Q8GZP6: 11S (Ana o 2); (B) B2KN55: 11S- pistachio like 11S protein; (C) Q8L5L6: 7S (Ana o 1) and (D) Q8H2B8:2S (Ana o 3).

e. Production of Polyclonal Antibodies Against Cashew 11S R/A and 2S Proteins

Both the partially purified cashew 11S R/A and 2S proteins were dialyzed against 0.01 M PBS, pH 7.4. The final protein concentration of both protein fractions was 0.50 mg/mL protein based on the 2-D Quant protein assay. The polyclonal IgG antibodies raised against 2S cashew proteins are intended to target the heat stable proteins present in cashew milk and other heat processed products (Figure 2.1). The 11S R/A cashew polyclonal IgG antibodies are expected to target the reduced cashew proteins (Abtahi, et al., 1997). For immunization purposes, these two proteins were injected into two different sets of rabbits (3 each) and titers were monitored for no less than 9 months. Titers were continued to be monitored until antibody levels were high enough (>10000) to be used for ELISA development.

Polyclonal IgG antibodies directed against both the cashew 11S R/A and 2S proteins in rabbits were monitored using titration curves. Titration curves were graphed by plotting log antibody dilution on the x-axis and absorbance on the y-axis. The log reciprocal of the mid-linear portion of the titration curve (ED50) was used to determine the titer value. If the bleed for a specific rabbit had a titer value of at least 4, or antilog >10000, antibodies from each rabbit were pooled separately for further ELISA development. Figure 3.10 gives a titration curve for Rabbits NE 384, 385 and 386 from bleed date 9/2/2019 for antibodies raised against cashew 2S proteins (provided as an example titer curve). To demonstrate the calculation, the corresponding ED50 value for the titer of NE 386 was -4.943 where the antilog titer value represented the antilog of 4.943, corresponds to 87,700 (Figure 3.10).

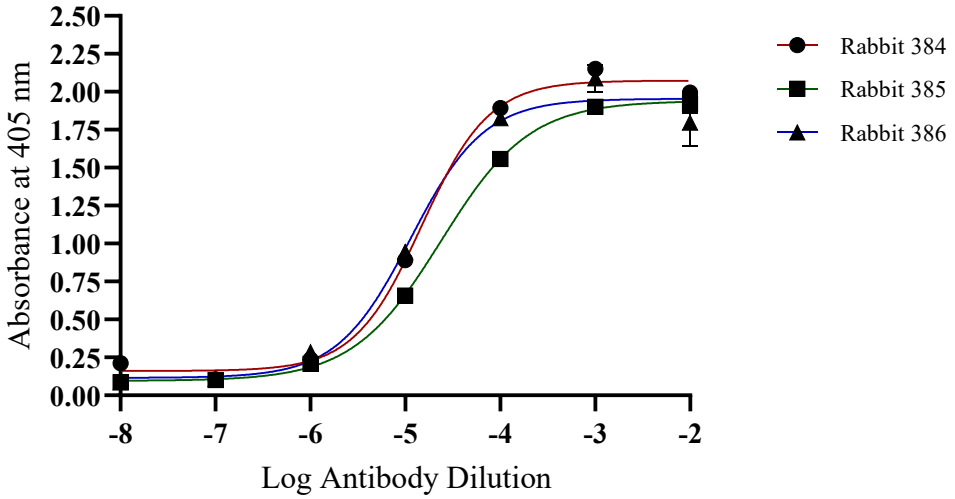


Figure 3.10. Titration curve from Rabbits NE 384, NE 385, & NE 386 (bleed date 9.2.19 (#5)) cashew 2S sera. Wells coated with 1µg/mL of cashew 2S. Each data point represents the mean of duplicate readings.

Production bleeds of each rabbit began after consistent titers >10000 were found for both the cashew 2S and 11S R/A antisera. Figure 3.11 gives the titer values for IgG antibodies raised against cashew 2S proteins (Rabbits NE 384, NE 385, NE 386) while Figure 3.12 gives the titer values for IgG antibodies raised against cashew 11S R/A proteins (Rabbits NE 378, NE 379, NE 380).

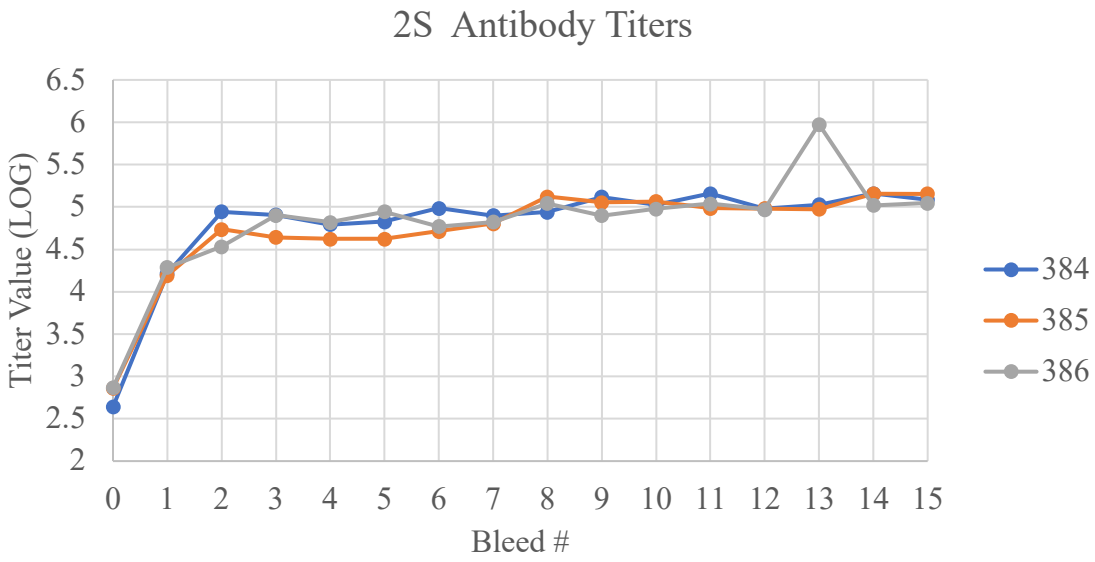


Figure 3.11. Immune response of Rabbits NE 384, NE 385 & NE 386 to cashew 2S immunogen. Each data point represents the mean of duplicate readings.

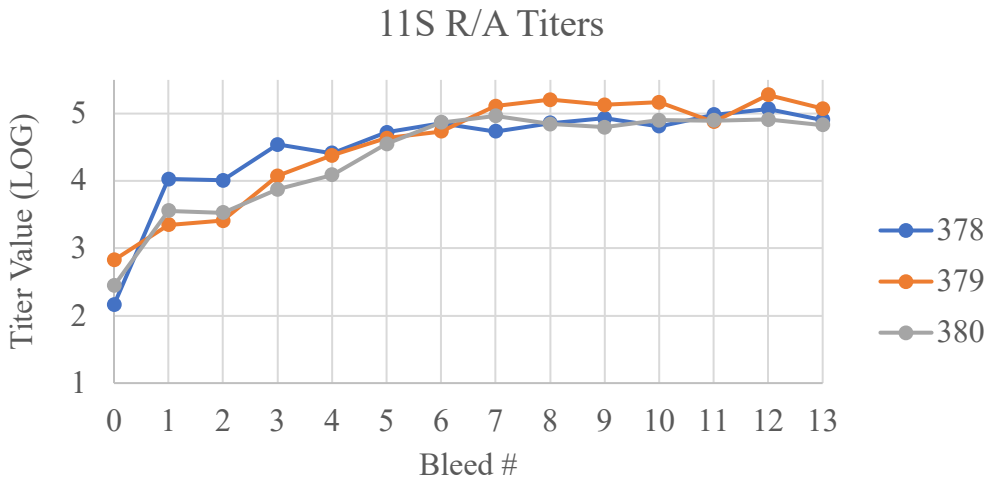


Figure 3.12. Immune response of Rabbits NE 378, NE 379 & NE 380 to cashew 11S R/A antigen. Each data point represents the mean of duplicate readings.

While the cashew 2S sera had titer values greater than 10,000 following the second bleed, the cashew 11S R/A sera did not reach a consistent high titer value until bleed 4 (Figure 3.11 & Figure 3.12). Both the cashew 11S R/A and 2S antisera performed well throughout production bleeds and were used in the further development of a cashew ELISA. For further ELISA development, the cashew 11S R/A antisera from bleed date 9/23/2019 (#4) was selected and for the cashew 2S antisera, from bleed date 9/16/2019 (#6).

IV. CONCLUSIONS

The glycine-HCl purification method gave the highest yield of partially purified cashew 2S proteins in comparison to other purification methods carried out including anion exchange. A pH precipitation followed by a low pH extraction with pH adjustment gave a high yield of partially purified cashew 11S proteins. The purified cashew 11S proteins were reduced and alkylated in order to target the reduced form of cashew 11S for antibody production. Mass spectrometry analysis confirmed the presence and enrichment of the cashew 11S and 2S proteins in the immunogens used for rabbit immunization. Titers of rabbits were monitored following immunization to ensure consistent antibody production. Both the cashew 11S R/A and 2S titers were established to be suitable (>10,000) and thus, using these antibodies, a cashew ELISA was developed and evaluated for robustness and sensitivity in detecting highly processed cashew residues as described in the following chapters.

V. REFERENCES

Abtahi, S., & Aminlari, M. (1997). Effect of sodium sulfite, sodium bisulfite, cysteine, and pH on protein solubility and sodium dodecyl sulfate−polyacrylamide gel electrophoresis of soybean milk base. *Journal of Agricultural and Food Chemistry, 45*(12), 4768-4772.

Bernard, H., Guillon, B., Drumare, M. F., Paty, E., Dreskin, S. C., Wal, J. M., Adel-Patient, K., & Hazebrouck, S. (2015). Allergenicity of peanut component Ara h 2: Contribution of conformational versus linear hydroxyproline-containing epitopes. *Journal of Allergy and Clinical Immunology, 135*(5), 1267-1274.

Boye, J. I., Aksay, S., Roufik, S., Ribéreau, S., Mondor, M., Farnworth, E., & Rajamohamed, S. H. (2010). Comparison of the functional properties of pea, chickpea and lentil protein concentrates processed using ultrafiltration and isoelectric precipitation techniques. *Food Research International, 43*(2), 537-546.

Gaskin, F. E., & Taylor, S. L. (2011). Sandwich enzyme-linked immunosorbent assay (ELISA) for detection of cashew nut in foods. *J Food Sci, 76*(9), T218-226.

Harlow, E., & Lane, D. (1988). *Antibodies: A Laboratory Manual*: Cold Spring Harbor Laboratory.

Hummel, M., Wigger, T., Höper, T., Westkamp, I., & Brockmeyer, J. (2015). Simple, rapid, and selective isolation of 2S albumins from allergenic seeds and nuts. *Journal of Agricultural and Food Chemistry, 63*(26), 6035-6040.

Khan, A., Weaver, C. M., & Sathe, S. (2006). Association of zinc with soy proteins as affected by heat and pH. *Journal of Food Science, 55*, 263-264.

Kinsella, J. E. (1979). Functional properties of soy proteins. *Journal of the American Oil Chemists' Society, 56*(3), 242-258.

Mattison, Bren-Mattison, Y., Vant-Hull, B., Vargas, A. M., Wasserman, R. L., & Grimm, C. C. (2016). Heat-induced alterations in cashew allergen solubility and IgE binding. *Toxicology Reports, 3*, 244-251.

Mattison, Vant-Hull, B., Bren-Mattison, Y., & Grimm, C. C. (2019). A cashew specific monoclonal antibody recognizing the small subunit of Ana o 3. *Toxicol Rep, 6*, 736-744.

Mukherjee, S. (2019). Chapter 2 - Isolation and Purification of Industrial Enzymes: Advances in Enzyme Technology. In R. S. Singh, R. R. Singhania, A. Pandey & C. Larroche (Eds.), *Advances in Enzyme Technology*, (pp. 41-70): Elsevier.

Nagano, T., Hirotsuka, M., Mori, H., Kohyama, K., & Nishinari, K. (1992). Dynamic viscoelastic study on the gelation of 7 S globulin from soybeans. *Journal of Agricultural and Food Chemistry, 40*(6), 941-944.

Neuhoff, V., Arold N Fau - Taube, D., Taube D Fau - Ehrhardt, W., & Ehrhardt, W. (1988). Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis, 9*(6), 255-262.

Robotham, J. M., Xia, L., Willison, L. N., Teuber, S. S., Sathe, S. K., & Roux, K. H. (2010). Characterization of a cashew allergen, 11S globulin (Ana o 2), conformational epitope. *Mol Immunol, 47*(9), 1830-1838.

Roux, K. H., Teuber, S. S., & Sathe, S. K. (2003). Tree nut allergens. *International Archives of Allergy and Immunology, 131*(4), 234-244.

Sathe, S. K. (1994). Solubilization and electrophoretic characterization of cashew nut (Anacardium occidentale) proteins. *Food Chemistry, 51*(3), 319-324.

Sze-Tao, K. W. C., & Sathe, S. K. (2000). Functional properties and in vitro digestibility of almond (Prunus dulcis L.) protein isolate. *Food Chemistry, 69*(2), 153-160.

Wang, F., Robotham, J. M., Teuber, S. S., Tawde, P., Sathe, S. K., & Roux, K. H. (2002). Ana o 1, a cashew (Anacardium occidental) allergen of the vicilin seed storage protein family. *Journal of Allergy and Clinical Immunology, 110*(1), 160-166.

Zhao, Y., Sun, X., Marquis, C. P., & Lee, N. A. (2019). Development of a sensitive sandwich ELISA specific to 2S albumin (Ana o 3) as a stable protein marker for cashew nut residue detection in pre-packaged food products. *Food Control, 96*, 432-440.

CHAPTER 4: DEVELOPMENT OF A SENSITIVE ELISA FOR DETECTION OF CASHEW NUT RESIDUE IN PLANT MILK MATRICES

I. INTRODUCTION

Raising antibodies against a protein(s) of interest is the first step in ELISA development (Harlow & Lane, 1988). ELISAs use either polyclonal or monoclonal antibodies for allergen detection. Some of the recently developed cashew ELISAs have used polyclonal antibodies, using a total cashew extract or purified cashew protein as the immunogen (Gaskin & Taylor, 2011; Zhao, et al., 2019). In the current study, rabbits were immunized separately with cashew Ana o 2 (11S) and Ana o 3 (2S) proteins as outlined in Chapter 3.II.e.

Sandwich ELISAs are a common method used for allergen detection (Wei, et al., 2003). A sandwich ELISA utilizes a capture and detector antibody. The capture antibody "captures" the antigen that may be present in a sample extract while the detector antibody binds to the antigen-antibody complex to make a "sandwich." The detector antibody can be bound to an enzyme-conjugated complex or have an additional enzyme-conjugated antibody added for detection. Once bound, a substrate is added and a change in color can be read colorimetrically (Harlow & Lane, 1988). The sandwich ELISA method requires two antibodies to detect any specific protein, resulting in high sensitivity and specificity (Aydin, 2015). To optimize and validate an ELISA, cross-reactivity and matrix interference studies are performed to ensure an assay's robustness (Gaskin & Taylor, 2011). Matrices incurred with known amounts of the protein in a pre- and post-processed matrix can be evaluated for recovery of protein residue of interest following processing. Plant milk is processed by high-temperature short-time (HTST) or ultra-high temperature

(UHT) processing thus, the matrix is suitable for pre- and post- processing evaluation of protein recovery. Recovery of protein from a matrix can validate the robustness and sensitivity of the ELISA (Gaskin & Taylor, 2011).

With highly processed plant milks on the rise, current allergen detection methods must adequately identify cross-contact between highly processed allergens to ensure the safety of the products for allergic consumers. Based on previous research, current cashew ELISAs are not meeting industry standards on detecting cashew protein residue from cashew milks (Chapter 2.III.b). This reinforces the need for a more sensitive cashew ELISA for highly processed cashew matrices. The optimization and evaluation of the developed cashew ELISA follows methods established by Engvall and others (1971), with the goal of improving cashew protein detection by ELISA in highly processed matrices (Engvall, et al., 1971).

II. MATERIALS AND METHODS

a. Recognition of Cashew Ana o 2 & Ana o 3 Proteins Using Polyclonal IgG Antibodies Raised Against Specific Cashew Proteins

Immunoblots were performed to determine the different cashew proteins being recognized by the cashew 11S R/A and 2S antibodies. Briefly, 1.0 g of roasted de-fatted cashew flour was extracted in 20 mL of both a non-reducing (0.01 M PBS, pH 7.4) and reducing buffer (0.01 M PBS with 0.01 M Sodium Sulfite and 1% SDS, pH 7.4) in a shaking water bath (200 rpm) at 60°C for 25 min. The protein concentration of the resulting supernatant following centrifugation at 12000 xg for 10 min was determined using the 2-D Quant protein assay (GE Healthcare, Chicago, IL). The protein profile of the supernatant was characterized using SDS-PAGE. Five different samples (cashew extracted in non-reducing buffer, cashew extracted in reducing buffer, cashew 2S immunogen, cashew 11S R/A immunogen, and cashew 11S immunogen before reduction and alkylation [native]) were prepared 1:1 (v/v) using 2X Laemmli sample buffer with or without DTT. SDS-PAGE was run as described previously in Chapter 2.II.a using protein loads ranging from 2-10 µg protein per lane. Immunoblotting procedures were followed according to Towbin et al. (1979) with minor modifications as discussed previously (Chapter 2.II.a). The antibodies from each rabbit and their respective dilutions used (diluted in blocking buffer) were:

- Cashew 2S antibody: diluted 1:10000
- Cashew 11S R/A antibody: diluted 1:10000

b. Antibody Preparation

Two sets of three rabbits each were used to generate polyclonal IgG antiserum against cashew 11S R/A and 2S proteins. The sera from the three rabbits (NE 378, 379, 380) immunized against cashew 11S R/A proteins were pooled. Independently, the sera from the three rabbits (NE 384, 385, 386) immunized against cashew 2S protein were pooled. Polyclonal IgG antiserum against roasted cashew was raised in sheep as discussed in previous work by Gaskin and Taylor (2011). The antibody purification procedure of Ivens (2018) was used to isolate and purify IgG antibodies from the rabbit antisera. Antibodies raised against roasted cashew from sheep were not purified. The Melon Gel IgG Spin Purification Kit (Thermo Scientific, Waltham, MA) was used to purify IgG present in the sera which contains the cashew 11S R/A and 2S IgG, using the instructions provided by the manufacturer. The purified IgG antibodies were subjected to buffer exchange into a carbonate-bicarbonate buffer at pH 9.4 using Zeba Desalt Spin Columns (Thermo Scientific, Waltham, MA) following the instructions provided by the manufacturer. This buffer exchange was performed to ensure buffer additives from the initial purification were removed from the purified sera. The concentration of the purified IgG antibodies was determined following buffer exchange by measuring the absorbance at 280 nm (NanoDrop 2000 Microvolume Spectrophotometer, Thermo Scientific, Waltham, MA). Following buffer exchange, the cashew 11S R/A and 2S IgG was conjugated to horseradish peroxidase (HRP) using EZ-Link Plus Activated Peroxidase Kit (Pierce, Rockford, IL) using the instructions provided by the manufacturer. HRP-conjugated IgG was purified further to remove unconjugated IgG using a Conjugate Purification Kit (Pierce, Rockford, IL) using the instructions provided by the

manufacturer. The purified IgG and purified HRP-conjugated IgG were pooled and stored as aliquots at -20°C until further analysis.

Tagging efficiency of the purified HRP-conjugated IgG was measured by using a direct ELISA format. Briefly, a 96-well polyvinyl microtiter plate (NUNC-Immuno™ MaxiSorp™ 96-MicroWell™ plates, Nagle Nunc Intl., Rockester, NY, USA) was coated with 100 µL/well of 1 µg/mL immunogen protein prepared in coating buffer (0.015 M Na_2CO_3, 0.035 M $NaHCO_3$, pH 9.6) and incubated overnight (O/N) at 4°C. Following incubation, the plate was washed with wash buffer (0.05% Tween 20 in 0.025 M PBS, pH 7.4) four times and blocked with blocking buffer (0.1% gelatin in 0.025 M PBS, pH 7.4) for 1 h at 37°C. The plate was washed four times with wash buffer followed by the addition of 10-fold dilutions of HRP-conjugated IgG from each IgG antibody pool (100 µL/well) in conjugate buffer (0.5% BSA in 0.025 M PBS containing 0.2% Tween 20, pH 7.4) and incubated for 2 h at 37°C. The plate was washed four times with wash buffer and developed using 3,3',5,5'-tetramethylbenzidine (TMB SigmaFast™ Tablets, Sigma Chemical Co., St. Louis, MO). The reaction was stopped by adding 1 M Hydrochloric acid (100 µL/well) and the absorbance was read at 450 nm using a plate reader (ELx808 Ultraplate, BioTek Instruments, Inc., Winooski, VT). GraphPad Prism® v8.0 software (GraphPad Prism® software, Inc., San Diego, CA) was used to generate the curves and analyze the data.

To visualize differences in protein profiles between crude and purified IgG, silver staining following SDS-PAGE was performed. SDS-PAGE was run as described previously in Chapter 2.II.a Samples of crude sera and purified IgG were prepared 1:1

(v/v) in 2X Laemmli sample buffer and loaded in 5 µL or 10 µL volumes into the wells. Silver staining of the SDS-PAGE gel was performed using the Silver Stain Plus kit (BIO RAD, Hercules, CA) according to instructions provided by the manufacturer.

c. Development of a Sandwich ELISA for Detection of Cashew Nut Residue

To optimize the ELISA for the desired sensitivity (at or below 0.2 ppm cashew protein based on current cashew ELISAs), different dilutions and combinations of capture and detector antibodies (checkerboard titration) were tested until the most sensitive antibody pairing and dilution was determined (Gaskin & Taylor, 2011; Zhao, et al., 2019). Table 4.1 lists the antibody pairings that were used for optimization.

Table 4.1. Capture and detector antibody pairs used for sandwich ELISA development.

Capture Antibody	**Detector Antibody**
11S R/A (Crude)	Whole Cashew (Crude)
2S (Crude)	Whole Cashew (Crude)
2S/11S R/A (Crude) 1:1	Whole Cashew (Crude)
11S R/A (Purified IgG)	2S (HRP-Purified IgG); 11S R/A (HRP-Purified IgG)
2S (Purified IgG)	2S (HRP-Purified IgG); 11S R/A (HRP-Purified IgG)
Whole Cashew (Crude)	11S R/A (Crude); 2S (Crude); 2S/11S R/A (Crude) 1:1

*1:1; indicates a pooled 1:1 ratio of both cashew 2S and 11S R/A crude sera

Capture and detector antibodies were tested at different dilutions where the purified rabbit antisera, crude rabbit antisera and crude sheep antisera were diluted 1:1000, 1:2500, 1:5000, 1:7500, 1:10000, 1:20000, and 1:30000 in both coating and conjugate buffer and vice versa. When used, a commercial conjugate antibody, alkaline phosphatase

(AP)-labeled rabbit anti-sheep IgG or goat anti-rabbit IgG, was diluted 1:5000 based on previous ELISA work (Gaskin & Taylor, 2011). All ELISA optimization procedures used the same ELISA steps and reagents as described below, except for varying the combination or dilution of the purified antibodies, crude antisera, or cashew protein. Due to the large number of assay optimizations performed, only select optimization data along with the final, optimized procedure are further discussed.

i. Standard Curve in Buffer

The initial standard curve was prepared using roasted defatted cashew flour extracted in buffer. Roasted defatted cashew flour was extracted 1:10 w/v in 0.01 M PBS with 0.1 M sodium sulfite and 1% SDS, pH 7.4 in a shaking water bath (200 rpm) at 60°C for 25 min. This buffer was chosen based on the information obtained from optimizing reducing buffers as discussed in Chapter 2.III.a. The protein concentration of the supernatant from centrifugation at 12500 xg for 5 min was determined using the 2-D Quant protein assay (GE Healthcare, Chicago, IL). The supernatant with a protein concentration of 27.3 mg/mL was used to prepare the standard curve by serially diluting the clarified supernatant in the optimized buffer, 0.01 M PBS, pH 7.4 (PBS). The standard curve was prepared by serially diluting 3-fold in PBS starting at a 200 ppm cashew protein concentration (200, 66.6, 22.2, 7.4, 2.5, 0.8, 0.3, 0.09, 0.03, 0.01, 0.003 ppm cashew protein) for the generation of a 12-point curve including 0 ppm cashew protein buffer blank.

ii. Standard Curve in Matrices

The second standard curve in matrix was generated due to the high background observed with the addition of SDS in preliminary tested sample extracts (discussed later

in Chapter 4.III.d). Two different processed matrices, HTST almond milk and baked cookie (prepared as described in Chapter 4.II.e and Chapter 5.II.a) were evaluated as a potential matrix in which to develop the standard curve. The cashew standard curve in matrix was prepared using a homogenized 200 ppm total cashew protein incurred matrix extracted 1:10 w/v in 0.01 M PBS with 0.1 M sodium sulfite in a shaking water bath (200 rpm) at 60°C for 25 min. The supernatant following centrifugation at 12500 xg for 5 min was serially diluted 3-fold starting with the direct extract (200, 66.6, 22.2, 7.4, 2.5, 0.8, 0.3, 0.09, 0.03, 0.01, 0.003 ppm cashew protein). A 0 ppm standard curve point (buffer blank) was also added, consisting only of the optimized ELISA sample buffer, PBS.

iii. Optimized Cashew ELISA Procedure

The optimized sandwich cashew ELISA was based on the procedure of the FARRP in-house assay as mentioned previously in Chapter 2.II.b with minor modifications. A 96-well polyvinyl microtiter plate (NUNC-Immuno™ MaxiSorp™ 96-MicroWell™ plates, Nagle Nunc Intl., Rockester, NY, USA) was coated with 100 µL/well of 1:10000 v/v crude rabbit anti-cashew 2S and 11S R/A antibody combined (1:1) prepared in coating buffer (0.015 M Na_2CO_3, 0.035 M $NaHCO_3$, pH 9.6) and incubated O/N at 4°C. Following incubation, the plate was washed with wash buffer (0.05% Tween 20 in 0.025 M PBS, pH 7.4) four times and blocked with blocking buffer (0.1% gelatin in 0.025 M PBS, pH 7.4) for 1.5 h at 37°C. The plate was washed four times with wash buffer and the protein standard (200 ppm cashew protein in baked cookie supernatant diluted 3-fold in PBS to 0.003 ppm cashew protein) and samples (requiring an initial 10-fold dilution in PBS of the clarified sample extract supernatant) were added (100 µL/well) and incubated for 1.5 h at 37°C. The plate was washed four

times with wash buffer, followed by the addition of 1:2500 v/v diluted crude sheep anti-roasted cashew antibody (100 µL/well) in conjugate buffer (0.025 M PBS containing 0.5% BSA and 0.2% Tween 20, pH 7.4) and incubated for 1.5 h at 37°C. The plate was washed four times with wash buffer and incubated with 1:5000 v/v diluted AP-labeled rabbit anti-sheep IgG in conjugate buffer (100 µL/well) for 1 h at 37°C. The plate was washed four times with wash buffer and developed using p-nitrophenyl phosphate substrate (p-NPP SigmaFast™ Tablets, Sigma Chemical Co., St. Louis, MO) dissolved in 0.2 M Trizma buffer (100 µL/well). The reaction was stopped by adding 1 M NaOH (100 µL/well) and the absorbance read at 405 nm using a plate reader (ELx808 Ultraplate, BioTek Instruments, Inc., Winooski, VT). The standard curve utilized a Sigmoidal Curve with quantitative results taken from the linear portion of the curve. The limit of detection (LOD) was determined from the blank absorbance mean (µ) plus three times the standard deviation (SD) (LOD= mean + (3*SD)) (Armbruster, et al., 2008). The limit of quantitation (LOQ) was determined from the blank absorbance mean (µ) plus ten times the standard deviation (SD) (LOD= mean + (10*SD)) (Armbruster, et al., 2008).

d. Cross-reactivity Studies on Commercial Plant Milks

The optimized cashew ELISA was targeted to detect cashew protein residue in a plant milk matrix. To verify that other plant milks did not show significant interference, cross-reactivity studies on plant milks were performed. Eight different commercial plant milks (pea milk, oat milk, coconut milk, soy milk, walnut milk, macadamia nut milk, almond milk, and hazelnut milk) commonly available in the market were purchased from local grocery stores in Lincoln, Nebraska. Each sample was extracted 1:10 w/v with 0.01 M PBS containing 0.1 M sodium sulfite in a shaking water bath (200 rpm) at 60°C for 25

min followed by centrifugation at 12500 xg for 5 min. The supernatants were stored at -20°C until further analysis. All plant milks were analyzed using the optimized cashew ELISA procedure and as (following an initial 10-fold dilution) direct, 10-fold, 100-fold, and 1000-fold diluted extracts in triplicate wells for each dilution. Results obtained from the cross-reactivity studies were expressed in ppm cashew protein.

e. Preparation of Incurred Almond Milk

All incurred model foods were made with washed, roasted cashew. Cashews were washed and roasted according to procedures outlined previously in Chapter 3.II.a. A 2000 ppm cashew protein spike was prepared in a granulated sugar base by grinding 1 g of washed, roasted cashew with 99 g of granulated sugar in a mini food processor (Cuisinart, Stamford, CT) for 15 min, mixing every 3 min to ensure thorough mixing. Homogeneity analysis was performed using the commercial cashew ELISA by 3M (Saint Paul, MN) on six varying quadrants of the 2000 ppm cashew protein sugar-spike to ensure a homogenous sample for consistent sampling.

i. Incurred Almond Milk Matrix Preparation

Almond milk was prepared using the formula listed in Table 4.2., with incurred levels of 0, 0.5, 2, 10, 20, 200 ppm cashew protein using the 2000 ppm cashew protein sugar-spike (Ferragut, et al., 2015).

Table 4.2. Incurred almond milk negative control (0 ppm cashew protein) formula based on commercial formula percentages (Ferragut, et al., 2015).

Ingredient	**Weight (g)**	**Percent (%)**
Sugar*	120	10
Almond Milk (1 part almond to 5 parts water)	1080	90
Total	**1200**	**100**

*Sugar containing a homogenous mixture of 2000 ppm cashew protein was incorporated into the final almond milk formulation to derived almond milk samples incurred with 0.5, 2, 10, 20, or 200 ppm cashew protein

Washed, hand-sorted, raw almonds were soaked in water for 24 h. Almonds were weighed and a 1:5 ratio of almonds to water was blended for 2 min in an Osterizer blender (Sunbeam Corporation, Delray Beach, FL). The almond meal/water mixture was filtered gravimetrically using a cheesecloth filter. The liquid collected (almond milk) was divided into six separate containers and spiked accordingly, adding 10% sugar (w/w) using the sugar and sugar-spike with the desired cashew protein level. The incurred almond milk samples were separated into two equal aliquots. One aliquot was stored at -20°C until further analysis and the remaining aliquot was subjected to high-temperature short-time (HTST) processing.

HTST processing of nut milks requires a time and temperature minimum of 90°C for 90 sec (Bogahawaththa, et al., 2018; Dhakal, et al., 2014). To mimic HTST processing, a heat-block was set to 90°C. Almond milk (1 mL/tube) was aliquoted into 1.5 mL test tubes and heated for 90 sec. Following HTST processing, almond milk was cooled rapidly in an ice bath. All incurred pre- and post-HTST processed samples were stored at -20°C until further analysis.

f. Extraction and Evaluation of Incurred Almond Milk

Incurred almond milk samples, both pre- and post-HTST were evaluated using the optimized cashew ELISA with the cashew standard curve prepared using a 200 ppm cashew protein cookie matrix (0.003 to 200 ppm cashew protein). The percentage recovery of cashew protein from the incurred almond milk samples was calculated as the recovered ppm concentration over the expected ppm concentration of cashew protein.

Following the required initial 10-fold dilution, additional dilutions of 10-fold, 25-fold, and 50-fold were evaluated in duplicate on two independent days to illustrate consistency and day-to-day variation. Each incurred cashew protein level (0, 0.5, 2, 10, 20, 20 ppm cashew protein) for both pre- and post-HTST processed almond milks, was extracted 1:10 w/v in 0.01 M PBS with 0.1 M sodium sulfite, pH 7.4 in a shaking water bath (200 rpm) at 60°C for 25 min. The supernatant following centrifugation at 12500 xg for 5 min was analyzed for recovery of cashew protein using the developed cashew ELISA.

For comparison, the commercial cashew ELISA from BioFront Technologies (Tallahassee, FL) was used to determine the recovery of cashew protein in the incurred almond milks both pre- and post-HTST. The BioFront ELISA was performed using the instructions provided by the kit manufacturer.

g. Extraction and Evaluation of Commercial Cashew Milks

The six commercial cashew milks (Forager Project, Silk, Pacific Foods, Dream, Elmhurst, SoDelicious) used in the initial phase of this project as described in Chapter 2.II.a were evaluated using the developed cashew ELISA. Each cashew milk was extracted 1:10 w/v with 0.01 M PBS containing 0.1 M sodium sulfite in a shaking water

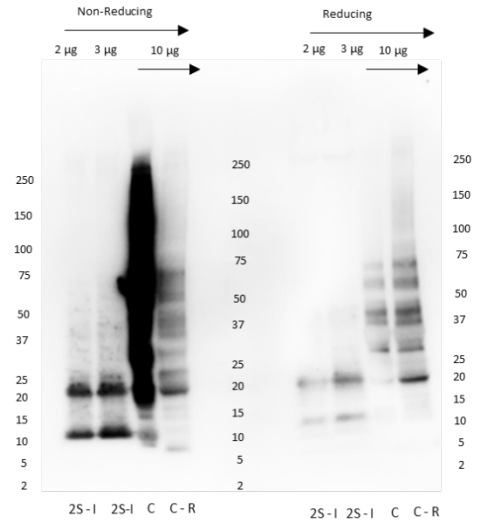
bath (200 rpm) at 60°C for 25 min. The supernatants following centrifugation at 12500 xg for 5 min were stored at -20°C until further analysis. All six commercial cashew milks were analyzed using the optimized cashew ELISA procedure and tested at dilutions of (following an initial 10-fold dilution) 5-fold, 10-fold, 50-fold, and 100-fold in PBS and tested in duplicate wells across two independent trials.

III. RESULTS AND DISCUSSION

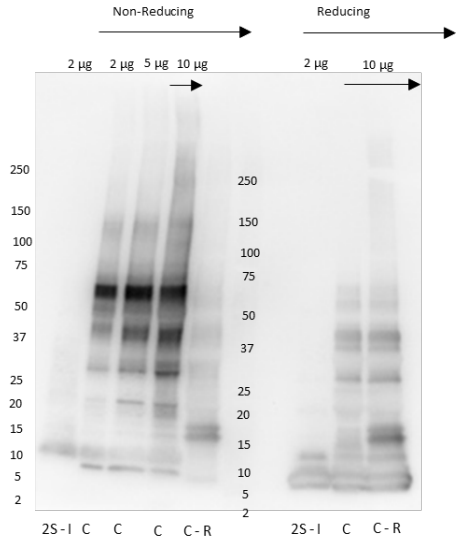
a. Protein Recognition by Immunoblotting

Western blotting was performed to determine binding affinity and specificity of the antibodies to proteins in varying cashew extracts. From 2-D Quant protein estimation, roasted ground cashew extracted in a non-reducing buffer had a protein concentration of 8.8 mg/mL while cashew extracted in a reducing buffer had a protein concentration of 9.6 mg/mL. The protein concentrations of the cashew 2S and cashew 11S R/A proteins were 0.5 mg/mL while that of the cashew 11S native was 30 mg/mL.

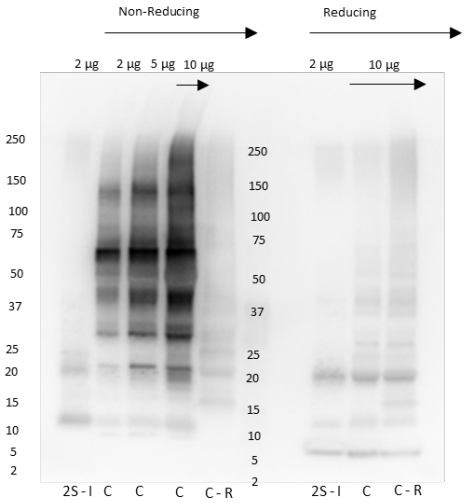
When the proteins were probed with the cashew 2S polyclonal sera (Figure 4.1), differences were observed in the antibody binding pattern to the different cashew samples tested.



[A] Rabbit NE 384



[B] Rabbit NE 385

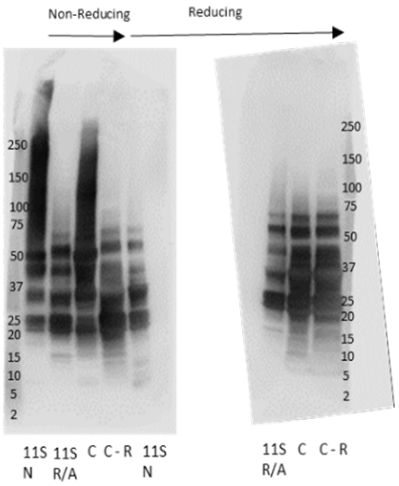


[C] Rabbit NE 386

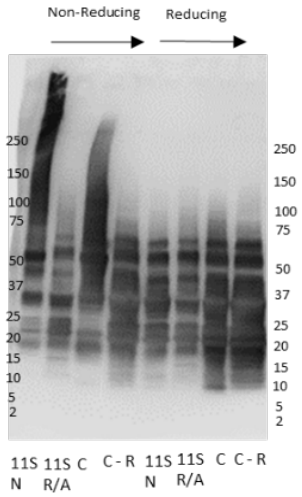
Figure 4.1. Immunoblot analysis of rabbit polyclonal sera raised against cashew 2S proteins of reduced and non-reduced cashew extracts and cashew 2S immunogen. [A] probed with Rabbit 384, [B] probed with Rabbit 385, and [C] probed with Rabbit 386 antisera. Each lane contains varying amounts of protein, ranging from 2-10 µg. The letters indicated in each lane correspond to the following samples from left to right: Lane M – Molecular Weight Marker (in kDa); Lane 2S – I – 2S immunogen; Lane C - Roasted, de-fatted cashew extracted in 0.01 M PBS at 60°C for 25 min; Lane C – R - Roasted, de-fatted Cashew extracted in 0.01 M PBS with 0.1 M sodium sulfite and 1% SDS at 60°C for 25 min.

Cashew proteins run under non-reducing conditions resulted in a stronger recognition of protein band(s) around 50 kDa, 33 kDa, and 12 kDa by each of the rabbits' antibodies (Figure 4.1). Since the cashew 2S sera was raised against a non-reduced form of the cashew 2S, this may have resulted in stronger recognition of proteins under non-reducing conditions (Figure 4.1). Sera from rabbit 384 showed a strong recognition of protein band(s) at 10 kDa and 20 kDa with the 2S immunogen. However, binding was weaker at 10 kDa and 20 kDa in both the non-reduced and reduced cashew extracts (Figure 4.1). Variation in the proteins that each rabbit recognizes as well as the differences in the binding intensity in a sample is expected with polyclonal antibodies (Harlow & Lane, 1988). With all the samples, multiple protein band(s) were recognized. This is expected since the cashew 2S immunogen was highly enriched with cashew 2S, allowing the polyclonal antibodies to recognize different protein band(s). Rabbit 385 had the strongest recognition of the lower molecular weight cashew 2S protein bands between ~5-12 kDa under both non-reducing and reducing conditions in comparison to rabbits 384 and 386 (Figure 4.1).

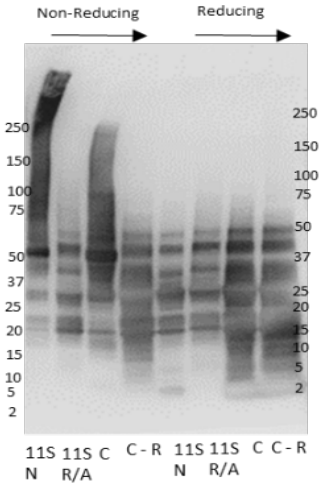
The polyclonal sera raised against cashew 11S R/A proteins in three rabbits resulted in very similar protein band(s) recognition across the different cashew samples (Figure 4.2).



[A] Rabbit NE 378



[B] Rabbit NE 379



[C] Rabbit NE 380

Figure 4.2. Immunoblot analysis of rabbit polyclonal sera raised against cashew 11S R/A proteins of reduced and non-reduced cashew extracts. [A] probed with Rabbit 378, [B] probed with Rabbit 379, and [C] probed with Rabbit 380 antisera. The protein load in each lane was 10 µg/lane except for 11S N and 11S R/A which had protein loads of 2 µg/lane. The letters indicated in each lane correspond to the following samples from left to right: Lane M – Molecular Weight Marker (in kDa); Lane 11S N - 11S Native; Lane 11S R/A -11S R/A used for immunization; Lane C - Roasted, de-fatted cashew extracted in 0.01 M PBS at 60°C for 25 min; Lane C – R - Roasted, de-fatted cashew extracted in 0.01 M PBS with 0.1 M sodium sulfite and 1% SDS at 60°C for 25 min.

The likely cashew 11S R/A protein band(s) around 20 and 30 kDa under reducing conditions were recognized strongly by the sera from all three rabbits and across all cashew samples (Figure 4.2). While there are multiple other protein bands being recognized as well, this only proves to be more beneficial in future ELISA development. There is less variation in the recognition of protein band(s) between the cashew 11S R/A sera from the three rabbits than in the cashew 2S sera (Figure 4.1 vs Figure 4.2). The strong recognition of protein bands(s) across cashew samples rather than stronger recognition towards the cashew 11S R/A protein band(s) near 20 kDa and 30 kDa may prove to be an advantage in future ELISA development (Figure 4.2).

For both the cashew 2S and 11S R/A rabbit sera, under reducing conditions, protein band(s) for the 2S (~8-15 kDa) and 11S R/A (20 & 37 kDa) were strongly recognized throughout all cashew samples and immunogens (Figures 4.1 and 4.2). This further ensures that the cashew 2S and 11S R/A proteins collectively are targets for the developed antibodies that will be recognized when using the more rigorous reducing extraction outlined in Chapter 2.III.a.

b. Antibody Purification

The tagging efficiency of the pooled and purified IgG-HRP conjugated cashew 2S and 11S R/A was evaluated by using a direct ELISA. The two antibody sets showed that there was a greater dynamic range for the cashew 11S R/A IgG than for the cashew 2S IgG (Figure 4.3).

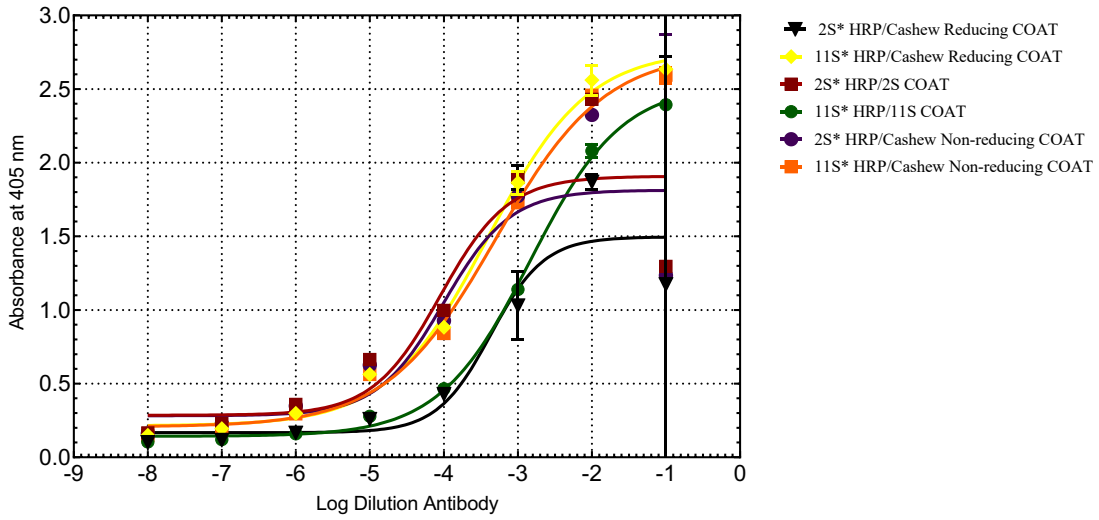


Figure 4.3. HRP tagging efficiency of purified antibodies (cashew 2S and cashew 11S R/A) using 2S immunogen, 11S R/A immunogen, cashew (extracted in 0.01 M PBS), and cashew reducing (extracted in 0.01 M PBS with 0.1 M sodium sulfite & 1% SDS) as the coating protein at 1µg/mL. * Indicates HRP tagged.

This observation was consistent and independent from the coating protein (cashew 11S R/A protein, non-reduced cashew protein, or reduced cashew protein) (Figure 4.3). Based on this data, the optimum dilution of the HRP-conjugated 11S R/A and 2S IgG was 10^4.

The purified IgG antibodies were first evaluated to determine the optimum dilution for the capture and detector antibody. Table 4.3 gives the protein content of the purified sera based on the measurement of the absorbance at 280 nm. Based on these protein concentrations, a silver stain of a reducing SDS-PAGE gel was performed to further ensure IgG purification efficiency (Figure 4.4).

Table 4.3. IgG concentrations (mg/mL) and volumes (µL) at each purification step for the cashew 2S (2S) and cashew 11S R/A (11S R/A) sera by measuring the absorbance at 280 nm.

Sera against Cashew Proteins	Sample	Concentration (mg/mL)	Volume (µL)
2S	Before Purification	52.0	3000
2S	After Purification and Buffer Exchange	1.0	885
2S HRP-Labeled	After HRP-Labeling and Buffer Exchange	0.32	1500
11S R/A	Before Purification	54.0	3000
11S R/A	After Purification and Buffer Exchange	0.7	1000
11S R/A HRP-Labeled	After HRP-Labeling and Buffer Exchange	0.15	1500

* HRP-Labeled indicates after HRP-labeling of the purified IgG.

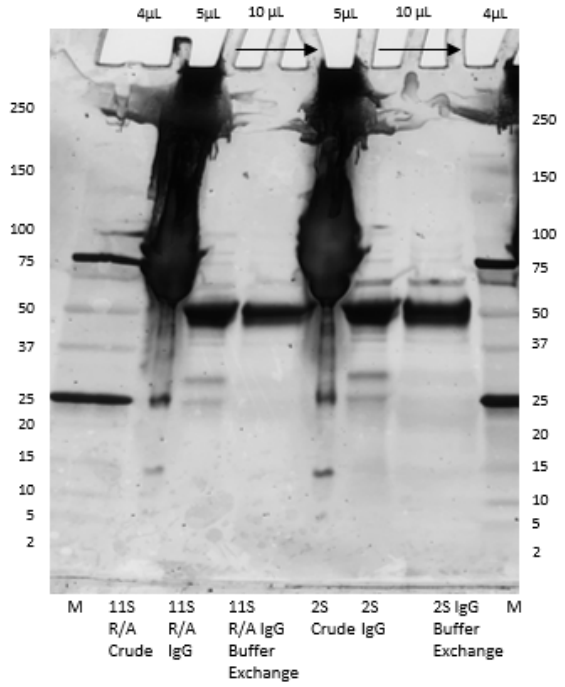


Figure 4.4. Silver Stain of cashew 11S R/A and 2S rabbit antisera at different purification stages following SDS-PAGE under reducing conditions. The letters indicated below each lane correspond to the following samples from left to right: M – Molecular Weight Marker (in kDa); 11S R/A Crude – 11S R/A pooled (NE 378, NE 379, NE 380) sera; 11S R/A IgG – 11S R/A pooled sera purified IgG; 11S R/A IgG Buffer Exchange – 11S R/A purified IgG in carbonate-bicarbonate buffer, pH 9.6; 2S Crude – 2S pooled (NE 384, NE 385, NE 386) sera; 2S IgG – 2S pooled sera purified IgG; 2S IgG Buffer Exchange – 2S purified IgG in carbonate-bicarbonate buffer, pH 9.6.

IgG has a molecular weight of 150 kDa under non-reducing conditions, splitting into two heavy chains at 50 kDa and one light chain near 25 kDa under reducing conditions (Janeway, et al., 2001). From left to right, for both the cashew 11S R/A and 2S sera, the crude sera shows the most smearing, indicating the presence of multiple proteins other than IgG (Figure 4.4). The purified IgG before buffer exchange showed some bands at other locations other than where IgG is expected at 50 kDa and 25 kDa (Figure 4.4). Following buffer exchange, the purified IgG showed the heavy chain of IgG (50 kDa) as

the most prominent band, and the light chain as a much fainter band at 25 kDa (Figure 4.4).

c. Cashew Sandwich ELISA Antibody Optimization

ELISA optimization began with different combinations of capture and detector antibody pairings. Initial checkerboard titration evaluating purified cashew 11S R/A and purified cashew 2S IgG paired with HRP-labeled 11S R/A and 2S IgG resulted in high absorbance values only for the most concentrated pairings, indicating that the presence of cashew proteins can be detected only with very low dilutions of the purified IgG (Tables 4.4 to 4.6). This is not optimal as a large quantity of sera would be needed for each ELISA. Additionally, the ELISA would not provide the desired sensitivity needed for a robust method to detect cashew nut protein residue.

Table 4.4. Purified cashew 11S R/A IgG coated antibody using 10 µg/mL cashew protein sandwiched with cashew 11S R/A HRP-labeled IgG as the detector antibody. Values shown are absorbance values measured at 405 nm.

		11S R/A HRP IgG											
		10	**30**	**90**	**270**	**810**	**2430**	**7290**	**21870**	**65610**	**196830**	**590490**	**1771470**
11S R/A IgG	**10**	0.933	0.402	0.178	0.089	0.061	0.05	0.049	0.05	0.048	0.048	0.048	0.049
	50	0.777	0.355	0.154	0.095	0.055	0.045	0.043	0.043	0.042	0.041	0.041	0.04
	250	0.576	0.285	0.131	0.075	0.053	0.046	0.044	0.045	0.045	0.046	0.045	0.045
	1250	0.201	0.127	0.077	0.054	0.045	0.044	0.041	0.04	0.041	0.041	0.041	0.04
	6250	0.098	0.069	0.052	0.044	0.041	0.041	0.041	0.039	0.041	0.04	0.04	0.039
	31250	0.084	0.062	0.05	0.045	0.042	0.042	0.051	0.046	0.041	0.041	0.041	0.044
	156250	0.075	0.055	0.046	0.041	0.04	0.039	0.039	0.039	0.041	0.04	0.042	0.042
	781250	0.073	0.054	0.047	0.041	0.042	0.044	0.04	0.04	0.039	0.038	0.041	0.042

***** Dilutions of the purified 11S IgG ranged from 10-781250 in coating buffer. Dilutions of the purified 2S HRP IgG ranges from 10-1771470 in conjugate buffer. The highlighted value indicates the highest observed absorbance reading.

Table 4.5. Purified cashew 2S IgG coated antibody using 10 µg/mL cashew protein sandwiched with purified cashew 2S HRP-labeled IgG used as the detector antibody. Values shown are absorbance values measured at 405 nm.

		2S HRP IgG											
		10	**30**	**90**	**270**	**810**	**2430**	**7290**	**21870**	**65610**	**196830**	**590490**	**1771470**
2S IgG	**10**	0.361	0.262	0.151	0.085	0.06	0.052	0.048	0.05	0.051	0.048	0.048	0.049
	50	0.28	0.201	0.124	0.075	0.053	0.044	0.044	0.041	0.044	0.042	0.042	0.041
	250	0.199	0.151	0.101	0.068	0.053	0.047	0.043	0.044	0.045	0.043	0.045	0.043
	1250	0.076	0.065	0.054	0.047	0.045	0.041	0.04	0.046	0.04	0.04	0.041	0.039
	6250	0.051	0.045	0.045	0.043	0.042	0.039	0.041	0.04	0.039	0.04	0.04	0.04
	31250	0.046	0.043	0.042	0.043	0.041	0.039	0.04	0.04	0.042	0.042	0.042	0.04
	156250	0.045	0.045	0.041	0.039	0.041	0.039	0.039	0.039	0.046	0.041	0.039	0.038
	781250	0.046	0.041	0.04	0.039	0.04	0.041	0.038	0.039	0.038	0.04	0.042	0.039

***** Dilutions of the 2S IgG ranges from 10-781250 in coating buffer. Dilutions of the purified 2S HRP tagged IgG ranges from 10-1771470 in conjugate buffer. The highlighted value indicates the highest observed absorbance reading.

Table 4.6. Purified cashew 2S and cashew 11S R/A IgG coated antibody using 1 µg/mL reduced cashew protein (extracted in 0.01 M PBS with 0.1 M sodium sulfite and 1% SDS) sandwiched with opposite cashew 2S and cashew 11S R/A HRP-labeled IgG as the detector antibody. Values shown are absorbance values measured at 405 nm.

11S R/A Purified IgG Coat				**2S Purified IgG Coat**			
		100	**500**			**100**	**500**
2S HRP	**10**	0.175	0.072	**11S R/A HRP**	**10**	0.266	0.182
	100	0.06	0.042		**100**	0.063	0.07

***** Dilutions of the purified 2S or 11S R/A IgG ranges from 100-500 in coating buffer. Dilutions of the purified 11S R/A or 2S HRP IgG ranges from 10-100 in conjugate buffer.

Table 4.4 provides the absorbance values of the purified cashew 11S R/A IgG coated plate using the HRP-labeled 11S R/A IgG as the detector antibody. Table 4.5 gives the absorbance values of the purified cashew 2S IgG coated plate using the HRP-labeled 2S IgG as the detector antibody. Table 4.6 lists the absorbance values of the purified 2S IgG or 11S R/A IgG coated plate using the opposite HRP-labeled 2S or 11S R/A IgG at select dilutions as the detector antibody. As Tables 4.4 and 4.5 indicate, an optimum 10-fold dilution of IgG for an optimally read plate with a high absorbance is problematic in the development of an ELISA. High dilutions of antibodies are necessary for efficient antibody application. Antibody dilutions ranging from 1000-fold to 10000-fold are common in an ELISA (Gaskin & Taylor, 2011). An optimum purified IgG dilution (10-fold) does not show commerciality or feasibility for an efficient ELISA. Switching the capture and detector antibody to an opposite pairing (i.e. 11S R/A with 2S HRP-labeled IgG) did not result in higher absorbance values or better detection of cashew proteins (Table 4.6).

Based on the above observations, it was determined that the concentration of the purified IgG was not sufficient for ELISA development. Consequently, pooled crude sera from the two sets of rabbits, against the cashew 11S R/A and the cashew 2S, was used for antibody pairing as the capture and detector antibody for all future ELISA optimization. Raising antisera against two different cashew proteins in rabbits was hypothesized to be a way in which the ELISA could use different antibody epitope binding sites for cashew protein detection. However, since both sets of antibodies were raised in rabbits, any AP or HRP-labeled anti-rabbit conjugate could potentially recognize both the capture and detector antibodies. The antibodies developed by Gaskin and Taylor (2011) were

evaluated as a way to use an alternate anti-cashew animal species as either the capture or detector antibody, in combination with either the developed cashew 11S R/A or 2S rabbit antisera. Any commercial anti-animal species could be used as the enzyme-labeled conjugate with no potential interference from both the capture and detector antibody raised in the same animal.

Optimization to determine the best capture and detector antibody resulted in the selection of crude rabbit anti-cashew 11S R/A or 2S sera as the capture antibody and crude sheep anti-roasted cashew sera as the detector antibody. Table 4.7 and Table 4.8 illustrate the optimization of the sheep anti-cashew detector antibody paired with the optimized rabbit cashew 2S and cashew 11S R/A sera as the capture antibody.

Table 4.7. Crude rabbit anti-cashew 2S sera as capture antibody using 10 µg/mL cashew protein sandwiched with sheep anti-cashew sera as detector antibody. Values shown are absorbance values measured at 405 nm.

Coat Crude 2S IgG Antisera													
Sheep anti-roasted cashew IgG antisera		100		1000		10000		15000		20000		30000	
	1000	1.859	2.179	2.744	2.883	3.187	3.266	3.325	3.328	3.356	3.293	3.303	2.958
	10000	1.736	1.779	2.204	2.229	2.336	2.386	2.486	2.55	2.595	2.564	2.357	2.381
	15000	1.621	1.821	2.065	2.061	2.175	2.285	2.222	2.3	2.248	2.283	2.342	2.378
	20000	1.469	1.656	1.816	1.844	1.951	2.039	2.09	1.872	2.048	2.153	2.164	2.163
	30000	1.336	1.422	1.635	1.582	1.736	1.75	1.811	1.825	1.805	1.893	1.858	1.928
	50000	1.349	1.414	1.596	1.635	1.75	1.71	1.767	1.764	1.794	1.831	1.83	1.838

* Dilutions of the crude 2S sera ranges from 100-30000 in coating buffer. Dilutions of the crude sheep anti-roasted cashew sera ranges from 1000-50000 in conjugate buffer. Highlighted values indicate the best detector antibody dilution range. Values in red indicate the best coating antibody dilution range.

Table 4.8. Crude rabbit anti-cashew 11S R/A sera as the coating antibody using 10 µg/mL cashew protein sandwiched with sheep anti-cashew sera as the detector antibody. Values shown are absorbance values measured at 405 nm.

Coat Crude 11S R/A IgG Antisera													
Sheep anti-roasted cashew IgG antisera		100		1000		10000		15000		20000		30000	
	1000	2.39	2.396	2.875	2.912	3.24	3.28	3.354	3.203	3.308	3.253	3.346	3.143
	10000	2.15	2.163	2.427	2.386	2.383	2.395	2.451	2.395	2.459	2.544	2.516	2.581
	15000	2.102	1.86	2.282	2.229	2.242	2.279	2.283	2.237	2.3	2.317	2.321	2.423
	20000	1.865	1.769	2.086	2.042	2.097	2.149	2.085	2.148	2.147	2.177	2.176	2.305
	30000	1.564	1.457	1.667	1.706	1.669	1.691	1.69	1.684	1.713	1.76	1.754	1.813
	50000	1.515	1.488	1.61	1.582	1.623	1.7	1.656	1.678	1.684	1.686	1.69	1.77

* Dilutions of the crude sera 11S R/A ranges from 100-30000 in coating buffer. Dilutions of the crude sheep anti-roasted cashew sera ranges from 1000-50000 in conjugate buffer. Highlighted values indicate the best detector antibody dilution range. Values in red indicate the best coating antibody dilution range.

From evaluating the absorbance values in Table 4.7 and Table 4.8, the optimum dilution for the capture antibody (cashew 11S R/A and cashew 2S) was a 10000-fold dilution in combination with the detector antibody (sheep anti-roasted cashew) diluted 1000-10000 in buffer.

In conclusion, the optimized cashew ELISA used the rabbit anti-cashew 2S and rabbit anti-cashew 11S R/A sera, separately and eventually pooled, as the capture antibody at a 1:10000 v/v dilution in coating buffer paired with the sheep anti-roasted cashew sera as the detector antibody at a 1:2500 v/v dilution in conjugate buffer. The use of cashew 2S and 11S R/A sera showed similar antibody affinity, indicating that both sets of antibodies are good targets for ELISA development.

d. Cashew Sandwich ELISA Standard Curves

The optimized ELISA antibody pair included the crude rabbit anti-cashew 11S R/A and/or 2S sera as the capture antibody and the crude sheep anti-roasted cashew sera as the detector antibody. Standard curves were evaluated for the two ELISAs until an optimized standard curve ranging from 0.003 to 200 ppm – cashew protein was developed.

i. Cashew Standard Curve Development in Buffer

The cashew standard curve in buffer was developed by extracting roasted defatted cashew 1:10 w/v in a reducing PBS buffer as previously described (Chapter 4.II.c). To have the benefit of an ELISA which targets different cashew proteins, both the cashew 11S R/A and 2S antibodies were combined 1:1 (v/v) in equal volume (2S/11S R/A) and tested against the two separate 11S R/A and 2S standard curves. Figure 4.5 illustrates an optimized standard curve with varying capture antibodies (2S, 11S R/A, 2S/11S R/A).

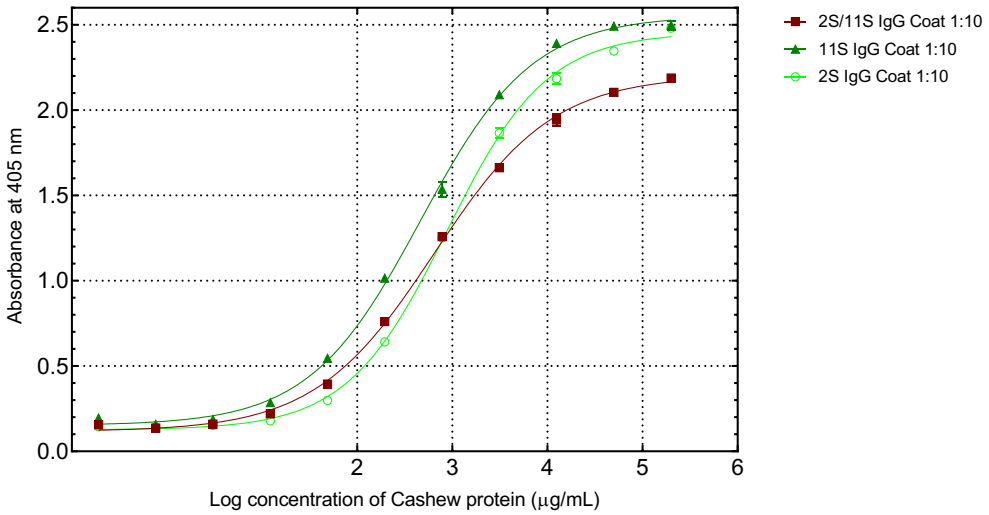


Figure 4.5. Optimized cashew standard curve in buffer ranging from 0.003 ppm cashew protein to 200 ppm cashew protein comparing the crude rabbit 2S and 11S R/A antisera combined curve to each separately. Each point represents the average of 2 wells.

No major differences could be found between the curves, with the pooled sera (2S/11S R/A) having a dynamic range between that of the cashew 11S R/A and 2S sera (Figure 4.5). Due to the potential for more antibody-protein targets with both sets of antibodies (11S R/A and 2S), the pooled sera from both the cashew 11S R/A and 2S were used as the capture antibody.

An example of standard curve optimization is shown in Figure 4.6.

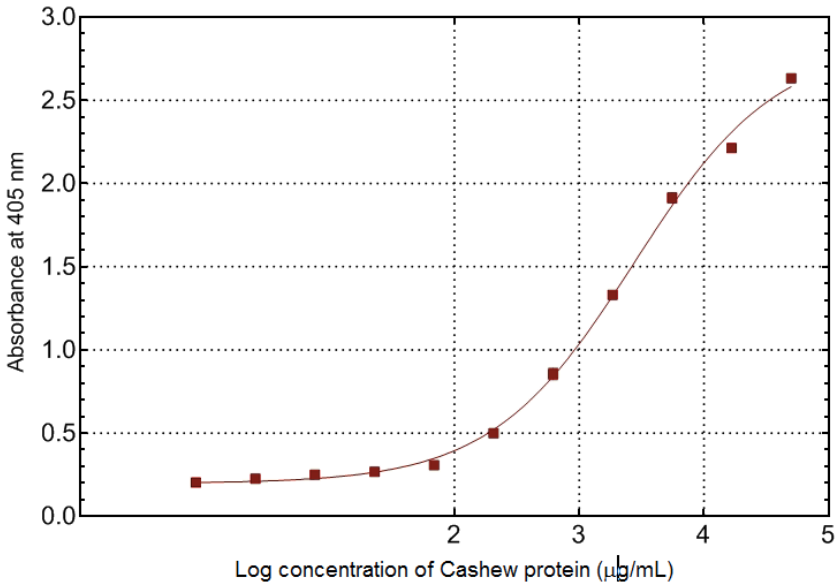


Figure 4.6. Cashew standard curve in buffer from 0.0008 to 50 ppm cashew protein of the rabbit 2S antisera. Each point represents the average of 2 wells.

Figure 4.6 illustrates the cashew 2S as the capture antibody at a 1:10000 v/v dilution in coating buffer paired with the sheep anti-roasted cashew antibody at a 1:2500 v/v dilution in conjugate buffer. The standard curve ranges from 0.0008 to 50 ppm cashew protein; however, the bottom plateau was further optimized. The curve was optimized by increasing the linear portion of the sigmoidal curve by varying the concentrations of the cashew standard curve until the curve had an optimized linear portion. Different 3-fold and 4-fold dilutions at different cashew protein concentrations were evaluated. The optimized standard curve had had a final range of 0.003 to 200 ppm cashew protein at 3-fold dilutions in PBS. The range of quantification of the standard curve was approximately 0.3 to 7.4 ppm cashew protein (Figure 4.7).

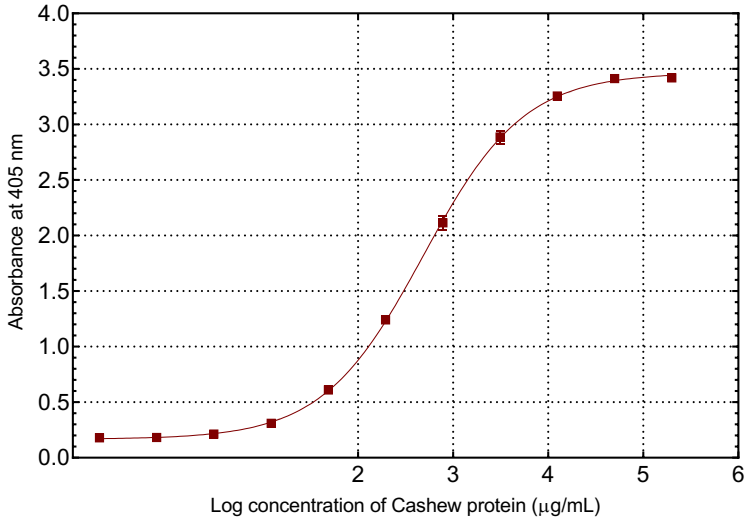


Figure 4.7. Optimized cashew standard curve in buffer from 0.003 to 200 ppm cashew protein using the combined 11S R/A/2S antisera as the coating antibody. Each point represents the average of 2 wells.

The optimized cashew standard curve in buffer was finalized using the pooled cashew 2S/11S R/A sera (Figure 4.7). However, preliminary evaluation of the incurred almond milk matrices and commercial cashew milks resulted in little to no recovery of cashew protein. Table 4.9 gives the cashew protein recovery from pre- and post-HTST processed almond milk using the standard curve in buffer.

Table 4.9. Average percentage recovery (% Recovery) from almond milks incurred with known amounts of cashew protein pre-HTST and post-HTST evaluated using a cashew standard curve in buffer.

Sample	Average (% Recovery)
Pre-HTST (0 ppm Cashew Protein) Almond Milk	BLQ
Pre-HTST (0.5 ppm Cashew Protein) Almond Milk	BLQ
Pre-HTST (2 ppm Cashew Protein) Almond Milk	BLQ
Pre-HTST (10 ppm Cashew Protein) Almond Milk	BLQ
Pre-HTST (20 ppm Cashew Protein) Almond Milk	BLQ
Pre-HTST (200 ppm Cashew Protein) Almond Milk	BLQ
HTST (0 ppm Cashew Protein) Almond Milk	BLQ
HTST (0.5 ppm Cashew Protein) Almond Milk	BLQ
HTST (2 ppm Cashew Protein) Almond Milk	BLQ
HTST 10 ppm (Cashew Protein) Almond Milk	BLQ
HTST 20 ppm (Cashew Protein) Almond Milk	BLQ
HTST 200 ppm (Cashew Protein) Almond Milk	BLQ

BLQ; Below the Limit of Quantification (1 ppm cashew protein)

The recovery of cashew protein was below the limit of quantification for all the incurred levels. Table 4.10 gives an example of the 200 ppm cashew protein HTST almond milk absorbance values across dilutions, all below the linear portion (1 to 2.6 AU at 405 nm) of the optimized standard curve in buffer.

Table 4.10. 200 ppm cashew protein incurred pre-HTST almond milk with recovery values determined using the standard curve in buffer. All absorbance values measured at 405 nm.

Sample	Absorbance at 405 nm (AU)	Cashew Protein (ppm)	% Recovery	Dilution
200 ppm Cashew Protein Pre-HTST Almond Milk	0.3905	BLQ	BLQ	1
	0.3175	BLQ	BLQ	10
	0.4165	BLQ	BLQ	100
	0.517	BLQ	BLQ	1000
	0.2165	BLQ	BLQ	10000

*BLQ; Below the limit of Quantification (1 ppm cashew protein). All values were outside the linear portion of the curve (1-2.6 AU) across dilutions.

Preliminary evaluation of a commercial cashew milk sample (Elmhurst) was also problematic with variable recovery and detection using a standard curve in buffer. With increasing dilutions of the extract, the absorbance value did not decrease as expected but instead increased until a 600-fold dilution was performed (Table 4.11).

Table 4.11. Absorbance values and protein values determined by a standard curve in buffer for Elmhurst cashew milk. All absorbance values measured at 405 nm.

Sample	Absorbance at 405 nm (AU)	Cashew Protein (ppm)	Dilution Factor
Elmhurst Milked	1.264	4.418	10
	2.1155	174.188	100
	2.126	**1062.758**	**600**
	1.7725	**1018.024**	**1000**
	1.0815	**752.789**	**2400**

* Values in bold are cashew protein concentrations calculated following the minimum 600-fold dilution.

With dilutions above a 600-fold dilution, the absorbance began to decrease and the protein concentration values appeared more reliable upon further dilution. The

hypothesized problem with the standard curve in buffer was that the SDS in the extraction buffer (0.01 M PBS with 0.1 M sodium sulfite and 1% SDS) affected detection and recovery in the ELISA format. SDS is known to interfere in ELISAs; however, no interference was shown in any previous standard curve optimization. The lack of interference in the standard curve was most likely due to the serial dilutions of the cashew extract, diluting the SDS to a concentration which wouldn't interfere with the ELISA. Diluting the SDS to a low concentration has been found to have negligible effects in an ELISA (McCabe, et al., 1988). However, in the sample extracts, the SDS concentration was not diluted to a sufficiently low concentration and thus, contributed to the interference observed with these samples (Tables 4.9-4.11).

Based on these observations, if SDS were to be included as an additive in the extraction buffer, a minimum 600-fold dilution would be required. A 600-fold dilution would dilute out the SDS and have an absorbance value within the linear portion of the curve. However, none of the incurred matrices contain cashew protein concentrations that are high enough to include a 600-fold dilution and still be within the linear portion of the curve, thus, a different extraction buffer without the addition of SDS was evaluated to reliably detect cashew protein.

To address this issue, lower SDS concentrations and another detergent, Tween 20, was tested at different concentrations as additives to the extraction buffer of PBS for the development of the standard curve. Sodium sulfite was not removed from PBS as an additive in the extraction buffer as preliminary testing of extraction buffers showed an increase in cashew protein extraction (in no matrix) with the use of sodium sulfite in comparison to no reducing agent (Chapter 2.III.a). In addition, the rabbit antisera were

raised against the reduced form of the cashew 11S, making the use of a reducing agent, 0.1 M sodium sulfite necessary for extraction. Extracting samples in PBS without the addition of sodium sulfite was not evaluated as the reducing agent was thought to further increase antigen-antibody binding and was previously shown to increase cashew protein extraction (Chapter 2.III.a).

No major differences in protein recovery from two different cashew matrices (Elmhurst Milked and a 200 ppm baked cookie) were observed utilizing the modified extraction buffers (Table 4.12).

Table 4.12. Recovery of cashew protein (ppm) from Elmhurst cashew milk and an incurred baked cookie (200 ppm cashew protein) following the addition of different extraction additives to 0.01 M PBS with 0.1 M sodium sulfite buffer.

Extraction Additive	Elmhurst Cashew Protein Recovery (ppm)	Cashew Protein Recovery from Baked Cookie incurred with 200 ppm cashew protein
0% SDS	29.2	2.7
0.002% SDS	19.6	1.6
0.0073% SDS	41.8	1.9
0.01% SDS	27.0	1.5
1% SDS	1649.5	BLQ
0.1% Tween-20	BLQ	BLQ
1% Tween-20	BLQ	BLQ

*BLQ; Below the Limit of Quantification (1 ppm cashew protein)

The low recovery from the incurred matrices was potentially due to the inefficient extraction of cashew proteins from a matrix (also described in Chapter 2.III.a). In Chapter 2.III.a, the extracted and evaluated cashew milk matrices showed no large increases in cashew protein extraction until SDS was incorporated to the extraction buffer. However, recovery of proteins from cashew nuts showed a significant increase following the addition of sodium sulfite (Chapter II.III.a). It is hypothesized that the optimized

reducing buffer (0.01 M PBS with 0.1 M sodium sulfite) efficiently extracts cashew protein from a ground cashew sample in the absence of a complex matrix (i.e. cashew milk). However, in processed foods, cashew protein extraction is not as efficient with the addition of sodium sulfite and may account for the low recoveries of cashew protein observed in a matrix.

ii. Cashew Standard Curve in Matrices

To address the low recovery of cashew protein from a matrix, a cashew standard curve in two different processed matrices was evaluated. The two matrices included a 200 ppm cashew protein in baked cookie (made as described in Chapter 5.II.a) and a 200 ppm cashew protein in HTST almond milk (made as described in Chapter 4.II.e). The standard curve in matrix was hypothesized to show a better recovery of cashew protein from plant milks and processed matrices than the standard curve in buffer. Both the standard curve in matrix and the incurred samples have undergone some type of processing, making the standard curve and samples more comparable during extraction in comparison to using a standard curve in buffer.

The baked cookie standard curve had a more significant mid-linear portion (from 0.3-1.6 AU in baked cookie compared to 0.5-1.3 AU in almond milk) than the almond milk standard curve, as seen in Figure 4.8.

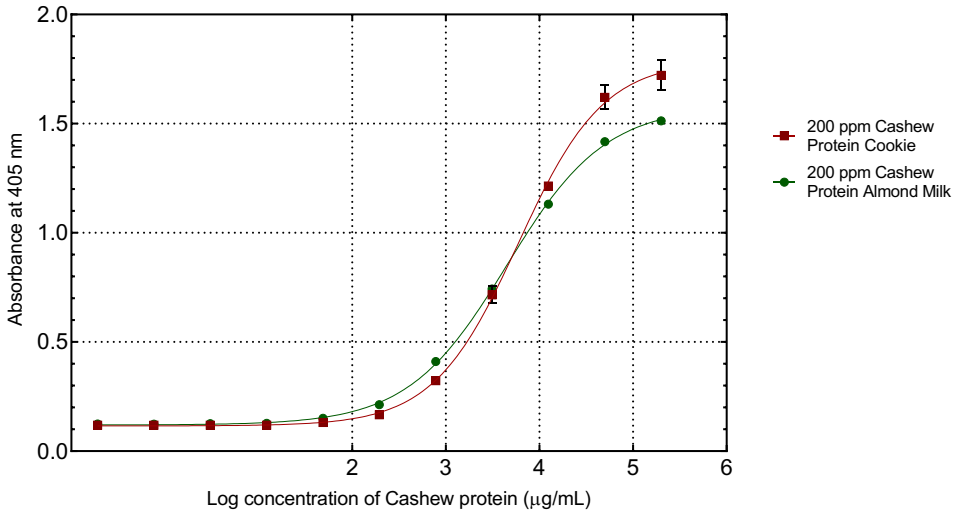


Figure 4.8. Standard curve in matrix using 200 ppm cashew protein incurred in baked cookie matrix or HTST almond milk in 0.01 M PBS with 0.1 M sodium sulfite. Each point represents the average of 2 wells.

The standard curve in baked cookie was also slightly able to better detect cashew protein from both raw and baked cookies (discussed in Chapter 5.III.a) and pre- and post-HTST almond milks (Figure 4.8). For this reason, the standard curve in baked cookie was used to determine percent recovery of cashew protein in pre-HTST and post-HTST almond milks.

The use of 0.1 M sodium sulfite in the extraction buffer used for both samples and standard curve development did give minor interfering background absorbance in the ELISA, similar to the addition of SDS (data not shown). Therefore, to minimize the background absorbance, an initial 10-fold dilution of all sample extracts was necessary. This initial 10-fold dilution is significantly less than the 500-fold dilution necessary for the standard curve in reducing buffer with SDS and was reasonable enough to compensate for the need to effectively reduce extracted cashew proteins (since rabbits

were raised against a reduced form of the 11S) while also increasing protein solubility during extraction (Abtahi, et al., 1997).

The final cashew standard curve in baked cookie matrix was optimized slightly to increase linearity (data not shown) to remove some of the points from the plateau towards the lower limit of detection. The final standard curve was serially diluted 3-fold from 200 ppm cashew protein to 0.003 ppm cashew protein (200, 66.6, 22.2, 7.4, 2.5, 0.8, 0.3, 0.09, 0.03, 0.01, 0.003) (Figure 4.9).

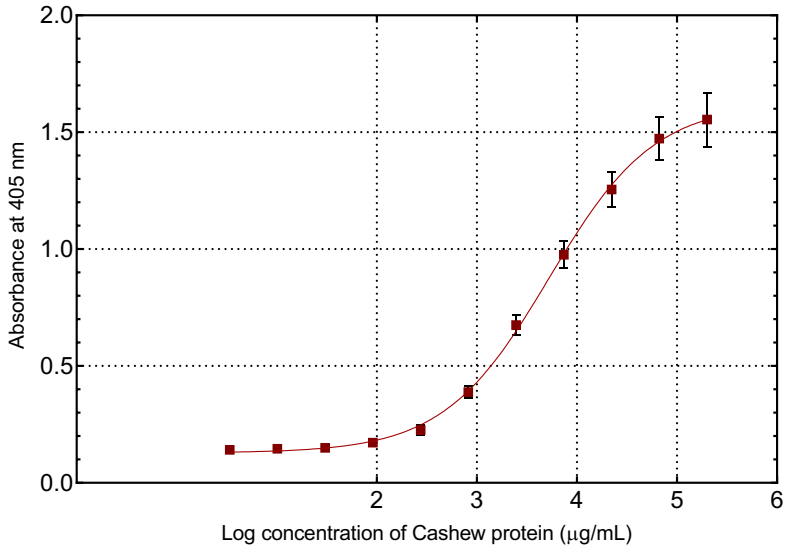


Figure 4.9. Standard Curve in matrix using 200 ppm cashew protein incurred baked cookie matrix extracted in 0.01 M PBS with 0.1 M sodium sulfite. The representative standard curve is of a combined 20 runs over 5 independent days. Each point represents the average of 40 wells.

A 0 ppm cashew protein standard was also included comprising of only PBS. The final cashew standard curve in a baked cookie matrix, of a combined 20 independent runs over 5 days, is illustrated in Figure 4.9. Data from all 20 curves were combined so as to

determine the LOD and the LOQ of the assay (Figure 4.9). The LOD for the cashew ELISA was calculated to be 0.09 ppm cashew protein while the LOQ was 0.3 ppm cashew protein. The LOD (0.04-0.06 ppm cashew protein) and LOQ (0.2 ppm cashew protein) of the most recently developed cashew ELISA was slightly more sensitive however, still fairly comparable (Zhao, et al., 2019).

e. Cross-Reactivity Studies of Plant Milks

To assess the potential cross-reactivity of different non-cashew plant milks, 8 different commercial plant milks (pea milk, oat milk, coconut milk, soy milk, walnut milk, macadamia nut milk, almond milk, and hazelnut milk) were evaluated (Table 4.13).

Table 4.13. Cross-reactivity analysis of different plant milks in the developed cashew ELISA.

Plant Milk	Cashew Protein Detected (ppm)
Pea	BLQ
Oat	BLQ
Coconut	BLQ
Soy	BLQ
Walnut	BLQ
Macadamia Nut	BLQ
Almond	BLQ
Hazelnut	BLQ

BLQ; Below the limit of quantification (0.3 ppm cashew protein)

Each plant milk type was evaluated in the developed cashew ELISA in triplicate (Table 4.13). Upon evaluation, no plant milk resulted in a cashew protein concentration higher than the LOQ (0.3 ppm cashew protein). This indicated that the plant milk matrix or processing condition does not have a matrix effect on the developed cashew ELISA. While little research has been carried out on plant milk matrices and cross-reactivity in an ELISA, lateral flow devices have shown little to no cross-reactivity with other plant milk matrices and is in agreement with our observations (Masiri, et al., 2016).

f. Recovery of Incurred Almond Milk

Homogeneity analysis of the cashew spike in sugar using the 3M cashew ELISA as well as homogeneity analysis of the 200 ppm incurred matrices using the BioFront cashew ELISA confirmed that the cashew spike was thoroughly homogenized following blending (data not shown).

High recovery of cashew protein was observed for both the pre- and post-HTST incurred almond milk matrices using the optimized cashew ELISA (Table 4.14).

Table 4.14. Average percentage recovery of incurred cashew almond milks pre-HTST and post-HTST determined using the developed cashew ELISA and the commercial BioFront cashew ELISA kit.

Sample	Developed ELISA Average % Recovery	BioFront ELISA Average % Recovery
Pre-HTST (0 ppm Cashew Protein) Almond Milk	BLQ	BLQ
Pre-HTST (0.5 ppm Cashew Protein) Almond Milk	BLQ	85 ± 6
Pre-HTST (2 ppm Cashew Protein) Almond Milk	BLQ	78 ± 2
Pre-HTST (10 ppm Cashew Protein) Almond Milk	129 ± 16	77 ± 7
Pre-HTST (20 ppm Cashew Protein) Almond Milk	121 ± 3	82 ± 5
Pre-HTST (200 ppm Cashew Protein) Almond Milk	79 ± 14	84 ± 4
HTST (0 ppm Cashew Protein) Almond Milk	BLQ	BLQ
HTST (0.5 ppm Cashew Protein) Almond Milk	BLQ	48 ± 5
HTST (2 ppm Cashew Protein) Almond Milk	BLQ	46 ± 1
HTST 10 ppm (Cashew Protein) Almond Milk	117 ± 6	46 ± 10
HTST 20 ppm (Cashew Protein) Almond Milk	114.8 ± 0.4	59 ± 2
HTST 200 ppm (Cashew Protein) Almond Milk	72 ± 7	67 ± 2

BLQ*; Below the Limit of Quantification. Developed ELISA LOQ (0.3 ppm cashew protein). BioFront ELISA LOQ (0.2 ppm cashew protein). Values expressed as average ± standard deviation (n=2).

Pre-HTST almond milk samples containing 10, 20, and 200 ppm cashew protein showed recoveries between 80-130% of the expected recovery (Table 4.14). Pre-HTST almond milk containing 2 ppm cashew protein was below the limit of quantification (LOQ; 0.3 ppm) however, it did fall above the limit of detection (LOD; 0.09 ppm) (Table 4.14). Therefore, the 2 ppm cashew protein pre-HTST almond milk can be qualitatively determined to contain cashew protein by the developed ELISA, although a quantitative result cannot be determined. Post-HTST almond milk samples containing 10, 20, and 200 ppm cashew protein showed similar results, with recoveries ranging between 70-120% of

the expected recovery (Table 4.14). Recovery values within 80-120% of the expected recoveries are considered within the acceptable range for ELISA recovery from incurred matrices (Andreasson, et al., 2015).

HTST processing did not have a significant effect on cashew protein detection, with little variation observed between pre- and post-processed samples other than a slight decrease (7% decrease) in detection of the 200 ppm cashew protein almond milk following HTST processing (Table 4.14). While low recoveries were observed at the 0.5 and 2 ppm cashew protein incurred levels in both the pre- and post-HTST processing, this could be due to a multitude of factors such as processing temperature protein degradation or low protein extraction of the sample (Abtahi, et al., 1997; Mattison, et al., 2014). However, the high recovery observed with both pre- and post- HTST treated samples suggest that this ELISA is robust in detecting HTST processed cashew protein and would be a reliable ELISA when validating plant milk matrices down to the lowest tested incurred almond milk, 10 ppm cashew protein.

To compare the performance of the developed ELISAs sensitivity, the commercial cashew ELISA kit from BioFront Technologies was also evaluated on its recovery of pre- and post-HTST almond milks incurred with cashew for comparison (Table 4.14). The BioFront kit was able to reliably detect cashew protein present in both pre- and post-HTST almond milk at the lowest incurred cashew level, 0.5 ppm cashew protein. This is more sensitive than what the developed ELISA was able to detect. In addition, the BioFront ELISA was very consistent in its recovery percentages, as the pre- (~81%) and post-HTST (~53%) incurred almond milks had similar percentage recoveries across all incurred levels.

The biggest difference between the developed ELISA and the BioFront ELISA was the change in percentage recoveries between pre- and post-HTST. BioFront had an average 35% loss in recovery post-HTST processing while the developed ELISA had an average 8% loss in recovery post-HTST processing. The BioFront ELISA did not detect cashew protein which had undergone high heat processing (HTST) as reliably as the developed ELISA, indicating that the developed ELISA has an increased robustness against HTST processing conditions. The developed ELISA is robust against highly processed matrices however, its consistency and sensitivity lacks in comparison to the BioFront cashew ELISA.

g. Recovery of Cashew Protein from Commercial Cashew Milks

The same six commercial cashew milks tested in Chapter 2.II.a were also evaluated using the developed cashew ELISA to determine if cashew protein in these UHT/HTST processed matrices can be detected. The developed cashew ELISA reliably detected cashew from the cashew milks Elmhurst, Dream, and SoDelicious with low variance between trials (Table 4.15).

Table 4.15. Recovery of cashew protein (ppm) from commercial cashew milks using the developed ELISA over two independent trials.

Sample	**Average Cashew Protein (ppm)**	**Coefficient of Variance** (%)
Elmhurst	485 ± 9	2
Forager Project	488 ± 74	15
Silk	43*	
Pacific Foods	591 ± 212	36
Dream	443 ± 53	12
SoDelicious	85 ± 19	22

*Only 1 trial for Silk was above the LOQ. Developed ELISA LOQ (0.3 ppm cashew protein). Values expressed as average ± standard deviation (n=2).

The cashew milks Forager Project and Dream showed more variation between trials however, this is not unexpected due to the high concentration of cashew protein in each cashew milk (Table 4.15). Pacific Foods had the most variation between trials (Table 4.15); however, this also may be due to the high concentration of cashew protein in the cashew milk or due to sampling variation.

When the developed cashew ELISA was compared to the previously tested commercial cashew ELISAs, depending on the cashew milk, the different ELISAs showed variation in the recovery of cashew proteins. Figure 4.10 illustrates the recovery of cashew protein from the six commercial cashew milks using two commercial cashew ELISAs (BioFront and R-Biopharm) and the developed cashew ELISA.

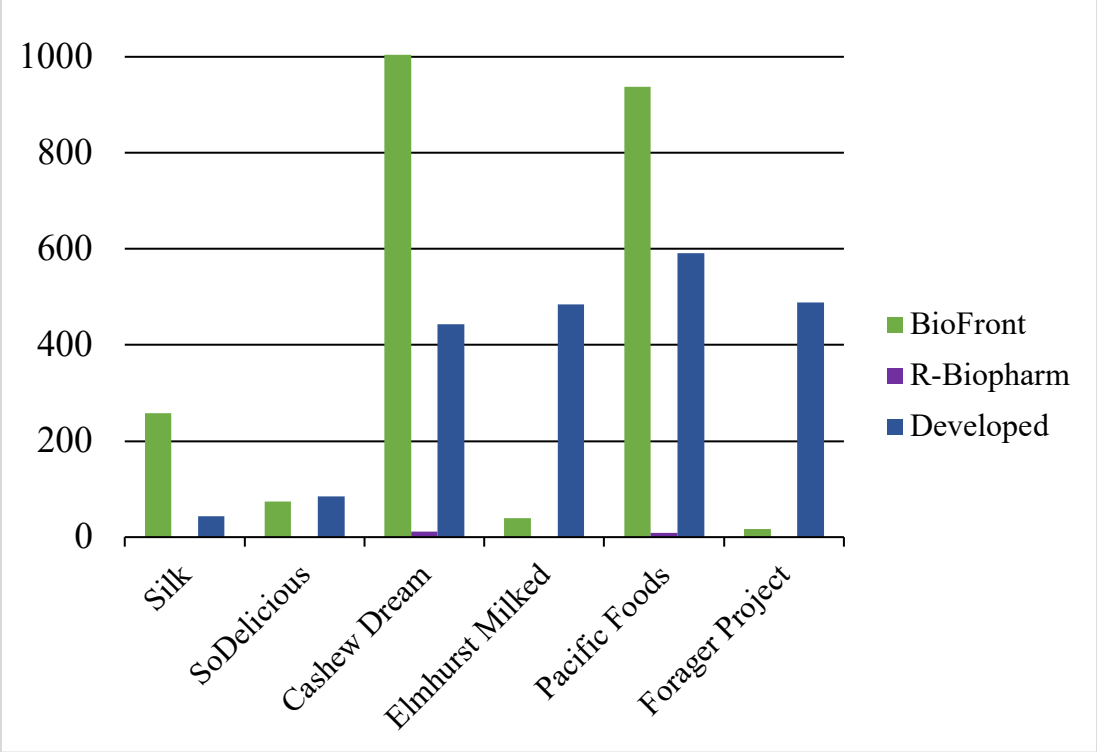


Figure 4.10. Average recovery of cashew protein (ppm) from commercial cashew milks using BioFront, R-Biopharm, and the developed cashew ELISA.

The BioFront kit detected more cashew protein in two cashew milks (Cashew Dream and Pacific Foods) while the developed cashew ELISA detected more cashew protein in two different cashew milks (Forager Project and Elmhust), with similar recovery values observed for two cashew milks (Silk and So Delicious) (Figure 4.10). R-Biopharm showed the least amount of cashew protein detection with all six cashew milks. This suggests that the developed cashew ELISA reliably identifies cashew protein from cashew milk matrices. While BioFront was able to recover more cashew protein from two of the cashew milks (Cashew Dream and Pacific Foods), the developed assay was able to recover more cashew protein from two other cashew milks (Elmhurst and Forager Project) (Figure 4.10). Since both the BioFront kit and the developed ELISA were able to

detect cashew protein from all six cashew milk matrices, both can be considered to be reliable in recovering cashew protein residue derived from cashew milks.

IV. CONCLUSIONS

Combining the crude rabbit anti-cashew 2S and 11S R/A sera as the capture antibody (1:10000) in a 1:1 pool paired with the sheep anti-roasted cashew sera as the detector antibody (1:2500) resulted in the development of a robust cashew ELISA. The developed cashew ELISA (LOD 0.09 ppm cashew protein; LOQ 0.3 ppm cashew protein) for plant milk matrices was reliable in recovering cashew protein from HTST processed almond milks incurred with known levels of cashew protein as well as commercial cashew milks. No cross-reactivity was observed with any of the 8 plant milks evaluated, indicating that this ELISA is not affected by the highly-processed plant milk matrices. When the developed ELISA was compared to a commercial cashew ELISA kit from BioFront Technologies, both showed similar capabilities to the developed assay in its evaluation of cashew milks and pre- and post-HTST almond milk incurred with cashew, with the exception of increased sensitivity of incurred almond milks with the commercial assay. However, the developed ELISA was more robust in detecting cashew protein from highly processed plant milk matrices, showing similar percentage recoveries pre- and post-HTST processing.

Further applications of the ELISA can include high pressure processing (HPP) products, baked products, or other potentially highly processed products which contain cashew. To evaluate the ability of the developed cashew ELISA to detect cashew protein from other heat processed matrices, the following chapter will evaluate another incurred matrix, cookie.

V. REFERENCES

Abtahi, S., & Aminlari, M. (1997). Effect of sodium sulfite, sodium bisulfite, cysteine, and pH on protein solubility and sodium dodecyl sulfate−polyacrylamide gel electrophoresis of soybean milk base. *Journal of Agricultural and Food Chemistry, 45*(12), 4768-4772.

Andreasson, U., Perret-Liaudet, A., van Waalwijk van Doorn, L. J., Blennow, K., Chiasserini, D., Engelborghs, S., Fladby, T., Genc, S., Kruse, N., Kuiperij, H. B., Kulic, L., Lewczuk, P., Mollenhauer, B., Mroczko, B., Parnetti, L., Vanmechelen, E., Verbeek, M. M., Winblad, B., Zetterberg, H., Koel-Simmelink, M., & Teunissen, C. E. (2015). A practical guide to immunoassay method validation. *Frontiers in Neurology, 6*(179), 1664-2295.

Armbruster, D. A., & Pry, T. (2008). Limit of blank, limit of detection and limit of quantitation. *The Clinical biochemist. Reviews, 29*(1), S49-S52.

Aydin, S. (2015). A short history, principles, and types of ELISA, and our laboratory experience with peptide/protein analyses using ELISA. *Peptides, 72*, 4-15.

Bogahawaththa, D., Buckow, R., Chandrapala, J., & Vasiljevic, T. (2018). Comparison between thermal pasteurization and high pressure processing of bovine skim milk in relation to denaturation and immunogenicity of native milk proteins. *Innovative Food Science & Emerging Technologies, 47*, 301-308.

Dhakal, S., Liu, C., Zhang, Y., Roux, K. H., Sathe, S. K., & Balasubramaniam, V. M. (2014). Effect of high pressure processing on the immunoreactivity of almond milk. *Food Research International, 62*, 215-222.

Engvall, E., & Perlmann, P. (1971). Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. *Immunochemistry, 8*(9), 871-874.

Ferragut, V., Valencia-Flores, D. C., Perez-Gonzalez, M., Gallardo, J., & Hernandez-Herrero, M. (2015). Quality characteristics and shelf-life of ultra-high pressure homogenized (UHPH) almond beverage. *Foods, 4*(2), 159-172.

Gaskin, F. E., & Taylor, S. L. (2011). Sandwich enzyme-linked immunosorbent assay (ELISA) for detection of cashew nut in foods. *J Food Sci, 76*(9), T218-226.

Harlow, E., & Lane, D. (1988). *Antibodies: A Laboratory Manual*: Cold Spring Harbor Laboratory.

Ivens, K. (2018). *Evaluating the digestion, absorption, and transport of food allergens.* Unpublished Dissertation, University of Nebraska-Lincoln.

Janeway, Travers, & Walport. (2001). The structure of a typical antibody molecule. In *Immunobiology: The Immune System in Health and Disease* 5 ed.). New York: Garland Science.

Masiri, J., Benoit, L., Meshgi, M., Day, J., Nadala, C., & Samadpour, M. (2016). A novel immunoassay test system for detection of modified allergen residues present in almond-, cashew-, coconut-, hazelnut-, and soy-based nondairy beverages. *Journal of Food Protection, 79*(9), 1572-1582.

Mattison, C. P., Desormeaux, W. A., Wasserman, R. L., Yoshioka-Tarver, M., Condon, B., & Grimm, C. C. (2014). Decreased immunoglobulin E (IgE) binding to cashew allergens following sodium sulfite treatment and heating. *Journal of Agricultural and Food Chemistry, 62*(28), 6746-6755.

McCabe, J. P., Fletcher, S. M., & Jones, M. N. (1988). The effects of detergent on the enzyme-linked immunosorbent assay (ELISA) of blood group substances. *Journal of Immunological Methods, 108*(1), 129-135.

Wei, Y., Sathe, S. K., Teuber, S. S., & Roux, K. H. (2003). A sensitive sandwich ELISA for the detection of trace amounts of cashew (Anacardium occidentale L.) nut in foods. *Journal of Agricultural and Food Chemistry, 51*(11), 3215-3221.

Zhao, Y., Sun, X., Marquis, C. P., & Lee, N. A. (2019). Development of a sensitive sandwich ELISA specific to 2S albumin (Ana o 3) as a stable protein marker for cashew nut residue detection in pre-packaged food products. *Food Control, 96*, 432-440.

CHAPTER 5: EVALUATION OF THE DEVELOPED CASHEW ELISA IN A BAKED COOKIE MATRIX

I. INTRODUCTION

Food manufacturers have multiple checkpoints in place in their allergen control programs to assess and mitigate potential allergen cross-contamination. Ensuring these checkpoints within their allergen control or cleaning programs are validated is an important aspect of food safety and control. Improved food safety regulations in recent years have required the implementation of more rigorous allergen labeling and handling practices aimed at decreasing the opportunity for improperly labeled allergenic foods products to reach consumers. This has resulted in an increased amount of food recalls for the respective food companies. As of 2014, allergens accounted for the highest number of food reportable food registry (RFR) entries (~44%) in the United States over any other individual pathogen contamination (FDA, 2014). The high number of RFR entries and associated recalls for allergens serves as a critical reminder that undeclared food allergens represent a critical food safety concern in our society. Food allergen detection methods have been an important tool for the food industry to assess cleaning procedures and aid in the mitigation of allergen cross-contact on shared processing equipment. While allergen detection methods are available for a number of priority allergenic sources, allergen detection methods need to be continually improved to protect the food-allergic consumer from consuming improperly labeled or foods with unintended allergen presence due to cross-contact.

Model foods incurred with the allergenic source of protein of choice are considered the best materials to use to validate the sensitivity and robustness of an ELISA

by determining the effects of processing conditions on protein recovery. Evaluating protein recovery prior to and following processing can help determine what impacts from processing may have on the recovery of proteins (Koppelman, et al., 2006). Many researchers have evaluated model foods as a way to confirm recovery of protein. These matrices have included ice cream, chocolate, cookies, and other baked goods (Downs & Taylor, 2010; Gaskin & Taylor, 2011). While most ELISAs are developed to ensure that heat processing will have less of an effect on protein detection and recovery, some heat processes still have a major effect. These heat processes have been determined to change the allergenic protein configuration (Masthoff, et al., 2013). After being subjected to heating, the immunogenicity of cashew proteins can be altered, leading to possible changes in conformational epitopes. This may alter the ability of an antibody to detect the protein (Masthoff, et al., 2013).

Some cashew proteins are more heat resistant than others, thereby heat processing (and other common forms of food processing) have less effect on the ability of an ELISA to detect cashew protein. To determine if the optimized cashew ELISA based on the cashew 2S and 11S R/A proteins was robust, two matrices, a cookie and an almond milk, were tested to determine recovery of incurred cashew at varying concentrations. Almond milk was previously evaluated to have reliable recovery by the developed ELISA (Chapter 4.III.f). Cookies were evaluated as an example of a baked matrix (Gaskin & Taylor, 2011). Baking is a common heat processing technique which cashews may be subjected to (Masthoff, et al., 2013). Five different incurred levels, in addition to a negative control, were evaluated for the recovery of cashew using the developed ELISA. Cashew protein recovery was evaluated pre- and post- baking.

II. MATERIALS AND METHODS

a. Preparation of an Incurred Cookie Matrix

All incurred model foods were formulated using roasted ground cashew. The cashew spike was developed and checked for homogeneity according to procedures outlined previously in Chapter 4.II.e.

Cookies were processed according to the AACC International Method 10-50.05 with minor modifications. The formulation utilized for this incurred matrix is listed in Table 5.1, with spiked levels including 0.5, 2, 10, 20, and 200 ppm cashew protein.

Table 5.1. Formulation (as adapted from AACC International Method 10-50.05 with minor modifications) for incurred baked cookies with cashew protein (0.5, 2, 10, 20, 200 ppm cashew protein).

Cashew Protein (ppm)	**0**	**0.5**	**2**	**10**	**20**	**200**	**Formula Percent (%)**
Ingredient	**Weight (g)**						
Shortening	64	64	64	64	64	64	13.5
2000 ppm Cashew Protein Sugar-Spike	0	0.1	0.5	2.4	4.7	47.3	X*
Sugar	130	129.9	129.5	127.6	125.3	82.7	27.5
Salt	2.1	2.1	2.1	2.1	2.1	2.1	0.4
Sodium bicarbonate	2.5	2.5	2.5	2.5	2.5	2.5	0.5
6% Dextrose solution	33	33	33	33	33	33	7
Water	16	16	16	16	16	16	3.4
Flour	225	225	225	225	225	225	47.6
TOTAL	**472.6**	**472.6**	**472.6**	**472.6**	**472.6**	**472.6**	**100.0**

X*; The percentage of spiked cashew protein will vary in the final formulation to arrive at the desired concentration of 0.5, 2, 10, 20 or 200 ppm cashew protein

An oven was preheated to 205°C (400°F) and a dark, non-stick half-sheet baking pan (46 x 33 cm) was lined with parchment paper. Shortening, sugar, sugar-spike (if adding), salt, and sodium bicarbonate were mixed at stir speed in a KitchenAid mixer (Benton Harbor, MI) for 3 min. The speed was increased to speed 6 and mixed for an additional 2 min. The dextrose solution and water were added and mixed for 1 min at stir speed, followed by additional mixing for 1 min at speed 2. The flour was added and mixed for 3 min at stir speed. Half of the raw cookie dough was stored at -20°C until further analysis. The rest of the cookie dough was divided into portions of 12 g each and flattened evenly to ensure even heat transfer. The cookies were baked for 10 min at 205°C (400°F) with the pans rotated half-way through baking. After baking, the cookies were transferred to a cooling rack and cooled for 20 min at RT. Both raw cookie dough and baked cookies at each spike level were ground using an Osterizer blender (Sunbeam Corporation, Delray Beach, FL), tested for homogeneity using the commercial cashew ELISA kit from BioFront Technologies (Tallahassee, FL), and stored at -20°C until further analysis.

b. Extraction and Evaluation of Incurred Dough and Baked Sugar Cookies

Raw dough and baked cookie samples from the five incurred cashew nut levels and the 0 ppm negative control, pre-and post-baking, were ground separately into fine particles using an Osterizer blender (Sunbeam Corporation, Delray Beach, FL) prior to extraction. One g from each batch of ground dough and baked cookie was extracted in 10 mL w/v of 0.01 M PBS with 0.1 M sodium sulfite, pH 7.4 in a shaking water bath (200 rpm) at 60°C for 25 min. Supernatants obtained after centrifugation at 12500 xg for 5 min at RT were stored at -20°C until further analysis.

The clarified supernatants were analyzed for recovery of cashew protein using the previously developed cashew ELISA (Chapter 4.II.c). The optimized standard curve in baked cookie matrix was prepared as mentioned previously in Chapter 4.II.c. Percentage recovery of cashew protein in cookie dough and baked cookie was calculated as the recovered ppm concentration over the expected ppm concentration of cashew protein added. The final result was based on one extraction of each incurred level analyzed in 2 independent trials.

c. Cross-Reactivity and Matrix Interference of Market Products

Since the developed assay showed no significant cross-reactivity in the evaluated plant milk matrices (Chapter 4.III.e), potential cross-reactivity or matrix interference was evaluated for 50 other food ingredients commonly used in the food industry. Ingredients were purchased from local grocery stores in Lincoln, Nebraska. Liquid samples were used without further processing, while non-liquid samples were ground into fine particles using an Osterizer blender (Sunbeam Corportation, Delray Beach, FL) or mortar and pestle. Each sample was extracted 1:10 (w/v or v/v) in 0.01 M PBS with 0.1 M sodium sulfite in a shaking water bath (200 rpm) at 60°C for 25 min. The supernatants obtained by centrifugation at 12500 xg for 5 min at RT were stored at -20°C until further analysis. The individual extracts were analyzed by the developed cashew ELISA at dilution levels of 1:1, 1:10, 1:100, and 1:1000 in 0.01 M PBS v/v in duplicate. Results obtained from ELISA analysis are expressed in ppm cashew protein.

Potential cross-reactivity was found with multiple matrices upon initial evaluation. The soluble protein content of these respective samples was determined using the 2-D Quant protein assay (GE Healthcare, Chicago, IL). These samples showing

potential matrix interference and/or cross-reactivity were re-extracted in the reducing buffer (0.01 M PBS with 0.1 M sodium sulfite) under the same conditions, except with the addition of 5% NFDM to both the standard and sample extract. Potential cross-reactive samples were re-evaluated with the same dilutions as used previously with results expressed in ppm cashew protein.

III. RESULTS AND DISCUSSION

a. Recovery of Incurred Cashew Nut from Raw Cookie Dough and Baked Cookie Matrices

The recovery of cashew protein from each of the incurred levels in cookie dough and baked cookie was evaluated using the same standard curve optimized previously in Chapter 4.II.c. Table 5.2 provides the recovery of cashew protein from the cookie dough and baked cookie at each incurred level from 2 independent trials.

Table 5.2. Percent recovery of cashew protein from cookie dough and baked cookie model foods incurred with known levels of cashew protein as determined by the developed cashew ELISA.

Incurred Model Food	Cashew Protein Level (ppm)	% Recovery
Raw Dough	0	BLQ
	0.5	BLQ
	2	BLQ
	10	209 ± 19
	20	193 ± 8
	200	177 ± 1
Baked Cookie	0	BLQ
	0.5	BLQ
	2	BLQ
	10	98 ± 8
	20	81 ±10
	200	74 ± 5

BLQ*; Below the limit of quantification (0.3 ppm cashew protein). Samples were extracted 1:10 w/v in 0.01 M PBS with 0.1 M sodium sulfite. Developed ELISA LOQ (0.3 ppm cashew protein). Values expressed as average ± standard deviation (n=2).

The 2 ppm cashew protein incurred cookie dough was determined to be below the LOQ (0.3 ppm cashew protein) but above the LOD (0.09 ppm cashew protein) of the ELISA (Table 5.2). The 2 ppm cashew protein incurred dough sample could thus be qualitatively determined by the ELISA to contain cashew protein, but not quantitatively determined (Table 5.2). The percentage recovery of cashew protein in cookie dough at incurred levels of 10, 20, and 200 ppm cashew protein was above the LOQ, giving recoveries of 200% on average (Table 5.2). The percentage recovery of cashew protein is dependent on what the standard curve is prepared in. The overestimation of cashew protein was seen here likely due to the large difference between the cookie dough matrix and the standard curve matrix. The overestimation of cashew present in cookie dough is not acceptable for ELISA recovery based on standard acceptance criteria (expected 80-120%) (Andreasson, et al., 2015); however, most matrices evaluated are processed, thus the relevance of a heat-treated standard curve is higher than that of a pre-processed, matrix-matched standard curve. Using a matrix matched standard curve (cookie dough) may help decrease the overestimation of recovery and can be evaluated in future work.

Recoveries for the baked cookie dough were 80-100% of expected cashew protein in each incurred level of 10, 20, and 200 ppm cashew protein cookies (Table 5.2). The protein recovery for post-baked cookies is within the acceptable range for food allergen detection methods (expected 80-120% recovery) (Abbott, et al., 2010). Processing under heat can affect the immunological properties of proteins including antibody binding (Mattison, et al., 2016). Lower recovery of protein can be observed when products undergo heat processing and our results show a slight decrease in protein detection. A potential explanation for low recovery at the lowest incurred levels (0.5 and 2 ppm

cashew protein) could be the extractability of cashew protein. The reducing extraction buffer was proven to be a more rigorous extraction buffer (Chapter 2.III.a) however, challenges may still be in effect at the low cashew protein concentrations. Even with this potential error, the detection was still within the acceptable range (80-120%) at concentrations of 10 ppm cashew protein or greater (Andreasson, et al., 2015). The low recovery observed with the 0.5 and 2 ppm cashew protein incurred levels are lower than what would be preferred; however, these levels are also closer to the limit of quantification (LOQ: 0.3 ppm) of the developed ELISA where variance may occur (Table 5.2). The developed cashew ELISA shows reasonable recovery in a baked cookie matrix, with overestimation seen in a dough matrix. However, both matrices showed quantitative cashew protein detection to 10 ppm cashew protein, with the cookie dough matrix showing qualitative cashew protein detection at 2 ppm cashew protein. Cookie dough cashew protein overestimation could be decreased in the future by using a spiked pre-processed cashew product (cookie dough) to prepare the standard curve so as to mimic pre-processing conditions.

b. Cross-Reactivity and Matrix Interference Evaluation of Market Products

A total of 50 food ingredients which are commonly used in the food industry were evaluated using the developed cashew ELISA for potential cross-reactivity (Table 5.3).

Table 5.3. Cross-Reactivity and matrix interference analysis of different food and food ingredients using the developed cashew nut ELISA.

Ingredient	Average Cashew Protein (ppm)	Ingredient	Average Cashew Protein (ppm)
Buckwheat	BLQ	Cinnamon	9.9 ± 0.05
Cornstarch	BLQ	Cumin	BLQ
Rice Flour	BLQ	Clove	20.4 ± 2
Oats	BLQ	Nutmeg	BLQ
Whole Wheat	BLQ	Vanilla Extract	BLQ
Skim Milk	BLQ	Oregano	50 ± 4
Brown Sugar	BLQ	Pecan	15 ± 3
Cocoa Powder	13 ± 2	Almond	BLQ
Cream of Tartar	BLQ	Pine Nut	BLQ
Maltodextrin	BLQ	Hazelnut	87 ± 36
Coconut	16 ± 1	Walnut	78 ± 7
Dried Chickpea	13 ± 0.07	Macadamia Nut	BLQ
Dried Green Split Pea	7 ± 0.6	Fresh Mango Seed	63 ± 3
Dried Lima Beans	BLQ	Pink Peppercorn	13494 ± 5345
Peanut	145 ± 37	Dried Mango	BLQ
Celery Seed	BLQ	Fresh Mango Flesh and Skin	BLQ
Poppy Seed	150 ± 26	Pistachio	132377 ± 8575
Fennel Seed	13 ± 0.06	Dried Cranberry	53 ± 0.4
Flax Seed	89 ± 14	Dried Cherry	BLQ
Mustard Seed	12 ± 0.4	Raisins	27 ± 4
Sesame Seed	710 ± 477	Dried Dates	BLQ
Sunflower Seeds	21 ± 1	Sumac	BLQ
Caraway Seed	34 ± 4	Fenugreek	BLQ
Almond Extract	BLQ	Brazil Nuts	74 ± 14
Chocolate	BLQ		

*BLQ; Below the limit of quantification (0.3 ppm cashew protein). Values expressed as average ± standard deviation (n=2).

Upon testing, a total of 24 food ingredients demonstrated a reading higher than the limit of quantification (0.3 ppm cashew protein) (Table 5.3). This number of potentially cross-reactive or interfering ingredients was unexpected. Some food ingredients common to the Anacardiaceae family such as pistachio, mango seed, sumac, and pink peppercorn have

been shown to be cross-reactive in cashew ELISAs due to the close botanical relationship with cashew and the high degree of protein homology (Bastiaan-Net, et al., 2019; Fong, et al., 2019; Gaskin & Taylor, 2011; Willison, et al., 2008). Consequently, some of the ingredients belonging to the Anacardiaceae family which tested for cross-reactivity are in agreement with previous observations, such as pistachio, pink peppercorn, and fresh mango seed (Table 5.3).

Some of the other potentially cross-reactive ingredients such as seeds, spices, baking ingredients, and other tree nuts may have had matrix interference rather than true cross-reactivity. To determine if matrix interference was responsible for these observations, 5% non-fat dried milk (NFDM) was added to the reducing extraction buffer to help bind potential polyphenols, tannins, or other interfering substances that may be contributing to the apparent positive detection (Yildirim-Elikoglu, et al., 2018). The ELISA was repeated with the results indicated in Table 5.4. Figure 5.1 compares the standard curves with (n=4 trials) and without 5% NFDM (n=20 trials).

Table 5.4. Soluble protein content (ppm) by 2-D Quant analysis of cross-reactive food ingredients and the apparent cashew protein equivalents (ppm) detected by the developed cashew ELISA, when extracted with and without 5% NFDM.

Ingredient	2-D Quant (ppm)	Average Apparent Cashew Protein Equivalents (ppm) No NFDM	Average Apparent Cashew Protein Equivalents (ppm) with NFDM
Cocoa Powder	726	13 ± 2	BLQ
Coconut	1667	16 ± 1	BLQ
Dried Chickpea	8478	13 ± 0.07	BLQ
Dried Green Split Pea	5401	7 ± 0.6	BLQ
Peanut	5770	145 ± 37	BLQ
Poppy Seed	5644	150 ± 26	27 ± 10
Fennel Seed	426	13 ± 0.06	BLQ
Flax Seed	7446	89 ± 14	87 ± 2
Mustard Seed	7224	12 ± 0.4	BLQ
Sesame Seed	14494	710 ± 477	19 ± 3
Sunflower Seeds	3181	21 ± 1	BLQ
Caraway Seed	486	34 ± 4	BLQ
Cinnamon	700	9.9 ± 0.05	BLQ
Clove	BLQ	20 ± 2	13 ± 0.8
Oregano	348	50 ± 4	20 ± 2
Pecan	BLQ	15 ± 3	23 ± 1
Hazelnut	11250	87 ± 36	72 ± 7
Walnut	1359	78 ± 7	48 ± 5
Brazil Nuts	10074	74 ± 14	12 ± 5
Fresh Mango Seed	BLQ	63 ± 3	10464 ± 279
Pink Peppercorn	500	13494 ± 5345	170460 ± 5945
Pistachio	9833	132377 ± 8575	682039 ± 52541
Dried Cranberry	BLQ	53 ± 0.4	BLQ
Raisins	BLQ	27 ± 4	BLQ

NFDM; Non-fat dried milk. Ingredients extracted in 0.01 M PBS containing 0.1M sodium sulfite with or without 5% NFDM at 60°C for 25 min. BLQ; Below the limit of quantification (0.3 ppm cashew protein). Values expressed as average ± standard deviation (n=2).

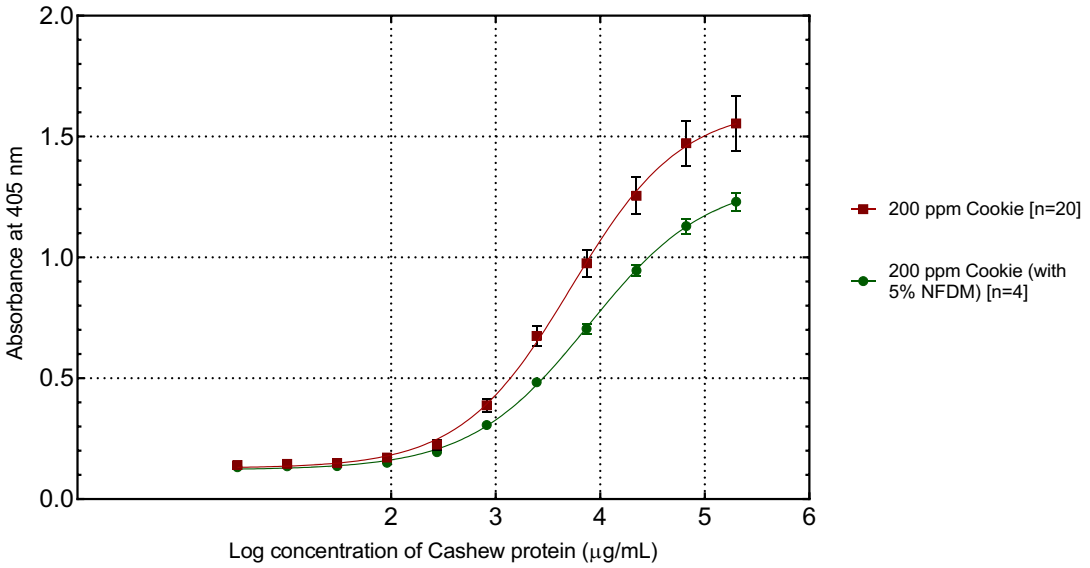


Figure 5.1. Standard curve in 200 ppm cashew protein incurred baked cookie matrix, diluted 3-fold in PBS. Extracted in 0.01 M PBS with 0.1 M sodium sulfite with (green curve) and without (red curve) 5% NFDM. n= number of independent standard curves.

The standard curve with 5% NFDM is shifted to the right, yet still has a similar dynamic linear range (Figure 5.1). Results of the potentially cross-reactive substances were analyzed using both the standard curves (with and without NFDM), with similar cashew protein equivalents determined for each. The similar linear ranges observed for the standard curves with and without NFDM suggest that NFDM is not needed for standard curve development. However, its use may help remove potentially interfering background in certain ingredients.

The addition of 5% NFDM in the extraction buffer did have a beneficial effect on 12 of the previous 24 potentially cross-reactive ingredients. As seen in Table 5.4, half of these ingredients resulted in being below the limit of quantification (BLQ) after extraction with NFDM. The ingredients which still gave positive results were poppy seed,

flax seed, sesame seed, clove, oregano, walnut, hazelnut, pecan, brazil nut, and the three cross-reactive ingredients determined previously (pink peppercorn, pistachio, and mango seed) (Table 5.4). While the ELISA assay detected cashew protein in all of these ingredients, it is likely due to matrix interference rather than true cross-reactivity. True cross-reactivity is indicated by a decrease in absorbance as the dilution of the ingredient extract is increased. However, with matrix interference the absorbance values across dilutions remain mostly consistent. Nine food ingredients (poppy seed, flax seed, sesame seed, clove, oregano, walnut, hazelnut, pecan, and Brazil nut) showed matrix interference with the developed cashew ELISA when analyzing the source of food/ingredient themselves. Most of these interfering foods are usually formulated in products at low levels. It is unlikely that the amount of these food ingredients will be as high when evaluating prepared food samples. However, future work on the ELISA should include more testing to lower potential matrix interference. This could include methods such as incorporating a different non-binding protein (fish gelatin) to the extraction buffer, using a different sample to extraction buffer ratio, or using purified antibodies in the cashew ELISA (Gan, 2016).

IV. CONCLUSIONS

The developed cashew ELISA showed consistent results in its ability to quantitatively detect the presence of cashew proteins from cashew milk as well as cashew protein from an incurred plant milk (almond milk) (Chapter 4). To determine if the cashew ELISA could be used to detect the presence of cashew protein in other processed matrices, raw cookie dough and baked cookies incurred with different levels of cashew protein (0, 0.5, 2, 10, 20, 200 ppm cashew protein) were evaluated. The recovery of cashew protein from incurred cookie dough was ~200%. Recovery was between 80-100% from baked cookie incurred with cashew protein. The developed cashew ELISA was able to detect cashew protein in the 10 ppm incurred dough and baked cookie matrices but was not able to detected cashew protein at the 0.5 and 2 ppm cashew protein concentrations. Fifty ingredients were evaluated for matrix interference and cross-reactivity. Three ingredients showed potential cross-reactivity (pistachio, pink peppercorn, and mango seed) while nine showed matrix interference after extraction with the addition of 5% NFDM.

The developed cashew ELISA recovered cashew protein from a processed cookie matrix reliably, with high recovery overestimation of the pre-processed cookie dough. Both standard and sample extracts should include NFDM as an additive for future testing, to further help decrease matrix interference. Future work on the ELISA should include steps to further validate the ELISA by decreasing matrix interference with other non-binding proteins, repeating sample trials, and assessing more ingredients for potential cross-reactivity.

V. REFERENCES

Abbott, M., Hayward, S., Ross, W., Godefroy, S. B., Ulberth, F., Van Hengel, A. J., Roberts, J., Akiyama, H., Popping, B., Yeung, J. M., Wehling, P., Taylor, S. L., Poms, R. E., & Delahaut, P. (2010). Validation procedures for quantitative food allergen ELISA methods: community guidance and best practices. *J AOAC Int, 93*(2), 442-450.

Andreasson, U., Perret-Liaudet, A., van Waalwijk van Doorn, L. J., Blennow, K., Chiasserini, D., Engelborghs, S., Fladby, T., Genc, S., Kruse, N., Kuiperij, H. B., Kulic, L., Lewczuk, P., Mollenhauer, B., Mroczko, B., Parnetti, L., Vanmechelen, E., Verbeek, M. M., Winblad, B., Zetterberg, H., Koel-Simmelink, M., & Teunissen, C. E. (2015). A practical guide to immunoassay method validation. *Frontiers in Neurology, 6*(179), 1664-2295.

Bastiaan-Net, S., Reitsma, M., Cordewener, J. H. G., van der Valk, J. P. M., America, T., Dubois, A. E. J., Gerth van Wijk, R., Savelkoul, H. F. J., de Jong, N. W., & Wichers, H. J. (2019). IgE cross-reactivity of cashew nut allergens. *Int Arch Allergy Immunol, 178*(1), 19-32.

Downs, M. L., & Taylor, S. L. (2010). Effects of thermal processing on the enzyme-linked immunosorbent assay (ELISA) detection of milk residues in a model food matrix. *Journal of Agricultural and Food Chemistry, 58*(18), 10085-10091.

FDA. (2014). The reportable food registry: a five year overview of targeting inspection resources and identifying patterns of adulteration. In FDA (Ed.), 5 ed.).

Fong, A. T., Du Toit, G., Versteeg, S. A., & van Ree, R. (2019). Pink peppercorn: A cross-reactive risk for cashew- and pistachio-allergic patients. *J Allergy Clin Immunol Pract, 7*(2), 724-725.e721.

Gan, C. (2016). *Development of a Sandwich Enzyme-Linked Immunosorbent Assay (ELISA) for Detection of Macadamia Nut Residues in Processed Food Products.* Unpublished Thesis, University of Nebraksa - Lincoln.

Gaskin, F. E., & Taylor, S. L. (2011). Sandwich enzyme-linked immunosorbent assay (ELISA) for detection of cashew nut in foods. *J Food Sci, 76*(9), T218-226.

Koppelman, S. J., & Hefle, S. L. (2006). 19 - Reference materials and method validation in allergen detection. In *Detecting Allergens in Food*, (pp. 348-356). Cambridge, England: Woodhead Publishing.

Masthoff, L. J., Hoff, R., Verhoeckx, K. C. M., van Os-Medendorp, H., Michelsen-Huisman, A., Baumert, J. L., Pasmans, S. G., Meijer, Y., & Knulst, A. C. (2013). A systematic review of the effect of thermal processing on the allergenicity of tree nuts. *Allergy, 68*(8), 983-993.

Mattison, Bren-Mattison, Y., Vant-Hull, B., Vargas, A. M., Wasserman, R. L., & Grimm, C. C. (2016). Heat-induced alterations in cashew allergen solubility and IgE binding. *Toxicology Reports, 3*, 244-251.

Willison, L. N., Tawde, P., Robotham, J. M., Penney, R. M. t., Teuber, S. S., Sathe, S. K., & Roux, K. H. (2008). Pistachio vicilin, Pis v 3, is immunoglobulin E-reactive and cross-reacts with the homologous cashew allergen, Ana o 1. *Clin Exp Allergy, 38*(7), 1229-1238.

Yildirim-Elikoglu, S., & Erdem, Y. K. (2018). Interactions between milk proteins and polyphenols: Binding mechanisms, related changes, and the future trends in the dairy industry. *Food Reviews International, 34*(7), 665-697.

CHAPTER 6: SUMMARY AND FUTURE WORK

Cashew nut allergy is the second leading tree nut allergy in the USA and corresponds to a high number of anaphylactic-related allergy deaths. It is essential that current cashew ELISAs have accurate detection of cashew protein in a variety of processed cashew products. With plant milk consumption on the rise, determining whether UHT (ultra-high temperature) or HTST (high-temperature short-time) processing has an effect on cashew protein detection in plant milk is important to the food industry and cashew-allergic consumers. Preliminary testing indicated that current cashew ELISAs were unable to accurately detect cashew protein from HTST/UHT treated cashew milk and therefore would likely struggle to accurately detect cashew protein residue that may be present in other plant milks produced on shared processing equipment.

To improve cashew ELISAs, a more robust ELISA method was developed, targeting the cashew 11S R/A and 2S cashew proteins for enhanced cashew protein detection across heat-treated matrices. The reduced form of cashew Ana o 2 (11S) was chosen as a target due to the reduced form increasing cashew 11S solubility during extraction and the relative stability of the acidic and basic subunits of the 11S protein. Cashew Ana o 3 (2S) was also chosen as a target due to cashew 2S protein's heat and pH resilience. A low pH extraction method isolated the cashew 2S protein while a pH extraction followed by low pH extraction method isolated the cashew 11S proteins. The cashew 11S proteins were subject to reduction and alkylation to produce targets against both the acidic and basic units of the cashew 11S. Antibodies were produced against the cashew 11S R/A and 2S for the development of a sandwich ELISA.

The developed cashew ELISA utilized a pooled coating antibody combining both the cashew 11S R/A and 2S rabbit antisera paired with a detector antibody containing roasted cashew sheep antisera. The LOQ of the ELISA was 0.3 ppm cashew protein and the LOD of the ELISA was 0.09 ppm cashew protein. The developed ELISA did not show cross-reactivity to any of the different plant milks that were evaluated. An optimized standard curve based on a 200 ppm cashew protein baked cookie resulted in recoveries ranging 70-130% of the incurred level in almond milks both pre- and post-HTST processing at the incurred cashew protein concentrations of 10, 20 or 200 ppm. This recovery range is within the acceptable limit of recovery for allergen detection methods. All commercial cashew milks were detectable by the developed cashew ELISA. The developed ELISA was robust in the detection of cashew protein from high heat processing methods including HTST.

To further validate the ELISA, another incurred matrix, cookie, was evaluated for the recovery of cashew protein pre- and post-processing. In cookie dough, an overestimation of 200% recovery at each incurred level was observed, indicating that pre-processing conditions result in an overestimation of cashew protein likely due to the development of the cashew ELISA standard curve based upon an incurred baked cookie matrix. Baked cookies resulted in recoveries between 80-100% at each incurred concentration from 10-200 ppm cashew protein, indicating that processed matrices gave more accurate recoveries. Cross-reactivity and matrix interference studies with 50 food ingredients were used to determine if these ingredients would interfere or cross-react with the ELISA. Following extraction with non-fat dried milk (NFDM) added as an additive, 9 different foods including seeds, tree nuts, and spices showed matrix interference with the

developed ELISA. Three food ingredients (pink peppercorn, pistachio, and mango seed) from the Anacardiaceae family showed cross-reactivity as expected.

The newly developed cashew ELISA reliably detects cashew protein in commercial cashew milks, pre- and post- HTST processed incurred cashew almond milks, and baked incurred cashew cookies. However, the developed ELISA overestimates cashew protein recovery from a raw cookie dough. Further validation of the cashew ELISA is necessary for better detection of cashew protein from processed food products.

The next steps for the developed ELISA include purifying IgG using a different purification method, matrix-matching the standard curve to a food sample, optimizing extraction buffer additives, and evaluating other highly processed cashew incurred matrices (high pressure processing (HPP), UHT, and freezing). While IgG purification was previously evaluated in the ELISA, it was discontinued due to low yield. If the IgG antibodies could be purified with larger yields, a more sensitive and robust ELISA may be developed, resulting in less matrix interference from food samples. The standard curve could be modified by matrix-matching the standard curve with the sample to decrease overestimation of cashew recovery, as seen in the incurred cookie dough. Matrix-matching may help ensure more accurate recovery for each food sample. However, a negative control of the matrix is not always feasible. Varying extraction buffer additives and concentrations, using a non-binding protein (fish gelatin) or sodium sulfite, may optimize cashew protein extraction with less background interference, similar to the use of NFDM. These extraction buffer additives may help further decrease background in the ELISA. A variety of other processed cashew matrices could be evaluated by the ELISA to validate the detection of cashew protein from processed matrices involving high

pressure and extremely low and high temperatures. Although HTST was used in this research, UHT, another common plant milk processing condition, could also be evaluated in an incurred matrix to further indicate the ELISA's robustness against high temperature processing.