

ABSTRACT

HECHT, ELIZABETH SARA. Characterization and Processing of Aflatoxin-Reduced, Whole Protein Soluble Extracts from Aflatoxin-Enriched Peanut Meal Using Response Surface Methods. (Under the direction of Dr. Jack P. Davis).

Mycotoxins are a global problem contaminating ~25% of the world's crops; these losses are not sustainable with world-wide food shortages (FAO and others 2012). Peanuts are a critical source of protein in the developing world yet are particularly susceptible to contamination by aflatoxins, a subset of the mycotoxin family. In developed countries, aflatoxin contaminated peanuts are diverted to oilstock, where any aflatoxin present does not fractionate with the oil, but is concentrated within the low oil, high protein (50% wt.) byproduct named peanut meal (PM). Previous work in our lab extracted aflatoxin-reduced, enzymatically-hydrolyzed protein from PM. However, whole-protein extracts could have functionality and processing benefits compared to hydrolysates. This thesis investigates three key research problems: the quantification of aflatoxin; the potential for producing an aflatoxin-reduced, whole-protein-rich soluble fraction derived from contaminated PM; and the interactions between aflatoxin and co-solutes under alkaline conditions.

Extraction and purification methods were improved to increase the recovery of aflatoxin quantified by HPLC from highly contaminated PM (373 ng/g). Solutions of 60% aqueous acetonitrile and 80% aqueous methanol were compared for extraction efficiency. The extract:PM ratio was considered for methanol extract systems and a 4:1 ratio extracted significantly more aflatoxin than from a 2:1 ratio. Acetonitrile solutions extracted significantly more aflatoxin than methanol solutions. When purified on AflaTest®

immunoaffinity columns, aflatoxin recoveries from both extracts were independent of load volume at dilutions equal or greater to 2.5x. When samples were purified on Florisil™ silica-gel columns, ~20% of aflatoxin loaded was detected in the flow-through. Furthermore, aflatoxin standards could be recovered by only ~80% after an eluent evaporation step from unsilicized glass vials; these losses made this purification method unsuitable to a highly contaminated PM system. The degradation of aflatoxins in HPLC solvent was documented as a function of time. At 24 hours, 80% losses in AFG₁ and AFG₂ were detected; degradation was prevented by addition of 1% acetic acid.

A central composite design study was used to optimize the extraction of protein and aflatoxin from PM aqueous dispersions based on four variables: NovaSil™ (a clay sorbent) (0.2 – 4%), calcium chloride (CaCl₂) (0 – 2%), pH (8 – 14), and time (15 – 60 min). The dispersion was centrifuged to isolate the soluble phase, and, after AflaTest® column purification, aflatoxin was quantified on an HPLC equipped with a photochemical reactor for enhancement detection system (excitation: 365 nm, emission: 440 nm). In soluble extracts, aflatoxin and protein ranged from 35 ng/g to 634 ng/g and 114 mg/g to 759 mg/g, respectively, on a dry weight basis. The quadratic models for log₁₀(AFB₁+1) and protein had excellent fits with R² equal to .97 and .94, respectively. Soluble nitrogen had the strongest correlation with pH and significant quantities of peptides were produced at a pH > 12. CaCl₂ showed a salting in effect for these alkaline hydrolysates and caused precipitation at lower pH values. AFB₁ had strong positive correlations with pH and CaCl₂. Furthermore, reversible and irreversible degradation of AFB₁ in soluble extracts was documented as a function of pH, with losses up to 90% occurring after treatment at pH 11. A desirability function to maximize

protein and minimize AFB₁ concentration yielded conditions of 0% NovaSil™, 2% CaCl₂, pH 10.88, 60 min and 1.11% NovaSil™, 2% CaCl₂, pH 6.0, 15 min, respectively. Isoelectric precipitation from an optimized extract resulted in a ten-fold reduction of soluble protein. Manganese peroxidase (MnP) was used as a secondary processing step to degrade aflatoxin in the soluble fractions. Degradation of AFB₁ in solutions could not be directly attributed to the enzyme; it is hypothesized that hydrogen peroxide, added as a cofactor, was the chemical agent responsible for degrading aflatoxin.

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Characterization and Processing of Aflatoxin-Reduced, Whole Protein Soluble Extracts from
Aflatoxin-Enriched Peanut Meal Using Response Surface Methods

by
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CHAPTER 1: LITERATURE REVIEW: PEANUT MEAL PROTEIN AND AFLATOXIN CONTAMINATION

1.1.0 Introduction

In 2012, 12.5% of the global population was estimated to be undernourished; simultaneously, pressure has been placed on agriculture to increase the nutritive quality, sustainability, and quantity of foods generated to meet a rising world population and address food security concerns (FAO and others 2012). The Food and Agricultural Organization (FAO) also estimates that each year, approximately 25% of crops are diseased by fungi and damaged by mycotoxins. Though mycotoxins are an unavoidable problem and occur naturally, better agricultural management practices and post-harvest amelioration strategies, particularly in developing countries, can help salvage existing food supplies and limit malnutrition.

Mycotoxins are the toxic secondary metabolites secreted by various soil and airborne fungi (Horn BW 2005). Many different types of mycotoxins are produced, including aflatoxins, fumonisins, and ochratoxins, for example (Abbas HK and Shier WT 2009). Human consumption leads to “mycotoxicosis,” which can result in mild symptoms, such as a weakened immune system, to severe hepatic, carcinogenic, and toxic damage (Williams JH and others 2004; Wild CP and Gong YY 2010). Animal consumption from feeds often results in sickly livestock with poor growth factors, or death in highly sensitized animals, such as turkeys or other birds (Bhat R and others 2010). Contamination of crops by fungi can occur at both the pre-harvest and post-harvest stage. The success of their colonization is dependent on a variety of factors, including substrate availability, quality of available substrate, plant

defenses, and opportunity (Bhat R and others 2010). The two most susceptible areas in plants for invasion are those already damaged from the natural environment or in the seeds (Abbas HK and others 2009).

Of all mycotoxins, aflatoxins are considered one of the most toxic metabolites and are regulated in over 100 developed countries (van Egmond HP and others 2007). In 1993, aflatoxin B₁ (AFB₁) was officially listed as a group I human carcinogen (IARC 1993). In the US, total aflatoxin levels in the food supply are required to be less than 20 ng/g, while in Europe, it is set at less than 4 ng/g (Gnonlonfin GJB and others 2013). In developing countries, aflatoxin contamination is a major problem, resulting from poorly enforced legal limits and limited infrastructure to reduce and detect fungal contamination (Groopman JD and others 2008). Consequently, in at least five different African countries, human subjects tested positive for aflatoxin markers at a rate of at least 98% (Gnonlonfin GJB and others 2013).

1.2.0 Aflatoxin

1.2.1 Environmental factors influencing field levels of aflatoxin

Aflatoxins are produced primarily by two species of *Aspergillus*: *A. parasiticus* and *A. flavus*. These metabolites are synthesized using cofactors and enzymes that are part of a polyketide pathway that overlaps and mimics steps of the lipid synthesis pathway (Sweeney MJ and Dobson ADW 1998). Colonization, secretion, and growth by these species on the peanut (*Arachis hypogaea L.*) thrive under harsh environmental conditions (Sweeney MJ and Dobson ADW 1998; Klich MA 2007). *Aspergillus* sp. may grow at temperatures between 10 - 43 °C, and a pH range of 2.1 – 11.2, with aflatoxin produced between 12 – 40 °C and a pH

of 3.5 – 8.0 (Wareing P and Fernandes R 2007). Aflatoxin may be synthesized by *A. flavus* at a water activity as low as 0.82 (Sweeney MJ and Dobson ADW 1998). Drought and heat exacerbate aflatoxin production, as these conditions weaken and damage plants, reducing their natural defenses (Sanders TH and others 1985; Holbrook CC and others 1994; Holbrook CC and others 2000; Girdthai T and others 2010). Furthermore, drought conditions are also associated with higher temperatures in the soil and leaves, which is correlated with increased aflatoxin production (Holbrook CC and others 1994; Guo BZ and others 2008).

1.2.2 Chemical properties of aflatoxin

The four most prevalent types of aflatoxin are named B₁ (AFB₁), B₂ (AFB₂), G₁ (AFG₁), and G₂ (AFG₂), (Figure 1.1). All are formed from a basic difuranocoumarin structure (Sweeney MJ and Dobson ADW 1998). The nomenclature was developed to stand for the color, blue or green, of photonic emission of the molecules after UV excitation.

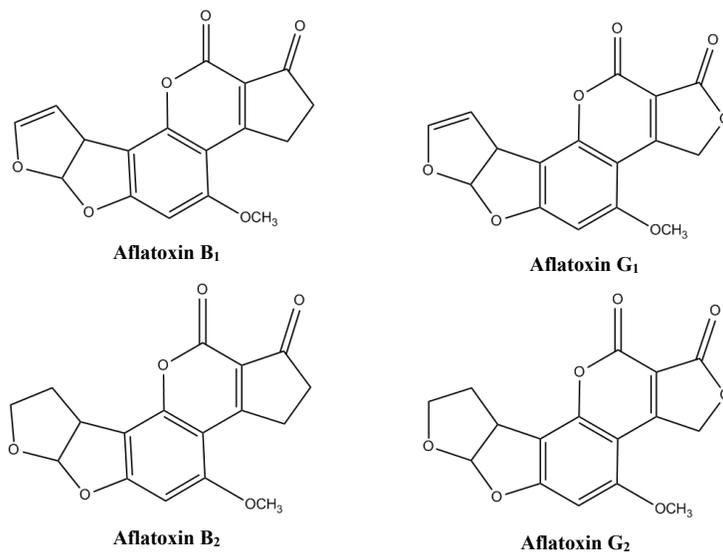


Figure 1.1. Structures of aflatoxin in its four primary forms.

1.2.3 Toxicology of aflatoxins

Aflatoxin was first identified for its toxigenic character during an outbreak of “Turkey X” disease in England in 1961 that was ultimately attributed to the presence of this compound in the peanut meal component of turkey feed (Richard JL 2008). Shortly thereafter, the first *in vitro* and *in vivo* characterization of the toxicity of these molecules was completed by Chang, Kader, Wick, and Wogen in 1963, where ducklings were fed doses of aflatoxin over eight days. Duck toxicity was evidenced by overall depressed growth and poor liver health, reflected by its reduced weight and the appearance of histopathologic lesions (Chang SB and others 1963). AFB1 has since been declared a group I carcinogen in 1993 by the World Health Organization (IARC 1993).

Of all aflatoxins, AFB1 and AFG1 are the most toxic (Khlangwiset P and others 2011). The toxigenic properties are derived primarily from the lactone ring. When aflatoxin was reacted with ammonia to achieve an open-ring structure, the derived metabolite, tested in chick embryos, was approximately 450 times less mutagenic and 18 times less toxic (Lee LS and others 1981). This may be chemically explained by aflatoxins’ susceptibility to form the highly reactive 8,9 epoxide (Wu Q and others 2009). Biologically, AFB1 may react in four different pathways (Figure 1.2): epoxidation (often initiated by the liver enzyme p450) and hydroxylation are likely to lead to highly toxic, mutagenic, and carcinogenic products, and O-dealkylation and ketoreduction are likely to result in non-toxic or mostly harmless byproducts (Wu Q and others 2009).

Aflatoxin M₁ (AFM1) is produced by the hydroxylation reaction (Wu F and others 2008) and may be secreted in animal byproducts. It is separately controlled by the FDA in

dairy milk to levels less than 0.5 ng/g (FDA 2005). The metabolites can react further and conjugate to DNA, forming adducts at the N⁷-guanine, or to proteins, particularly at methionine, lysine, and histidine sites, which results in toxicity (Roebuck BD 2004; Hanioka N and others 2012; Yang XJ and others 2012). Non-toxic byproducts generally only result if metabolites become conjugated to nucleophilic molecules, such as glutathione, though these events are not as kinetically favored (Wu Q and others 2009).

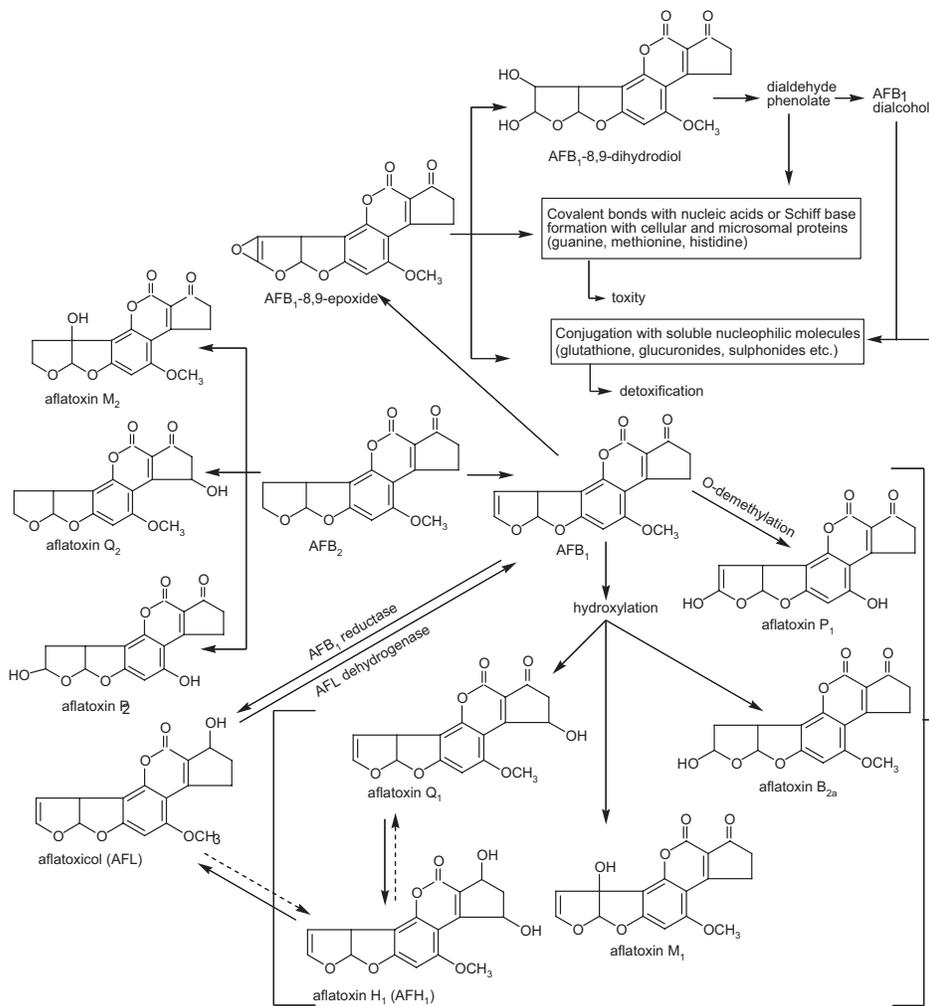


Figure 1.2. Possible pathways for aflatoxin degradation by biological metabolism (Wu Q and others 2009).

1.3.0 Aflatoxin Quantification Techniques

1.3.1 Purification of aflatoxin B1

Most protocols for quantification of aflatoxin rely on a series of steps to remove interfering compounds. One of the most popular cleanup procedures relies on antibodies to isolate aflatoxin from its matrix. Immuno-affinity columns use a gel scaffold to bind antibodies, which will subsequently bind aflatoxin as the crude material flows through. After washing, the aflatoxin can be eluted by a solvent for which it has a high affinity, such as methanol. The Journal of AOAC International has approved this method, originally proposed in 1990, for official use, and columns are commercially available, such as those under the name AflaTest® (Patey AL and others 1990; Trucksess MW and others 1991; VICAM 1999).

Other protocols validated for aflatoxin cleanup generally involve silica-gel columns. These were first approved for use by the AOAC in 1977 (Shotwell OL and Goulden ML 1977). Since then, these procedures have been streamlined to minimize the number of protocol steps and improve their efficiency. In one of the most recently published papers, Florisil™ columns were used to bind aflatoxins via magnesium cations embedded in a silica matrix. Background molecules in the crude extract are washed off with a series of three solvents, with decreasing polarity, ending with chloroform, before elution with an acetone/water/formic acid solvent (Sobolev VS 2007). It is important to note that during a study in this lab to optimize this protocol (data presented in Chapter 2), it was determined that this method may not be appropriate for highly contaminated peanut meal.

1.3.2 Fluorescence-based assays

Just as various cleanup columns take advantage of aflatoxins immuno-affinity properties, a variety of assays have been developed using this property to directly quantify aflatoxin. Enzyme-linked immunosorbent assay (ELISA) are commonly used in research and industry to quantify aflatoxin. These assays have a detection limit of about 1 ng/g total aflatoxin (Trombley A and others 2011). Generally, aflatoxin purified from a sample competes with an aflatoxin-horseradish peroxidase enzyme conjugate to bind to a well-bound aflatoxin-antibody. The wells are subsequently coated with anti-rabbit IgG binding antibody, and the amount of blue color produced (determined by an absorbance assay) is proportional to the amount of aflatoxin. In one of the most recent AOAC International ELISA protocols, peanut paste quantified using this protocol showed recoveries of 73-100%. Furthermore, the data correlated well ($R^2 = 0.99$) with high pressure liquid chromatography (HPLC) data. While the ELISA does offer speed and a low detection limit, it provides only a total aflatoxin value (Trombley A and others 2011). As this value depends on the sum of the fluorescence of all aflatoxin present, it is important that the standard contain a similar AFB1:AFB2:AFG1:AFG2 ratio to the sample, as this is a source of potential extra random error (Trombley A and others 2011).

In a similar type of analysis, a fluorometer can measure purified aflatoxin and report a total aflatoxin value (VICAM 2010). The VICAMTM fluorometer is one of the most commonly used instruments in the industry for its speed and ease, and takes advantage of the native fluorescent properties of aflatoxins. Unfortunately, due to the proprietary commercial nature of these instruments, they return values for aflatoxin on a wet meal basis directly and

do not provide the exact method/equation for calculating aflatoxin.

1.3.3 HPLC

Over the last two decades, numerous HPLC procedures to quantify aflatoxin have been published. HPLC is often preferred over other quantification techniques because it can measure each type of aflatoxin individually, versus a total aflatoxin count, and has improved sensitivity. Methods vary in the type of pre-column cleanup method, solvents used, type of HPLC column, and detection techniques. Recently, data from six inter-laboratory certification studies, of which three were conducted in the early 1990s and three in the late 2000s, for peanut butter, peanut meal, and animal feed studies were re-evaluated and compared by Garhard Buttinger, part of the Institute for Reference Materials and Measurements (IRMM), Joint Research Centre, European Commission (Buttinger G 2010). All methods showed virtually identical recovery rates, between approximately 70-110%, for both spiked and naturally contaminated substrate. Nearly all labs used reverse phase HPLC, with variation occurring in extraction solvent (methanol, acetonitrile, or chloroform based), cleanup column (immuno-affinity or silica-gel), and type of derivitization for detection (halogenation or tri-fluoroacetic acid). In peanut meal, the limit of detection improved ten times and the root mean squared deviation was approximately 30% better over the course of twenty years (Buttinger G 2010). The author concludes by stating that technology advancements across the field have improved reproducibility, sensitivity, cost, and efficiency (Buttinger G 2010).

An important innovation to HPLC aflatoxin methods was the advent of the photochemical reactor for enhanced detection (PHRED) system. This in-line post-

derivitization technique uses UV light to alter the aflatoxin structure to one that can excite at 365 nm and emit at 435 nm (Joshua H 1993). Unlike tri-fluoroacetic acid or halogenation derivitization, which disrupts the double bond of the dihydrofuran moiety by adding a hydroxyl group, this procedure is easier to carry out (though not necessarily more efficient) and minimizes the background matrix by exciting the aflatoxin at a relatively uncommon wavelength using a mercury lamp (Joshua H 1993). When compared to other derivitization techniques, such as bromination or iodination, there was no significant difference in reported aflatoxin values, and it was thus incorporated into AOAC methods in 2006 (Waltking AE and others 2006).

1.4.0 Control of aflatoxin in peanuts

Mycotoxin contamination of peanuts, like many legumes, is an unavoidable problem that may be managed through good agricultural practices and post-harvest methods. The success of these practices has made aflatoxicosis in humans an obsolete illness in most developed countries. However, the success of these practices is estimated to cost \$0.5 – \$1.5 billion each year in the US (Lamb MC and Sternitzke DA 2001) and represents yet another loss of food-grade protein.

1.4.1 Pre-harvest control of aflatoxin in peanuts

Pre-harvest control of aflatoxin in peanuts focuses on two main areas: plant breeding (section 1.4.2) and good agricultural practices. As described in section 1.2.1, the main environmental conditions affecting *Aspergillus* growth includes heat, drought and soil contamination. Farming advances to irrigate fields helps control aflatoxin contamination, however, it has been shown that certain types of irrigation, such as subsurface irrigation, can

in fact increase contamination instead of limit occurrence (Holbrook CC and others 1994). Harvesting early, before peak pod maturation, particularly in drought conditions, can be helpful if harvesting occurs before the aflatoxin is produced in the first place. In Australia, an aflatoxin risk-prediction website helps farmers choose the ideal harvest date based on environmental data (Queensland Government 2012). Furthermore, crop rotation of resistant crops (such as wheat) with peanut can help minimize *Aspergillus* from spreading year-to-year (Kabak B and others 2006).

1.4.2 Development of aflatoxin-resistant peanut lines

Plant breeding of peanut aims to create an *A. parasiticus* and *A. flavus* resistant plant. To date, no viable *A. flavus* resistant lines have been developed that are also able to meet growers' demand for high yield crops. Strategies have primarily centered on crossing certain lines that demonstrate good natural defenses against drought and other environmental stresses. These mechanisms include the upregulation of proteins that inhibit fungal growth and increased tryptophan production/uptake, which has been linked to limiting aflatoxin production (Holbrook CC and others 2000; Guo BZ and others 2008). Plants' natural anti-toxin metabolites, such as volatile aldehydes, certain anthocyanins, or antifungals like phytoalexins, may have the ability to suppress aflatoxin synthesis (Bhatnagar D and others 2006; Girdthai T and others 2010). Other ideas have focused on selecting for plants that naturally limit the amount of available lipid or free-tyrosine available, both of which have been shown to promote aflatoxin synthesis (Guo BZ and others 2008).

1.4.3 Post-harvesting control

Despite the best agricultural practices, some aflatoxin contamination is inevitable at harvest, especially in years with high heat and/or drought. Accordingly, post-harvest control practices are the next major stop point to preventing aflatoxin contamination in the food supply. While it is important to control pre-harvest contamination, it is as important to segregate those that have become contaminated. At peanut buying points, locations that farmers bring their crops for sales, all lots are sampled under USDA supervision and inspected visually for fungal contamination. Because of the difficulty associated with evenly sampling a lot, and the propensity for small, random pockets of high aflatoxin, about 200 lb. is sampled per load (Whitaker TB 2003). This sample is also used to determine the grade of peanuts. Any lot with even one peanut visually contaminated with fungus is entirely directed for oil stock. As damaged, small, and dislodged peanuts have increased risk for aflatoxin, screening by size and density is completed after lot-segregation by the sheller (Dorner JW 2008). Finally, the best technique to identify peanuts concentrated with aflatoxin is to blanch the nuts, and then sort kernels electronically post-shelling. This method can result in about a 90% reduction in aflatoxin (Cole R and others 1995). The composite of peanuts identified as aflatoxin contaminated, damaged, or of too small grade is diverted to oil stock. Aflatoxin does not fractionate in the food-grade oil produced, and is concentrated in the insoluble byproduct remaining. This material, referred to as peanut meal, is the low-oil, high protein (typically ~50% wt.) byproduct of this process.

It is also important to ensure that aflatoxin-negligent peanuts remain aflatoxin-free under warehouse storage conditions by ensuring that the moisture level of the seed is

maintained at safe levels. It has been determined that *A. flavus* will not invade peanuts in storage with a moisture of 7% or less (in equilibrium with a relative humidity of 70% or less) (Bulaong SSP 2002).

1.4.4 Chemical control of aflatoxin

A variety of chemical methods have been proposed to degrade aflatoxin to nontoxic end products. Ammonia application via ammonium hydroxide or gaseous ammonia to eliminate the lactone ring carbonyl group is a US industry approved technique for peanut meal, with a high pressure/high temperature method being most effective. Reaction products are considered safe for humans and animals, though it is used solely in animal applications (Park DL 1993). Other solvents have been shown in small-scale bench studies to degrade aflatoxin. In 1977, a survey of weak bases showed that nearly 100% degradation of aflatoxin in peanut protein isolate (extracted at pH 8) and protein concentrate (extracted at pH 4), with an initial natural contamination of 451 ng/g and 713 ng/g, respectively, could be achieved with 65-90% acetone, 0.5-1% benzoyl peroxide, 0.2 - 0.4% sodium hypochlorite, or 80% isopropyl alcohol solutions (Rhee KC and others 1977). Methylamine was found to be less effective, with the maximum solution (1.25%) tested only achieving about a six-fold reduction. Finally, while the chemicals tested generally showed the same degradation trend for peanut protein isolate and concentrate, testing with hydrogen peroxide showed major differences in the degradation of each substrate. Low levels of hydrogen peroxide (.5%) degraded 100% of aflatoxin in the concentrate, although under the basic conditions of the isolate, a solution of 2% hydrogen peroxide degraded less than half of available aflatoxin (Rhee KC and others 1977).

1.4.5 Processing control of aflatoxin

Although there are a number of options, due to high capital costs, very few processing techniques, besides ammoniation, are employed in industry for the directed purpose of degrading aflatoxin. Gaseous ozonation, for example, can achieve reductions in aflatoxin levels in peanut meal extremely quickly. After 10 minutes at 75 °C, peanut kernels (spiked at 20 ng/g) and peanut flour showed a 77% and 50% aflatoxin reduction, respectively (Proctor AD and others 2004). However, its disadvantages include cost and a dearth of toxicity studies related to its byproducts (Proctor AD and others 2004). Thermal applications are practical but the least efficient in independently achieving significant aflatoxin reductions. To achieve a 78% AFB1 reduction from a 237 ng/ml spiked sample, researchers had to hold peanuts at 150 °C for 120 min (Arzandeh S and Jinap S 2011).

One of the more successful processing operations to degrade aflatoxin for certain peanut products (such as meals or flours) is extrusion. The combined high heat and shear breaks the aflatoxin structure and conjugates it to non-toxic products. Using a single screw extruder, spiked peanut meal (480 ng/g) processed at a pH of 7.5 (20 % moisture) achieved greater than a 60% reduction in aflatoxin levels (Saalia FK and Phillips RD 2011). This study went on to show that at high alkalinity and low moisture (~25%), the addition of nucleophiles (lysine or methionine) to the system significantly increased the amount of aflatoxin degraded. They also demonstrated that calcium chloride increased the amount of aflatoxin degraded, to approximately 90% reduced. They hypothesized that calcium made aflatoxin interactions with native peanut meal proteins, facilitated through a nucleophilic attack, less enthalpically favored (Saalia FK and Phillips RD 2011).

Irradiation has also been considered for mycotoxin decontamination. Though there continues to be consumer backlash against irradiated foods, it nevertheless remains a safe tool up to an energy level of 5 MeV and 10 MeV for electrons or x-rays, respectively, generated from machines, to sterilize foods (FAO 2003). While many mycotoxin-irradiation studies have been published on other crops, few have directly involved the peanut. In one recent 2008 study, a 10^6 spore culture of *A. flavus* was inoculated on peanuts for a period of 10 days at 27°C and subsequently subjected to gamma radiation from a ^{60}Co radiation source (1.440 kGy/hr dose rate). At the maximum dose of 10 kGy, peanuts with an initial aflatoxin level of 16 ppm, saw a 58% reduction in aflatoxin levels (Ghanem I and others 2008). However, like that of ozone technology, radiation techniques tend to have high associated costs. Furthermore, radiation degradation of mycotoxin is highly dependent on the crop or food-source (Ghanem I and others 2008). Irradiation can also result in undesirable reactions in the peanut such as lipid oxidation (Mexis SF and Kontominas MG 2009).

1.4.6 Biological control of aflatoxin

In model systems, antifungals produced *in vitro* by a variety of lactic acid bacteria (LAB) and *Bacillus* sp. have been shown to be successful at decreasing the *Aspergillus* population grown on media. These antifungals include various peptides, polyphenols, and various acids (Kugler M and others 1990; Roy U and others 1996; Munimbazi CI and Bullerman L 1997; Munimbazi and Bullerman 1998). Generally, these antifungals are considered to be highly stable, and those from *Bacillus pumilus* were shown to maintain their structure/function up to 121°C for 15 min and between a pH of 2 - 10 (Munimbazi and Bullerman 1998). Furthermore, they are effective at low levels; 0.3 mg antifungals/ml of *A.*

parasiticus (10^6 - 10^7 spores per culture) inhibited aflatoxin production by 93% on culture plates (Munimbazi and Bullerman 1998).

Another strategy has focused on identifying bacteria and yeasts that can directly bind and sequester any aflatoxin secreted. Two strains of *Lactobacillus rhamnosus* were the first reported to behave through such a mechanism, and have thus been most thoroughly studied both *in vitro* and *in vivo* (El-Nezami H and others 1998). Aflatoxin binds directly to the cell wall polysaccharide and peptidoglycan, which, depending on the strain, can be a reversible or irreversible reaction depending on the solvent matrix (Haskard CA and others 2001; Lahtinen SJ and others 2004). Yeast has long been known to control against various mycotoxins through its role in beer fermentations (Scott PM and others 1995). Different species such as *S. cerevisiae* and *Candida krusei* were found to bind greater than 60% (w/w) of toxins *in vitro* (Shetty PH and Jespersen L 2006). Like LAB species, mycotoxins are predicted to bind to the yeast cell walls. However, yeast species are less strain-specific and bind primarily through their mannan components (Shetty PH and Jespersen L 2006).

Another biologically based strategy that has been investigated is competitive exclusion, an extremely promising recent advancement. The principle of this method, which has been successful with other crops such as cotton, is that other fungi, bacteria, and yeast that do not secrete toxins can outcompete *A. flavus* for resources (Dorner JW 2008). In peanuts, a number of select *Aspergillus* sp. lines have demonstrated effective control of *A. flavus* and *A. parasiticus* in the laboratory. However, only fieldwork can truly assess the effectiveness of this strategy. A recent April 2013 article evaluated the effect of applying a native Argentinian *Aspergillus* strain (AFCHG2) to peanut field fertilizer (50 kg/harvest)

over two years. In the treated versus untreated plot, 27% compared to 80% of isolates, respectively, were toxigenic at pod maturation. At harvest, however, this difference was eliminated, and both groups were equally contaminated. Only under drought and high heat field conditions did this treatment confer a significant protective effect, with strain-treated groups averaging 71% less aflatoxin (Alaniz Zanon MS and others 2013). This study demonstrated the aggressive nature of *A. flavus* strains under normal conditions, and showed that it can only be out-competed under harsh conditions. Though this line of research has been conducted in the hopes of identifying an effective biological control and eventually introducing “helpful” strains in the fields through the use of sprays and pesticides, this strategy has not seen widespread use to date.

1.5.0 Enteroabsorbant control of aflatoxin

Evidence of the efficacy of clay sorbents to remove AFB1 from substrates was demonstrated as early as 1979 (Masimango N and others 1979). These sorbents are most commonly used as feed additives, where they actively sequester aflatoxin in the gastrointestinal tracts of animals and are excreted in a bound form. The aflatoxin-clay complexes are biologically unavailable, preventing toxic effects (Kabak B and others 2006). Since that time, hundreds of papers have sought to study the safety of clays for animal and human use, synthesize more efficient sorbents, develop industrial scale processes, and use clays for animal feed improvement (Mishra HN and Das C 2003).

1.5.1 Chemical structure and binding characteristics of clays

Aluminosilicates are structurally the primary class of clay agents used to sequester aflatoxin from feeds. Within this class, a number of different groups exist, such as bentonites

and smectites. While all of these clays are primarily composed of montmorillonite (a type of phyllosilicate clay) and have high surface area, they differ in the type of ions they contain, the spacing of their molecular sheets, and the number of layers (West AR 1999).

Commercially, these clays are sold under names such as NovasilTM plus or Astra Ben 20A and generic names such as Na²⁺-bentonite or zeolite, for example. The basic structure of aluminosilicates is built from SiO₄ tetrahedra, and the Si:O ratio may be used to translate the precise conformation. Within the clay, certain Si⁴⁺ ions are substituted for by Al³⁺ ions and an additional ion such as Na⁺, K⁺, or Ca²⁺ that fills an open octahedral site to balance the charge (West AR 1999). Metal ions within the aluminosilicate clay chelate to the dicarbonyl groups of AFB1 (Arvide MGT and others 2008; Deng Y and Szczerba M 2011). While tangential binding to the clay surface does occur in a single layer, a majority of the AFB1 molecules are absorbed between the layers of smectite and thus are highly heat resistant and stable over a wide pH range (Arvide MGT and others 2008; Deng Y and others 2010). In hydrated smectite clay, as the aflatoxin binds in the interlayer space, about 50% of the ions migrate to the outer edge of the clay or into the solution, and the resulting clay that contains AFB1-H₂O bridged bonds is approximately 14% wider (Figure 1.3) (Deng Y and Szczerba M 2011). Finally, the type of cation used to dope the clay highly affected the aflatoxin binding ability of the clay based primarily on charge/radius ratio, with Mn²⁺, Mg²⁺, Ca²⁺, Na⁺, then K⁺ showing the strongest interactions.

Aluminosilicate clays have also been shown to associated with protein in a variety of vegetable protein aqueous extract systems. Proteins, much like aflatoxin, bind primarily to clays via electrostatic interactions. Due to the sensitivity of these interactions, absorbance

and desorption are heavily influenced by the cation with the clay and pH of the system (Harter RD and Stotzky G 1971; Ralla K and others 2010). Interestingly, whole proteins, versus peptides, were shown to bind with a higher affinity to these clays. Researchers proposed that large oligomers or aggregates could in essence prop open the clay and increase the amount of protein binding in the inter-layer space, versus surface region (Harter RD and Stotzky G 1971). However, they also saw that proteins of too large molecular weight could be limited in binding from steric hindrance (Harter RD and Stotzky G 1971). Furthermore, this effect was concentration dependent, with a 1:5 milk protein: clay ratio required to see binding (Harter RD and Stotzky G 1973).

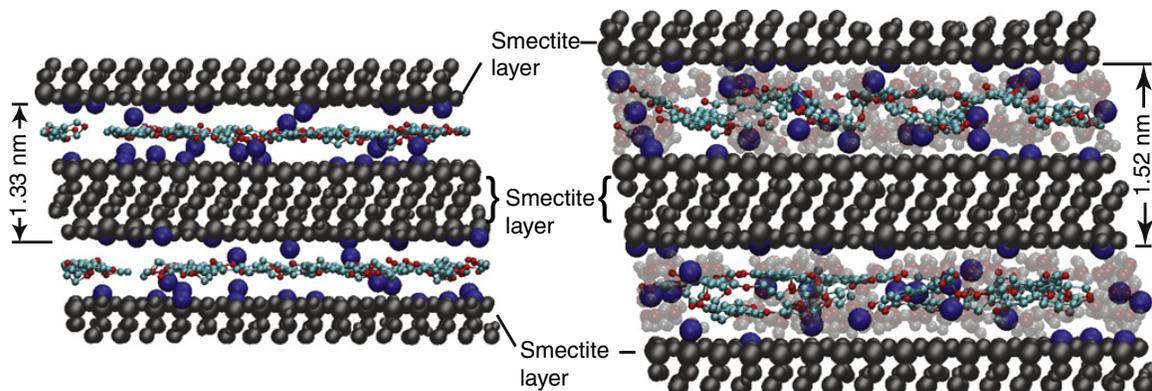


Figure 1.3 Model of a smectite clay binding aflatoxin in the (a) dehydrated and (b) hydrated form (Deng Y and Szczerba M 2011). Na^+ ions are in blue, oxygen molecules are in red, and water molecules are in grey.

1.5.2 Clay additives in animal feeds

Aluminosilicates and similar types of clays were originally introduced into animal feeds as a flow agent and are still used today for this purpose; however, it was quickly noticed that these additions resulted in animals that were less sickly and had better growth

performance (Jaynes WF and Zartman RE 2011). Even today, though clays are not federally approved for use in feeds as a growth agent, they are consistently added under the title of a flow agent to achieve these benefits (Jaynes WF and Zartman RE 2011). Of all animals, poultry and turkeys are most sensitive to aflatoxicosis. In 1987, a study by Timothy Phillips and others looked at the ability of clays to bind aflatoxin *in vitro* and within Leghorn chicks. It was showed that although various clays had different efficacy levels, of the 38 aluminas, zeolites, silicas, phyllosilicates, and modified-phyllosilicates tested *in vitro*, all significantly reduced the amount of ³H-AFB1 available from an aqueous solution (Phillips TD and others 1988). From this group, hydrated sodium calcium aluminosilicate (HSCAS), had reduction efficiencies of over 80%, and was selected for the *in vivo* chick studies. This was one of the first tests to show that not only did the clay improve body weight growth factors significantly, but it had anti-hepatocarcinogen effects; chicks treated with aflatoxin-feed supplemented with HSCAS had healthy livers versus those without, who had “friable and pale” livers (Phillips TD and others 1988). It also hinted at the remarkable ability of these clays to, as is now known, bind aflatoxin within the gut strongly enough to eliminate its bioavailability.

Other studies have looked extensively at the effects of clays using similar feed-based approaches in a wide range of animals, such as chickens, turkeys, dairy cows, goats, pigs, dogs, and rats (Ellis RW and others 2000; Bingham AK and others 2004; Pimpukdee K and others 2004; Afriyie-Gyawu E and others 2005; Thieu NQ and others 2008; Prvuloviae D and others 2009; Manafi M 2012). In one highly sensitive animal system, pregnant Sprague-Dawley rats were fed a control diet or a diet with 0.5% HSCAS over 20 days (Mayura K and

others 1998). Aflatoxin was introduced to one treatment group at 2 ppm by gavage between days 6 to 13. Maternal body weights and feed intake of HSCAS-treated rats were comparable to the control, whereas 64% of those fed diets with only aflatoxin died. Of those who survived, 50% had 100% embryonic resorptions, and of the group, only three fetuses, with significantly lower body weights, survived. None of these effects were seen in the group given feed containing AFB1 and HSCAS. Furthermore, all histological signs in the liver and kidney of aflatoxicosis were eliminated with inclusion of HSCAS. This paper lent further support to gastrointestinal binding of aflatoxin in the gut of the animals by HSCAS, as levels of AFM1 (a metabolite derived from AFB1 after processing in the liver) detected in the urine was significantly decreased (Mayura K and others 1998). A follow-up study in 2005 looked at the health effects of adding NovaSil™ independently to the diet of male and female Sprague-Dawley rats over a period of six months, the longest-term study in animals to date (Afriyie-Gyawu E and others 2005). The differences in organ weights and organ abnormalities at necropsy, various hematological parameters, and serum biochemistries were examined. While there were very slight significant differences found in certain serum biochemistries, none suggested that NovaSil™ could be considered to be toxic or harmful when ingested (Afriyie-Gyawu E and others 2005).

1.5.3 Use of aluminosilicates in humans

Using the evidence reported in the long-term rat trial as justification for human trials, in 2005, the results of a phase 1 clinical trial assessing tolerance to NovaSil™ clays in humans was the first of its kind to be published (Wang JS and others 2005). Ghana was selected as the location site as over 90% of its citizens suffer from chronic aflatoxicosis, it is

a peanut producing country, and it has large reserves of clay deposits (Phillips TD and others 2008). After two weeks, the clay was tolerated by all participants, with some gastrointestinal side effects in 25% of the group. Most importantly, hematological analysis of the blood and serum levels of vitamins A and E were not significantly different in patients pre and post study (Wang JS and others 2005).

As a follow-up, researchers conducted a 3-month double-blind placebo trial in 2007 in Ghana to further assess the biochemical effects of ingested clay on biomarkers of aflatoxicosis (Wang JS and others 2005). Participants were considered healthy, but all initially had serum AFB1-albumin adduct levels $> 0.5 \text{ pmol AFB1 mg}^{-1} \text{ albumin}$. After three months, a significant decrease in the biomarkers of aflatoxin exposure were documented for groups treated with a high dose (1.5 g/day) and low dose (.75 g/day) of NovaSilTM compared to a placebo group. Though some significant differences in blood chemistries were documented, such as overall levels of strontium and vitamin E in males, overall, the treatment was considered a safe and cost-effective way to treat aflatoxicosis arising from contaminated aflatoxin agricultural products in developing countries (Afriyie-Gyawu E, Ankrah NA, and others 2008; Wang P and others 2008).

1.6.0 Enzymatic control of aflatoxin

Enzymatic degradation of aflatoxin provides a promising alternative to more traditional processing methods, such as ammonia application, shunned in places such as Europe. White-rot fungus was selected for processing studies because of its ability to (1) synthesize metalloenzymes, such as manganese peroxidase (MnP) that have the capacity to oxidize complex aromatic compounds and (2) grow as a monokaryotic cell line with a high

capacity for genetic modifications in lab environments (Lomascolo A and others 2011). In 2010, Wang and others investigated the detoxification of aflatoxin by MnP. In pure solutions, about a 70% reduction in the concentration of AFB1 was achieved after ~13 hours of incubation at 5 standard units of catalytic activity of purified MnP (Wang J and others 2011). Likewise, the mutagenic activity, assessed using the umu operon test, was reduced approximately 50% in a 5 nkat or 70% in a 20 nkat MnP system (Wang J and others 2011). Earlier studies using horseradish peroxidase, a cheap and highly available enzyme, achieved 30-50% reductions in aflatoxin spiked peanut meal (Das C and Mishra HN 2000). While spiked studies are flawed in that they do not account for interaction effects between the matrix (primarily native proteins) and aflatoxin, they do provide a foundation for future work.

1.7.0 Peanut meal

Aflatoxins, like many mycotoxins, affect a range of crops from legumes to maize to tree nuts, but constitute a large problem in peanuts (Abbas HK and Shier WT 2009). In the US, damaged and contaminated nuts are diverted for oil crushing and extraction, and it is estimated that only 1-3% of the original peanut aflatoxin content fractionates into peanut oil, which is subsequently removed during refining, leaving a valuable, high quality lipid product (Natarajan KR and others 1975). In the crushing/extraction process, the insoluble byproduct, composed of high protein, non-lipid solids, are enriched in any aflatoxin present in the oilstock, and these solids are referred to as peanut meal; this material is used in feeds or fertilizer, depending on the aflatoxin levels; its market price is directly proportional to its application and hence its aflatoxin content. Peanut meal is a typically low-fat (1-3%), low

moisture (~10%), and high protein (~50%) material. Our lab and others have developed a number of techniques, reviewed in section 1.7.3, to add value to peanut meal via processing to isolate protein while minimizing aflatoxin.

1.7.1 Peanut/Peanut meal proteins

Peanuts contain a distribution of proteins grouped generally by size and solubility into two fractions, albumins and globulins. Within the globulin family, proteins are grouped into the arachin or conarachin family (Arthur JC 1953). Peanut proteins are considered to have a good amino acid profile, though lacking in methionine and lysine, and will precipitate at a pH of approximately 4.5 (Pattee HE and Young CT 1982).

1.7.2 Peanut meal protein and aflatoxin

Sequestration of aflatoxin from peanut proteins is hindered by the natural affinity of peanut proteins for aflatoxin (Mishra HN and Das C 2003). As already mentioned in the discussion regarding the toxigenic and carcinogenic properties of aflatoxin, it has a high affinity for histidine, methionine, and lysine sites (Roebuck BD 2004). Specifically in peanuts, direct interaction of aflatoxin with the arachin, conarachin I and conarachin II fractions has been detected *in vitro* at temperatures as low as 26°C with binding constants between 3.5-23 M⁻¹ (Monteiro PV and others 1996)

1.7.3 Value-added processing of peanut meal

Extensive work has been completed in our lab to isolate enzymatically hydrolyzed peanut protein from peanut meal while simultaneously sequestering aflatoxin with aluminosilicate clays in an effort to add value to peanut meal (Seifert LE and others 2010; Oakes AJ and others 2012; White BL and others 2013). Peanut meal aqueous dispersions

were prepared with commercial brand AB20 clay (for aflatoxin binding) and proteases (to maximize the amount of soluble protein/peptide). From this dispersion, two phases were prepared: an insoluble phase containing aflatoxin bound AB20 complexes and other insoluble constituents, and a soluble phase of protein/peptide, dissolved nutrients, and some remaining free aflatoxin. With this method, Seifert and others (2010) reduced the level of available aflatoxins from an initial 110 ng/g to levels less than 20 ng/g in the final soluble protein hydrolysate rich extract on a dry weight basis (Seifert LE and others 2010). As the aflatoxin reduced hydrolysate fraction is most valuable, Oakes and others (2012) built on this work and developed a spray-dry process to isolate the hydrolysates in a powder form. Maltodextrin was required for processing reasons and powders with up to 26.4% protein were achieved (Oakes AJ and others 2012). Further work by White and others (2012) investigated the adaption of this method at the pilot scale for contaminated peanut meal (191 ng/g) (Figure 1.4) (White BL and others 2013). Aflatoxin levels were reduced to less than 20 ng/g for all soluble fractions, with NovaSil™ significantly more effective in sequestering the toxin compared to AB20. Turkey feed studies are currently under investigation to understand the feed potential of the dried insoluble fraction (aflatoxin levels equal to ~20.8 ng/g) compared to an aflatoxin contaminated starting feed supplemented with clay additives.

1.8.0 Conclusion

Peanut meal is an excellent source of protein, but the typically high aflatoxin levels associated with this material limit its applications. In the first chapter of this thesis, work to improve the quantification of aflatoxin by HPLC from highly contaminated peanut meal will be studied. In the second chapter of this thesis, a process to isolate whole protein fractions

from peanut meal while controlling aflatoxin with clay enteroabsorbants and enzymes will be explored. It primarily differentiates itself from previous work in our group by focusing on whole protein isolations versus hydrolysates. Such whole protein concentrates/isolates offer different functionality benefits and improved isolation and concentration processing properties compared to hydrolysates. However, whole protein isolations do lead to new solubility and optimization challenges. Furthermore, by focusing on whole protein isolations, this work will provide new insights into fundamental interactions among peanut protein and aflatoxin.

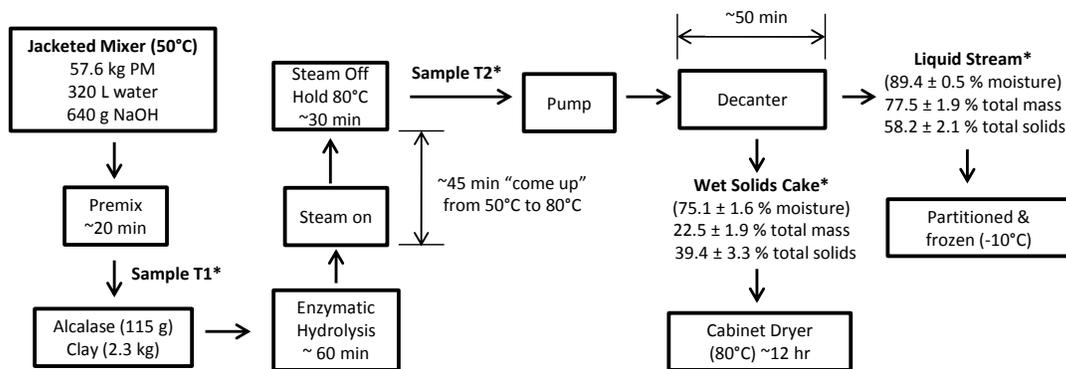


Figure 1.4. Diagram of pilot-scale process to extract protein hydrolysates from peanut meal (White BL and others 2013).

1.9.0 Research Objectives

The goals of the following work are as follows:

- 1) Improve purification and extraction methods of aflatoxin from highly contaminated peanut meal to increase recovery rates of aflatoxin.
- 2) Optimize a process to extract whole protein and minimize aflatoxin from aflatoxin-

enriched peanut meal.

- 3) Characterize the soluble protein-rich extract to gain insight into protein-aflatoxin interactions.

CHAPTER 2: QUANTIFICATION OF AFLATOXIN: METHOD DEVELOPMENT

2.1.0 Introduction

Aflatoxins are regulated in over 100 developed countries to levels between 1 – 30 ng/g on a dry weight basis due to their toxic and carcinogenic nature (van Egmond HP and others 2007). It is therefore vital that crops be sampled and accurately quantified for aflatoxin content prior to entering the food supply. In peanuts, where aflatoxin contamination can be rampant under harsh, drought-like environmental conditions, methods for quantification have been developed and refined over the last 50 years (Lamb MC and Sternitzke DA 2001). In developed countries, an elaborate infrastructure functions to successfully segregate contaminated peanuts from the food supply, and those peanuts are diverted to oilstock. Peanut meal, the insoluble byproduct from peanuts directed for oil fractionation, is enriched in aflatoxin because aflatoxin does not fractionate with the lipid layer. This material is valuable as it contains ~50% protein by weight; however, it too must be accurately quantified for the amount of aflatoxin present is correlated with its application. Peanut meal with aflatoxin levels less than 20 ng/g required in the most sensitive or selective animals, such as dairy cows or broilers, whereas with concentrations greater than 300 ng/g it is commonly directed to fertilizer.

The Journal of AOAC International has published many officially approved methods for aflatoxin quantification, with the most common including high pressure liquid chromatography (HPLC) and fluorometry analysis. Methods for quantification of any contaminated material begin with extraction of aflatoxin in acetonitrile, methanol, or, more rarely, chloroform solvents (Moller TE and Nyberg M 2004). These extracts are subsequently

loaded onto clean-up columns to remove the background matrix. The two most common types of purification columns are silica-gel based or immuno-affinity based. In the former, cations (generally magnesium) embedded in the silica matrix bind aflatoxin as it flows through the column (Sobolev VS 2007). In the latter, antibodies are used to select specifically for the four primary types of aflatoxin (B₁, B₂, G₁, G₂) (Kussak A and others 1995). For HPLC methods, purified aflatoxins can be separated into their individual components on a column, derivatized with trifluoroacetic acid, bromine, or UV light into compounds that fluoresce at desirable wavelengths (Lupo A and others 2011). Alternatively, aflatoxins can be quantified in their totality using a fluorometer, without separation on a column or derivitization, since they naturally fluoresce at a wavelength of 365 nm (VICAM 1999). Fluorometers are commonly used throughout the US industry, though they have more variability and error than HPLC procedures (Buttinger G 2010).

Despite an abundance of standardized AOAC approved procedures, a literature survey revealed that numerous studies deviate from these standards. Consequently, variability can be found among a number of key isolation and analytical procedures for aflatoxin. The main body of work presented in this thesis focuses on processing of peanut meal to isolate whole protein and remove aflatoxin, and thus accurate quantification of aflatoxin was vital. To ensure the best results, a new HPLC detected to aflatoxin analysis was obtained in the lab, and, as a result, verification of all aspects of existing aflatoxin quantification procedures was warranted. Variables considered included the extraction solvent, the solvent to peanut meal ratio, the purification procedure, and the HPLC conditions. The following data documents validation procedures and improves on current extraction and

purification methods.

2.2.0 Results and Discussion

2.2.1 Extraction ratio of solvent to peanut meal

The extraction ratio of solvent to peanut meal is expected to have a large effect on the amount of aflatoxin quantified. As small molecules have a saturation concentration, increasing the volume of solvent to peanut meal would be expected to increase the amount of aflatoxin detected until the maximum aflatoxin extracted reached a plateau. In the literature, it is most common to find papers using a 2:1 or 4:1 extraction solvent, and less common to see a 1:1 or 6:1 extraction method. The 2:1 and 4:1 extraction ratios were compared using 80% aqueous methanol as the extraction solvent. Aflatoxin was quantified using a VICAM fluorometer (Watertown, MA) because the HPLC protocol was not yet developed. The AflaTest® (Watertown, MA) column, a commercial immuno-affinity column, recommended by the VICAM manufacturer was employed to purify the aflatoxin from the matrix (VICAM 1999). The 4:1 solvent:peanut meal ratio extracted significantly more aflatoxin than the 2:1 system (Table 2.1). While this was a relatively simple experiment, it emphasizes the importance of the extraction ratio. It is concerning that studies are published using 2:1 or 1:1 solvent: meal ratios, particularly in highly contaminated peanut meal, as they could be underestimating the aflatoxin present. While the extraction ratio may not be as relevant for peanuts with low levels of contamination (< 20 ng/g), this data indicates that it is a critical parameter in certain systems.

Table 2.1. Effect of 80:20 methanol: water solvent: peanut meal ratio on aflatoxin quantification (n=4).

	2:1 ratio	4:1 ratio	P-value ($\alpha=.05$) using Paired 2-way T-test
Aflatoxin (ng/g)	158.0	218.5	.01*

2.2.2 Optimization of a silica-gel clean-up procedure for HPLC analysis

A silica-gel clean-up procedure was selected for purification of aflatoxin prior to HPLC analysis due to its speed and economic advantages compared to the immuno-affinity column procedure. The procedure followed initially was as is reported in Sobolev and others 2007. Briefly, 1 ml of extract was loaded onto the column, pulled through drop-wise by vacuum, washed with solvents of decreasing polarity, and eluted with 1 ml of acetone/formic acid/water solvent. The eluent was evaporated in glass vials under nitrogen stream and resuspended in 63:37 water: methanol prior to injection on the HPLC.

Comparison of aflatoxin values calculated by the VICAM fluorometer after AflaTest® purification and by the HPLC after Florisil™ purification revealed that recovery on the HPLC was significantly diminished. To try and account for these losses, the clean-up procedure (Sobolev VS 2007) on the Florisil™ column was examined at each stage. First, a significant increase in the amount of aflatoxin detected was observed when the amount of eluent used on the silica-gel column was tripled from 1 ml to 3 ml. Secondly, the flow through was collected and quantified to determine if any aflatoxin was being washed off the column. The mean aflatoxin detected in the starting peanut meal was significantly higher in samples calculated with the addition of the flow-through (Figure 2.1). While it is possible that the column was over-saturated, levels used in these experiments were equivalent to those

reported by Sobolev (2007). Work is underway to confirm these results with mass spectrometry and to determine the precise saturation limit of the column. Finally, the loss of aflatoxin during the evaporation step was considered. It has been reported in the literature that aflatoxin has a higher affinity for glass than non-polar solvents, and, without a silicanized coating, will stick to glass vials (VICAM 1999). In the Sobolev (2007) procedure, this step was completed with standard glass vials. When pure aflatoxin standard mix (Sigma-Aldrich, St. Louis, MO) was evaporated in these glass vials, resuspended, and quantified, losses of up to 20% were observed (Figure 2.2).

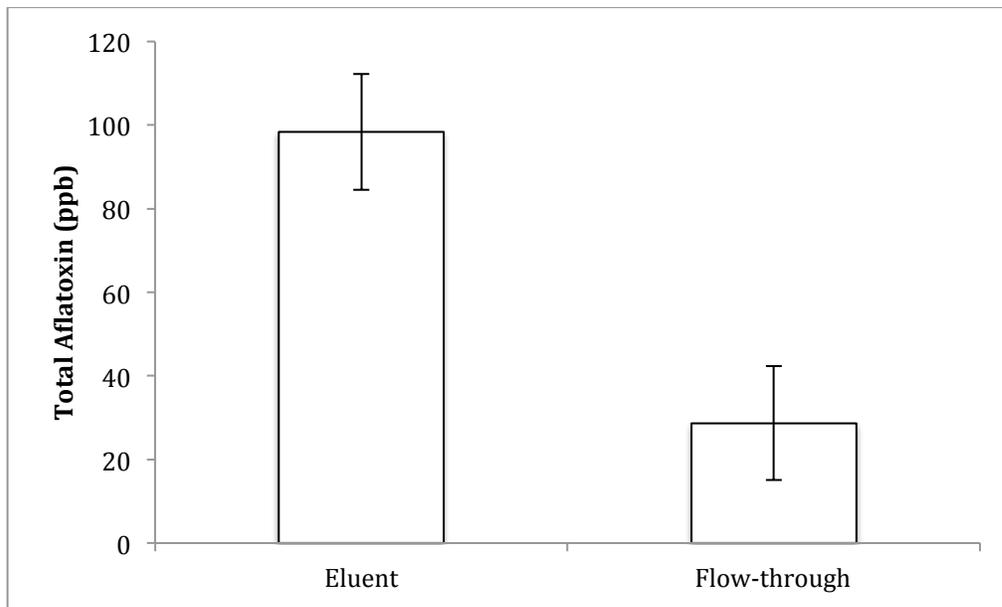


Figure 2.1 Comparison of flow-through and eluent fractions generated from clean-up of peanut meal extract using FlorisilTM columns for aflatoxin analysis (n=6). 1 ml of crude extract was loaded onto columns and pulled through using a solid phase extraction vacuum manifold. Columns were washed with .5 ml of methanol: water (80:20, v/v), 0.5 mL of methanol, and then 0.5 mL of chloroform: methanol (90:10, v/v). These components comprised the “flow through,” that were then evaporated with a nitrogen stream at 45 °C and resuspended in 2 ml of methanol. The aflatoxin was eluted from the column with 3 ml of acetone/water/formic acid (96:3.7:0.3, v/v), evaporated with nitrogen, and resuspended in 2 ml of methanol. Samples were quantified on the HPLC.

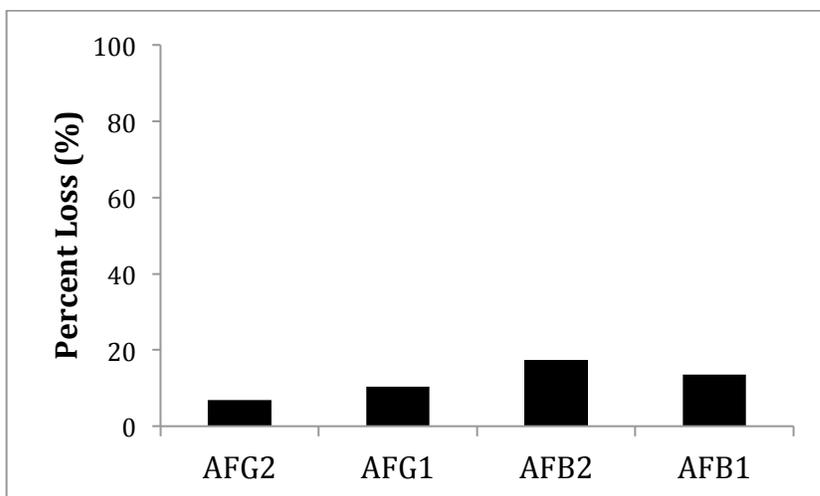


Figure 2.2. Losses of standard aflatoxin-mix post-evaporation and suspension in 63:37 water: methanol quantified on the HPLC (n = 6).

2.2.3 Optimization of an immuno-affinity clean-up procedure for HPLC analysis

Due to the large quantification errors originating from multiple sources in the Florisil™ column procedure, a new clean-up procedure using the commercial immuno-affinity column AflaTest® was investigated. The type of extraction solvent used to extract aflatoxin was considered. In the VICAM test manual, extraction with 80:20 methanol: water is recommended, and thus was used previously. However, 60:40 acetonitrile: water has been reported as a superior extraction solvent in the literature (Moller TE and Nyberg M 2004). Acetonitrile was confirmed as a more efficient solvent (Figure 2.3). The analysis also revealed new effects related to the load volume and dilution of the extract in the case of both acetonitrile and methanol (Figure 2.3). It was observed that in samples with no aqueous dilution, the quantity of aflatoxin in the extracts was highly dependent on the amount loaded. With a dilution of at least 2.5x, this load volume effect was eliminated and significantly more aflatoxin was detected. This suggests that the AflaTest® column matrix is highly sensitive to

the effects of a relatively non-polar solvent, such as acetonitrile, being passed through it. Mechanistically, a non-polar solvent could decrease the free energy change of aflatoxin binding to the scaffolded antibodies. Practically, this means that perhaps the most important consideration related to purification procedures is the extract dilution, versus the total amount of aflatoxin.

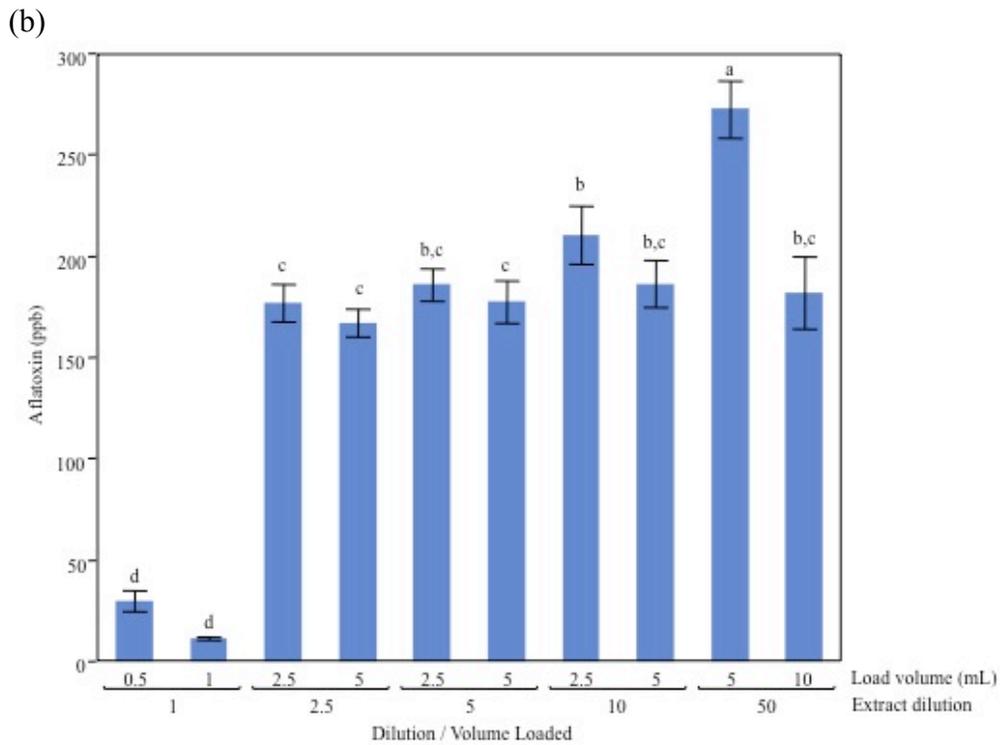
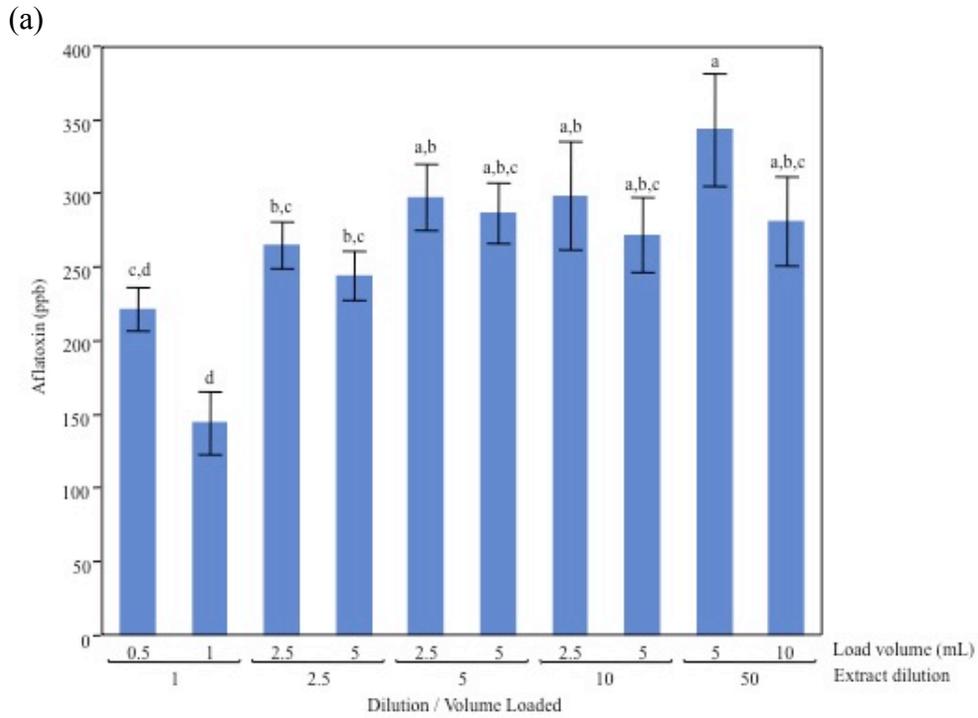


Figure 2.3. Effect of load volume and dilution of (a) 60:40 acetonitrile: water and (b) 80:20 methanol: water extracts purified on an AflaTest® column and quantified by HPLC.

2.2.4 Degradation of aflatoxin samples in the HPLC

Even with the HPLC procedure optimized for extraction ratio, type of solvent, and clean-up column parameters, aflatoxin concentration was highly variable based on the time of analysis. To see if degradation was occurring as a function of time and affecting the standard curve, the standard aflatoxin mix was quantified between 0-24 hours (Figure 2.4). Significant degradation was seen nearly immediately for the AFG1 and AFG2 samples, with degradation starting for the AFB1 and AFB2 samples at approximately four hours. A literature search revealed that this had been previously reported and suggested adding acetic acid at a 1% concentration to counteract this problem. The addition of acetic acid did prevent degradation of all aflatoxins over 24 hours. Standards run with acetic acid on the HPLC had a standard curve with a fit of 0.9999; previously, low and high concentrations of standards could not fall on the same curve. A survey of current aflatoxin methods suggested that well over 50% of quantification procedures do not specifically report adding acetic acid to samples prepared in HPLC buffer. It is unclear if this is a lapse in methods or documentation.

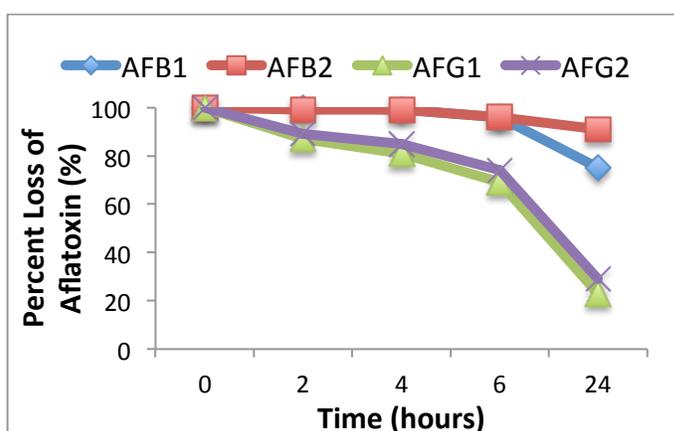


Figure 2.4. Degradation of standard mix in 63:37 water: methanol with time.

2.3.0 Conclusion

Ultimately, the procedures to optimize aflatoxin extraction and purification pre-HPLC quantification were successfully standardized and produced highly reproducible, accurate, and sensitive results. However, the variability in procedures throughout the literature is a cause for concern. This work reported new findings on the recovery of aflatoxin related to the extraction solvent: peanut meal ratio, AflaTest® extract load volume, and AflaTest® extract dilution and confirmed results regarding the best extraction solvent and the preventative effects of acetic acid on aflatoxin degradation. By presenting a holistic view of extraction and purification procedures of aflatoxin from peanut meal, this paper hopes to clarify some of the variability found among aflatoxin HPLC method papers. It also emphasizes the necessity of careful optimization and validation of procedures to ensure accurate and consistent aflatoxin measurements.

CHAPTER 3: EXTRACTION AND CHARACTERIZATION OF PROTEIN AND AFLATOXIN FROM PEANUT MEAL USING RESPONSE SURFACE METHODS

3.1.0 Introduction

Plant protein is becoming increasingly important world-wide as the growing energy costs of meat-based protein are placing an ever increasing strain on the world's stressed food supply (Aiking H 2011; de Boer J and Aiking H 2011). In most developing countries, peanuts serve as one of the most important protein sources and play a large role in the agro-economy (Prasad PVV and others 2010). Of the global peanut supply, 75% is crushed for oil due to worldwide economic demand (Prasad PVV and others 2010). In the U.S., peanuts selected for oil crushing are typically damaged, poorly sized, and/or contaminated by aflatoxin (Dorner JW 2008). Peanut meal, the byproduct of this process, while an excellent source of protein, becomes enriched in any aflatoxin present and is therefore limited to applications such as animal feed and fertilizer. A typical peanut meal contains 50% protein and very low levels of residual oil, making it an excellent candidate for processing to extract the protein, degrade or remove the aflatoxin, and overall add value to this product.

Previous work in our lab has shown that protease hydrolyzed protein can be extracted efficiently with water from highly contaminated peanut meal. Specialized aluminosilicate clays were added to these extractions to remove aflatoxin that was simultaneously solubilized, however, some residual aflatoxin remained in the soluble hydrolyzed protein fractions (Seifert LE and others 2010; Oakes AJ 2011; White BL and others 2013). These specialized aluminosilicate clays have high surface area and sequester and render aflatoxin biologically unavailable by binding aflatoxin via electrostatic interactions in the interlayer

space (Deng Y and others 2010). In a pilot scale process using a starting peanut meal aflatoxin concentration of 191 ng/g, the maximum reduction in aflatoxin in the soluble phase was achieved using 4% NovaSil™ (a commercial clay sorbent), giving a final aflatoxin concentration of 21 ng/g on a dry weight basis (White BL and others 2013). Further refining of the soluble fractions was required to reduce aflatoxin to levels less than 20 ng/g, which could be suitable for sensitive applications, such as dairy cattle feed.

The following work looks at maximizing the extraction of whole protein from aflatoxin-contaminated peanut meal. Whole protein, unlike hydrolysates, is significantly more difficult to solubilize, yet confers a number of different advantages. In this form, protein may be isolated more easily through techniques such as isoelectric precipitation or ultracentrifugation. Spray-drying of whole protein, versus hydrolysates, may require fewer aids, such as maltodextrin, and result in a more concentrated product (Ahmed EM and Schmidt RH 1979). Furthermore, whole protein can have different functional properties versus hydrolysates, such as improved foaming or surface activity abilities (McWatters KH and others 1976; McWatters KH and Cherry JP 1977; Kim N and others 1992; Zhao G and others 2013). Hydrolysates are also known to produce bitter tastes that hinder their use in high levels in feeds (Fujimaki M and others 1970). Finally, analysis of a system containing whole protein and aflatoxin may shed added light onto their chemical interactions. To date, very few, if any, studies have specifically evaluated aflatoxin binding within the complex peanut matrix, and have instead focused on interactions of aflatoxin with biological protein (such as liver protein p450 or gut proteins) (Yang XJ and others 2012). Accordingly, optimization of a process to maximize extracted protein and minimize extracted aflatoxin

from highly contaminated peanut meal will be accomplished using response surface analysis of a central composite design study with four factors: percent calcium chloride, percent clay, pH of the dispersion, and time for extraction.

The percentage of adsorbent clay used in aqueous-based aflatoxin systems has been shown to positively and linearly correlate with the percentage of aflatoxin sequestered (Seifert LE and others 2010). As these clays are thought to bind aflatoxin irreversibly, their efficacy is limited primarily by the number of binding sites available (Deng Y and others 2010). For this study, the commercial clay NovaSil™ was selected for a number of reasons. First, compared to other commercially available options, NovaSil™ has been shown to bind aflatoxin with an extremely high efficiency (Phillips TD and others 1988; Seifert LE and others 2010). Secondly, NovaSil™ has an extremely thorough and positive safety record throughout the literature. A Web of Science (Thomson Reuters) search (October 2013) with “aflatoxin and NovaSil™” as the keyword returns at least 34 studies. It was the clay of choice for a short-term study on pregnant rats (a highly sensitive model) and for the sole long-term study with male/female rats examining the effect of clay added to the diet over six months. Most importantly, it was safely and effectively used in a human study during which participants were fed clay to ameliorate existing aflatoxicosis (Afriyie-Gyawu E and others 2005; Afriyie-Gyawu E, Ankrah NA, and others 2008; Afriyie-Gyawu E, Wang Z, and others 2008). Finally, while clays may only be technically marketed as flow agents in feeds according to the current FDA regulations, the state of Texas recently passed a bill permitting NovaSil™ specifically to be used as an “aflatoxin binder” (Office of the Texas State Chemist 2011).

Calcium chloride has been shown in a number of studies to influence the amount of aflatoxin sequestered by aluminosilicate clays or degraded in processing steps such as extrusion (Deng Y and Szczerba M 2011; Saalia FK and Phillips RD 2011). Like any ion, calcium will fill the interlayer space of clays and greatly influence its electrostatics (West AR 1999). Calcium is divalent and has a good charge to size ratio, enhancing the clay's coordination to aflatoxin compared to monovalent ions (Deng Y and Szczerba M 2011; Jaynes WF and Zartman RE 2011). In one experiment in which high-aflatoxin peanut meal was extruded, calcium was thought to potentially directly disrupt the interactions between aflatoxin and peanut proteins (Saalia FK and Phillips RD 2011). The effects of calcium on clay electrostatics as well as protein interactions could benefit a clay/peanut meal system by increasing the probability of clay encountering free aflatoxin and potentially enhancing the binding efficacy of the clay. However, calcium is known to cause protein precipitation above the isoelectric point (pH ~4.5), and this effect must also be considered.

Selections of the final two variables, pH and time, are for reasons a bit more straightforward. Time will directly influence the amount of protein that can be extracted, as well as the amount of aflatoxin that can enter into the aqueous solution. Thus, the ideal time to maximize protein and minimize aflatoxin will be a fine balance between the two conflicting goals. Furthermore, minimization of extraction time is desirable in a process that has the potential to be adapted to industry. pH is a variable known to influence all aspects of the process. In terms of peanut protein, generally, as the pH moves farther away from the isoelectric point (~pH 4.5), an increase in solubility is observed, with peanut protein being 5-11% more soluble at a pH of 9 versus 7, for example (Pattee HE and Young CT 1982).

However, in the high alkaline regions, peanut protein is at risk for alkaline hydrolysis. Extreme alkaline extraction can also result in racemization or derivitization of amino acids, such as the formation of lysinoalanine (Damodaran S and others 2008). Likewise, pH may have a large effect on the form in which aflatoxin is extracted. Although the ability of aflatoxin to be degraded under alkaline conditions has only been shown in corn studies related to the nixtamalization process, there is no reason to expect a different trend in peanuts; this degradation is selectively irreversible (Anderson RA 1983; Dearriola MD and others 1988; Mendez-Albores JA and others 2004).

While enteroabsorbants are an efficient and safe first step to reduce aflatoxin during protein extractions, it is currently not possible to completely eliminate aflatoxin using this approach while extracting a high quantity of protein. Enzymatic degradation of aflatoxin shows promise for its efficiency, cost-effectiveness, and safety (Wong DWS 2009; Lomascolo A and others 2011). For this study, manganese peroxidase (MnP) from white rot fungus (*P. chrysosporium*) was evaluated for reduction of aflatoxin in peanut meal extractions (Figure 3.1). The end-products resulting from this type of aflatoxin enzymatic degradation have been verified as non-toxic (Wang J and others 2011). MnP has long been regarded for its capacity to degrade branched, phenolic compounds and has thus been extensively studied (Wong DWS 2009). Like other lignolytic enzymes, MnP contains the heme group iron protoporphyrin IX, which facilitates the oxidation of Mn^{2+} in the overall reaction:
$$2Mn^{2+} + 2H^+ + H_2O_2 = 2Mn^{3+} + 2H_2O$$
The enzyme is 46 kDa and is comprised ten main α -helices, stabilized by five disulfide bonds, that help correctly position the Mn^{2+} binding site (Figure 3.2) (Wong DWS 2009).

MnP is dependent on manganese and requires multiple cofactors to reach peak efficiency (a redox potential of ~ 0.8 V at pH 4.5) (Wong DWS 2009). Certain α -hydroxy acids and protein can increase the efficiency of MnP, with malate and lactate being most efficient; this activity is sensitive to concentration and peaks at approximately 50 mM, with activity decreasing on either side (Glenn JK and Gold MH 1985). In one example of a protein acting as a cofactor, MnP reached maximal activity with 3 mg/ml of gelatin in solution (Glenn JK and Gold MH 1985). Selection of optimal levels for cofactors can be dependent on the substrate for MnP. Hydrogen peroxide (H_2O_2) is necessary in some quantity to facilitate the initial oxidation of Mn^{2+} . In a series of experiments on different dyes, peak activity was established from anywhere between 0.2 – 1 mM H_2O_2 (Aitken MD and Irvine RL 1990; Young L and Yu J 1997). Likewise, while it is known that this enzyme works optimally at a pH of 4-5, its functionality at higher pHs is highly dependent on the conditions of the system (Young L and Yu J 1997).

Peroxidases are highly versatile enzymes and have been shown to have unique functionality with various proteins. Though experiments to polymerize proteins have not been done with manganese dependent peroxidase directly, other peroxidases in its family, such as horseradish peroxidase (HRP), have been shown to have this capacity. This was first shown in a simple system where poly-tyrosine chains of up to 12,000 monomers were formed from a buffered solution with HRP (Fukuoka T and others 2002). Application of this technology has been so successful that in 2005, it was demonstrated that this process could be used with HRP to create the coating for a heart valve made of the hyperbranched polymer poly(hydroquinone) and poly(resorcinol) (Benson AM and others 2005). Likely due to this

protein polymerization property, peroxidase has been shown in peanut systems to affect the allergenicity of proteins. After treatment of protein extracts from roasted peanuts with peroxidase in phosphate buffer at a pH 8, Ara h1 and Ara h2 visualized on an SDS-PAGE gel saw a significant loss in band intensity (Chung S-Y and others 2004). This coincided with the appearance of large molecular weight bands likely corresponding to the formation of polymers. To assess changes in allergenicity, IgE binding western blots and competitive IgE binding ELISA assays were conducted and revealed a significant loss in allergenicity in the treated samples (Chung S-Y and others 2004).

The potential for MnP to be an inexpensive, highly effective means of degrading aflatoxin in the soluble extracts while producing non-toxic end-products made it a logical selection as a secondary tool to maximize the value of the extracted protein. While it is unclear if MnP will demonstrate polymerization and anti-allergenic properties in this system, those potential added benefits set it apart from other methods.

The goal of the following work is to optimize the extraction of an aflatoxin-minimized protein extract from peanut meal using response surface methods and assess the efficacy of MnP aflatoxin degradation as a function of time and pH in a high protein/high aflatoxin and low protein/low aflatoxin peanut meal extracted soluble system.

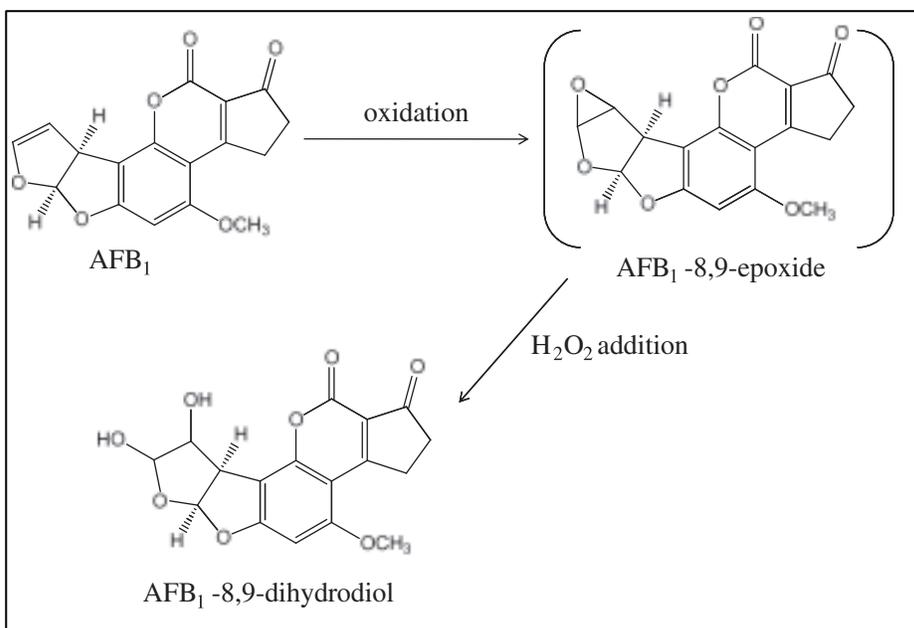


Figure 3.1. Proposed mechanism of AFB₁ degradation by MnP (Wang J and others 2011).



Figure 3.2. Ribbon diagram of MnP (Wong DWS 2009).

3.2.0 Methods

3.2.1 Materials

All chemicals used were analytical grade. Calcium chloride (CaCl_2), manganese sulfate (MnSO_4), hydrogen peroxide (H_2O_2), malic acid, and manganese peroxidase from white-rot fungus (*Phanerochaete chrysosporium*) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Hydrochloric acid (HCl), sodium hydroxide (NaOH), acetic acid, acetonitrile, and methanol were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Defatted peanut meal was gifted from Golden Peanut Co. (Alpharetta, GA, USA). NovaSil™ Plus was gifted from Trouw Nutrition (Jackson, MS, USA). AflaTest® Columns were obtained from VICAM (Watertown, MA, USA). A standard aflatoxin mix was obtained from Supelco (Bellefonte, PA, USA).

3.2.2 Experimental Design

A rotatable central composite design with seven center replicates to account for random error was generated using SAS v. 9.3 (Cary, NC, USA) (Table 3.1). Four variables were considered: percent CaCl_2 (0-2% wt./wt. peanut meal), percent NovaSil™ (0.2-4% wt./wt. dispersion), time (15-60 min), and pH (8-14). Each variable was coded and evaluated over five levels. The 30 runs generated were statistically randomized and evaluated without block effects. Two continuous quantitative variables, aflatoxin and protein on dry weight bases, were used as the response variables.

The design of the model and the regression analysis was evaluated in JMP Pro 10 (Cary, NC, USA) by analysis of variance (ANOVA). Data were transformed, if necessary, to obtain a Gaussian fit. Post-hoc analysis included the F-test at a 95% confidence level to

evaluate the significance of linear, interaction, and quadratic response models. Each model parameter was also evaluated for significance within the model using the t-test ($\alpha < .05$). Outliers were assessed by calculating the Cook's distance estimates and leverage values. Optimized formulations were generated using the desirability function as a part of the prediction profiler.

Table 3.1. Rotatable central composite design runs

Sample	Type	Clay (%) ¹	Time (min)	pH	CaCl ₂ (%) ²
1	Axial	2.1	37.5	8	1
2	Factorial	1.15	26.3	12.5	0.5
3	Factorial	3.05	48.8	9.5	0.5
4	Center	2.1	37.5	11	1
5	Factorial	1.15	26.3	12.5	1.5
6	Center	2.1	37.5	11	1
7	Factorial	1.15	48.8	12.5	1.5
8	Center	2.1	37.5	11	1
9	Axial	4	37.5	11	1
10	Center	2.1	37.5	11	1
11	Axial	2.1	37.5	11	2
12	Factorial	1.15	26.3	9.5	1.5
13	Factorial	1.15	48.8	9.5	1.5
14	Axial	2.1	15	11	1
15	Factorial	3.05	26.3	12.5	0.5
16	Factorial	3.05	26.3	9.5	1.5
17	Axial	2.1	37.5	14	1
18	Factorial	3.05	48.8	12.5	0.5
19	Axial	2.1	37.5	11	0
20	Axial	0.2	37.5	11	1
21	Factorial	3.05	26.3	9.5	0.5
22	Center	2.1	37.5	11	1
23	Factorial	3.05	48.8	12.5	1.5
24	Factorial	1.15	48.8	9.5	0.5
25	Factorial	1.15	26.3	9.5	0.5
26	Factorial	3.05	48.8	9.5	1.5

Table 3.1 continued.

Sample	Type	Clay (%) ¹	Time (min)	pH	CaCl ₂ (%) ²
27	Factorial	3.05	26.3	12.5	1.5
28	Center	2.1	37.5	11	1
29	Axial	2.1	60	11	1
30	Factorial	1.15	48.8	12.5	0.5

¹NovaSil™ percentage calculated as wt./wt. of dispersion

²CaCl₂ percentage calculated as wt./wt. of peanut meal

3.2.3 Dispersion preparation

All dispersions were prepared according to Table 3.1. 35 g of defatted peanut meal (starting aflatoxin concentration, 373 ng/g, n = 3) was combined with CaCl₂ in a 250 ml centrifuge tube. The amount of 4 M NaOH by weight required to adjust the pH of the dispersion was predicted using the equation: $\text{pH} = 1.93 \cdot \ln(\text{NaOH}) + 6.97$ (Figure 3.3). A total of 157.5 ml of water (including the NaOH solution) was added together with the NovaSil™ to initiate the extraction for a given amount of time. All dispersions were prepared at room temperature (20 °C) and mixed using a 360-degree rotator (Roto-Rack, Fisher Scientific) during extraction.

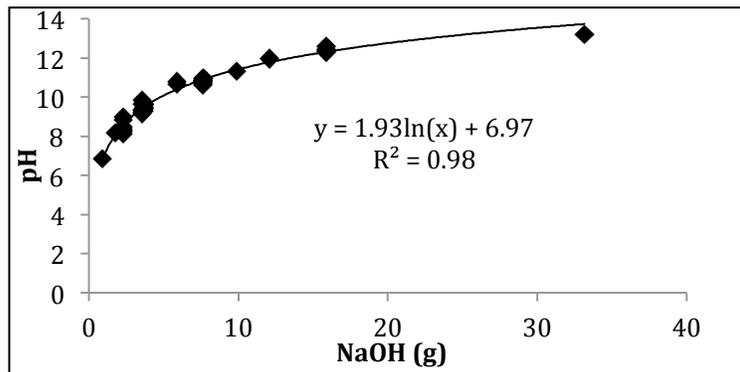


Figure 3.3. pH prediction profile based on amount of NaOH (g) added to aqueous peanut meal dispersions.

Upon completion of the extraction, the dispersions were placed in a Sorvall RC-5B centrifuge for 30 min at 4300 x g. The soluble fraction was decanted, filtered through grade 4 Whatman qualitative filter paper and immediately stored at -10 °C.

3.2.4 Moisture analysis

The gravimetric moisture contents of the soluble and insoluble fractions were determined using approximately 3 g and 5 g of the sample, respectively. Soluble fractions were dried overnight to equilibration in an oven (47 °C) under vacuum and insoluble fractions were dried for 6 hours at 130 °C. All moistures were measured in triplicate.

3.2.5 Density analysis

The density of the soluble fraction was measured at 20°C using a DMA 5000 oscillating tube density meter (Anton-Paar, Austria). The detection limit of the instrument is $\pm 5 \times 10^{-6}$ g/ml at $\pm .01^\circ\text{C}$. Densities expressed are the average of two measurements.

2.2.6 Protein Quantification

The protein of the soluble fraction was quantified by two methods, the bicinchoninic acid (BCA) assay using the Pierce BCA Protein Assay Kit (Rockford, IL, USA) and total soluble nitrogen. Briefly, for the BCA assay, samples were appropriately diluted and loaded into microplates. 25 μl of sample was reacted with 200 μl of fluorescent working reagent, incubated at 37 °C for 30 min, shaken for 30 s, and finally read in a Tecan Safire 2 (Switzerland) microplate reader for absorbance at a wavelength of 562 nm. Samples were analyzed for nitrogen content with a 2400 CHN Elemental Analyzer (Perkin Elmer, Norwalk, CT) by an outside lab, and converted to percent protein using a nitrogen conversion

factor of 5.46.

3.2.7 Protein characterization

The protein from the soluble fraction was characterized using SDS-PAGE run on a Criterion™ Cell system (BioRad Laboratories, Hercules, CA, USA). Samples were prepared in a 1:1 ratio with 2x Laemmli sample buffer containing 5% 2-mercaptoethanol. Ready 10-20% Tris-HCl gels were loaded with 20 µl of sample containing 20 µg of protein or 10 µl of BioRad Precision Plus Protein lane markers. Gels were run in Tris/glycine/SDS running buffer at 200 V for 55 min. Bands were visualized by staining with Bio-Safe Coomassie G-250 for 30 min followed by an overnight wash. Gels were analyzed using ImageLab 4.1 software provided by BioRad Laboratories.

3.2.8 Aflatoxin extraction and quantification

For solid material, 50 g of peanut meal was extracted with 200 ml of 60:40 acetonitrile: water. It was gravity filtered through a Whatman number 4 filter before being diluted at a 2.5:1 ratio prior to loading on the AflaTest® column. For the aqueous soluble fractions, between 5-10 ml of crude solution was adjusted from its native pH (Table 3.1) to a pH between 6-7 and left to equilibrate while shaking for 1.5 hours. Acetonitrile was then added in a 60:40 ratio to the soluble extract, then the mixture was vortexed, shaken for 5 min, and centrifuged at 160 x g for 5 min. The supernatant was decanted, filtered through a microfiber filter if necessary, and diluted 2.5 times with water. 10 ml of diluted acetonitrile extract was loaded onto an AflaTest® column, which was then washed with 10 ml of water, and aflatoxins were eluted with 1 ml of methanol. Samples were diluted 1:1 with 2% acetic acid solution and remained in queue for HPLC analysis no longer than 10 hours. Soluble

fraction extractions were run at least in duplicate. The HPLC procedure was adapted from methods published by Sobolev (2007). Briefly, samples were run on a Shimadzu HPLC equipped with a DGU-20A degasser, RF-20A fluorescence detector, and a photochemical reactor for enhanced detection (PHRED) (Aura Industries, NY, NY, USA). 20 μ l of sample was injected onto a 50 x 4.8 mm i.d., 2.5 μ m XTerra MS C18 analytical column (Waters, Raleigh, NC) held at 42 °C. An isocratic gradient using 63:37 water: methanol at a flow rate of 0.6 ml/min was used to separate individual aflatoxins at a pressure of approximately 2200 psi. Peaks were detected by fluorescence with an excitation wavelength of 365 nm and emission wavelength of 440 nm. Aflatoxin G2, G1, B2, and B1 were quantified using an external aflatoxin mix standard curve generated daily. The limit of detection (LOD) was determined to be 0.01 ng/ml.

3.2.9 Enzyme solution preparation

Soluble fractions of peanut meal dispersions were prepared as described in section 3.2.3. Selected soluble fractions were diluted due to limited sample supply between 5 to 10-fold and to aflatoxin levels no less than 20 ng/g. 50 ml of the diluted soluble fraction was adjusted to a pH of 6 or 8 with 1 N HCl. The solution was brought to 31 °C \pm 1 °C in a water bath and stirred with a Wheaton overhead stirrer. Cofactors were added just prior to the addition of enzyme at levels of 50 mM malonate, 1 mM MnSO₄, and 0.1 mM H₂O₂. MnP was resuspended in DI water, kept on ice while thawing, and added at 0.001 U/ml to initiate the reaction. 10 ml of sample were taken at various time points and immediately placed in a water bath at 80 °C for 20 min to stop the reaction.

3.3.0 Results

3.3.1 Quantification of aflatoxin

The starting peanut meal had a total aflatoxin concentration of 373 ± 30 ($n = 3$). As shown in the spectrum in Figure 3.4, AFG2 and AFG1 are virtually undetectable, and the ratio of AFG2:AFG1:AFB2:AFB1 was 1: 3.5: 20.4: 1467.9. For the central composite design experiment, aflatoxin data was modeled only for AFB1, as at very low levels, AFG2 and AFG1 degrade rapidly in solution and thus may not be accurately quantified by HPLC. Furthermore, in both peanut meal and nearly all other contaminated commodities, AFB1 is the predominant aflatoxin detected, and the different forms of aflatoxin are found in a generally consistent ratio (Trombley A and others 2011). AFB1 is the most toxic of the aflatoxins and is even independently regulated in certain countries, such as throughout the European Union (van Egmond HP and others 2007). The choice to model solely AFB1 was reasoned to introduce the least random error and qualitatively be the most important response.

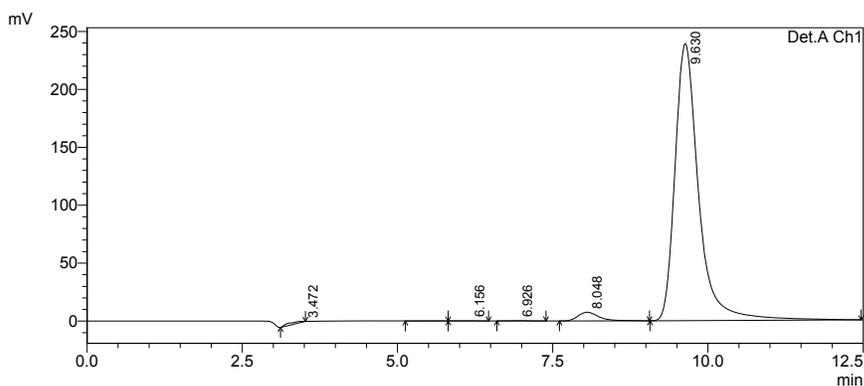


Figure 3.4. HPLC spectrum of starting peanut meal, peaks from left to right include: baseline subtracted solvent (methanol/acetic acid), AFG2 (6.156 min), AFG1 (6.926 min), AFB2 (8.048 min), and AFB1 (9.630 min).

Initial attempts to quantify aflatoxin in the soluble extracts at a high alkaline pH values gave surprisingly low results or undetectable values, despite concentrating samples up to 20-fold during the pre-HPLC clean-up procedure (data not shown). To assess if this was a real effect, the soluble fraction from a dispersion of 35 g peanut meal in 157.5 ml water was adjusted, post-decanting, with 4 M NaOH to varying pH values between 6.1 (native pH) and 10.5. After equilibrating for 1.5 hours, each fraction was analyzed for aflatoxin on the HPLC (Figure 3.5a). As the pH increased, the level of aflatoxin detected decreased in an exponential fashion. The most likely explanation for this behavior is that the alkalinity of the solution causes the aflatoxin ring to open and lose its fluorescence properties (Kiermeie F and Ruffer L 1974). The intersection of the two linear regions on the plot represented the point (pH 8.79) where it was deemed a significant loss in aflatoxin quantified occurred (18.23% loss). This is a reasonable loss as there is usually a large error (at least 10%) intrinsic to most aflatoxin analysis.

A second experiment was conducted to determine if the loss associated with pH was reversible or irreversible. The soluble fraction was analyzed at its native pH (6.1) and then adjusted to pH 11.5 and equilibrated for 1.5 hours. Half of the sample was analyzed at pH 11.5 and half was re-adjusted down to a pH of 6.1 (additional 1.5 hour incubation) (Figure 3.5b). When the solution was analyzed initially at pH 6.1, AFB1 was detected at approximately 280 ng/g. At a pH of 11.5, no aflatoxin could be detected (LOD .01 ng/ml). However, after dropping the pH back to 6.1 from 11.1, approximately a 90% reduction in aflatoxin was seen after the alkaline treatment. A possible reason that aflatoxin was no longer detected in high quantities was that it conjugated to certain nucleophiles in the system

(lysine, histidine, or methionine) or to the hydroxyls attached to the reducing ends of any sugars extracted from the peanut meal (Dearriola MD and others 1988). This type of irreversible derivitization would likely prevent it from fluorescing during HPLC analysis.

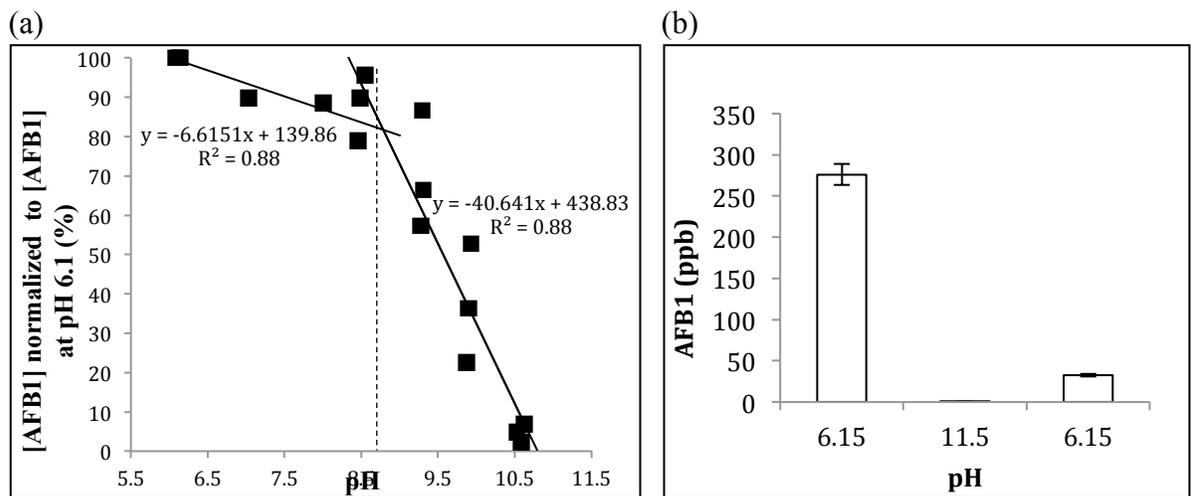


Figure 3.5. Quantification of AFB1 in the soluble fraction of a 35 g peanut meal/157.5 ml water dispersion (a) adjusted from a pH of 6.1 to 10.5 and normalized to the starting aflatoxin concentration (n=3) and (b) adjusted from a pH of 6.1, to 11.5, and back down to 6.1 (n=3).

3.3.2 Dry fractionation of peanut meal

Peanut meal typically contains a variety of ground components beyond the seed, including some small fraction of hull, skin, and foreign contaminants, including bag fibers, or stones. As this study focuses on high protein components, dry fractionation of the peanut meal according to particle size was initially considered in the hopes of finding a protein-rich fraction. The starting peanut meal was dry fractionated using standard sieves into fractions A, B, and C of a particle size > 1.18 mm, 1.00 mm $<$ particle size < 1.18 mm, and a particle size < 1.00 mm, respectively. The peanut meal was comprised of $35.5\% \pm .1\%$ fraction A, 10.0

% \pm .3 % fraction B, and 54.3 % \pm .3 % fraction C by weight (n = 3, 200 g samples). It was analyzed by an outside lab for proximate analyses of protein, fiber, fat and moisture content (Table 3.2). Though the measurements were precise enough to produce significant differences between the fractions, meaningful differences in the amount of protein per fraction were not observed. This was somewhat surprising as peanut meal has not been previously examined on the basis of particle size and expectations were that protein would be low in the fraction of particle size > 1.18 mm, which, based on visual analysis, seemed comprised of relatively large amounts of hull fragments and debris. Due to the small changes in proximate composition among samples, unfractionated peanut meal was selected for the remainder of the study. From an industry standpoint, this decision had economic merit as well.

Table 3.2. Proximate analysis of peanut meal fractions.

Fraction	Moisture (%)	Fat (%)	Protein (%)	Fiber (%)
A > 1.18 mm	10.28	2.01	51.24	6.98
1.00 mm < B < 1.18mm	10.29	1.68	54.31	5.70
C < 1.00 mm	10.38	1.47	52.05	5.72

3.3.3 Protein and aflatoxin trends

Data for the 30 soluble fractions prepared from the central composite design were evaluated for their final pH, protein content, and aflatoxin content (Table 3.3). Despite efforts to obtain precise factorial, axial, and center points, the buffering capacity of the system made it extremely difficult to extract dispersions at a specific pH, even with a prediction equation. In general, the pH is approximately \pm 0.3 from the desired level. The experimental and

theoretical mass balance of the system was computed to ensure the accuracy of all measurements related to the inputs and outputs of the system (Table 3.3). The experimental mass was derived from the weight of the insoluble and soluble fraction and their respective moistures, versus the theoretical weight, which was calculated as the sum of each added reagent. The soluble extracts showed a range in aflatoxin and nitrogen concentrations on a dry weight basis from 35 ng/g to 634 ng/g and 126 mg/g to 759 mg/g, respectively. Increasing nitrogen in soluble extracts correlated to a polynomial increase of aflatoxin in solution (Figure 3.6). It is important to note here the influence of the other factors affecting this correlation. For example, in the 500 mg/g protein range, the data segregates into two groups of with seemingly high or low aflatoxin off the trend line. On closer examination, the points with too high aflatoxin were all extracted at high pH (>12) and those that were too low contained high levels of clay (>2%). The effect of added clay becomes increasingly important at extreme protein levels and high pH. Had a series of dispersions been made without clay and calcium chloride, an excellent correlation with an extremely steep slope would be expected between protein and aflatoxin, though the correlation would not necessarily be linear. There was also a Langmuir-type fit between protein and total percent solids (Figure 3.7a) and density (Figure 3.7b), respectively. This confirmed that while primarily protein was being extracted in dispersions, some soluble fiber and sugar do likely comprise a significant percentage of solutions with high densities and total solids.

Table 3.3. Mass balance, protein, and aflatoxin analysis of central composite design runs.

Sample #	Clay (%) ¹	CaCl ₂ (%) ²	Time (min)	pH	Moisture (%)	Density (g/ml)	Experimental Mass (g)	Theoretical Mass (g)	BCA Protein (mg/g)	N-analysis (mg/g)	Aflatoxin B1 (ng/g)
1	2.06	1.01	37.5	8.14	98.06	1.0135	196.6	196.9	114.8	126.4	45.5
2	1.14	0.49	26.3	12.62	94.44	1.0366	194.6	194.9	582.7	539.1	574.5
3	2.96	0.50	48.8	9.66	97.17	1.0203	198.5	198.6	403.2	508.4	67.7
4	2.07	1.00	37.5	10.85	95.84	1.0279	195.7	188.6	435.6	523.8	124.8
5	1.14	1.50	26.3	12.56	94.37	1.0378	195.0	203.0	609.1	515.2	634.3
6	2.06	1.01	37.5	10.88	95.50	1.0273	196.4	188.6	502.6	537.0	71.7
7	1.14	1.50	48.8	12.42	87.17	1.0386	194.8	195.5	805.8	538.4	414.3
8	2.06	1.01	37.5	10.87	91.59	1.0282	196.7	196.8	441.1	506.7	172.6
9	3.87	1.00	37.5	10.64	92.25	1.0262	199.1	200.0	440.9	497.7	164.6
10	2.07	1.00	37.5	10.81	91.75	1.0276	195.5	196.5	478.7	519.7	108.4
11	2.06	2.00	37.5	10.71	92.57	1.0259	196.8	197.4	376.7	454.0	170.6
12	1.14	1.50	26.3	9.63	95.02	1.0178	195.5	195.3	245.1	305.0	48.9
13	1.14	1.50	48.8	9.40	94.70	1.0182	195.0	195.2	242.4	759.3	272.1
14	2.06	1.00	15.0	10.98	91.79	1.0265	196.2	196.9	473.1	227.6	42.5
15	2.97	0.50	26.3	12.46	89.79	1.0360	197.7	198.5	411.3	520.5	456.6
16	2.96	1.50	26.3	9.48	95.68	1.0164	198.9	199.0	181.7	207.5	41.2
17	2.06	1.00	37.5	13.18	86.67	1.0508	196.2	196.9	492.7	505.8	553.5
18	2.98	0.50	48.8	12.30	88.76	1.0382	197.6	198.6	493.6	496.5	265.7
19	2.06	0.00	37.5	10.83	90.73	1.0304	195.9	196.4	874.6	516.8	59.9
20	0.20	1.00	37.5	10.94	91.10	1.0296	192.6	193.1	562.0	531.2	190.3
21	2.96	0.50	26.3	9.63	94.03	1.0200	198.8	198.5	370.1	421.7	35.0
22	2.07	1.00	37.5	10.76	91.71	1.0274	195.9	196.8	568.2	480.2	124.8
23	2.97	1.50	48.8	12.29	89.44	1.0370	198.1	199.0	601.4	571.1	517.9
24	1.14	0.50	48.8	9.46	93.07	1.0228	195.1	195.0	422.6	453.1	53.6
25	1.14	0.50	26.3	9.65	93.24	1.0219	194.9	195.0	441.9	412.4	39.1
26	2.96	1.50	48.8	9.29	95.32	1.0133	199.0	198.9	168.4	201.6	116.8
27	2.97	1.50	26.3	12.35	89.40	1.0359	198.6	198.9	540.3	511.0	490.8
28	2.07	1.00	37.5	10.76	91.69	1.0272	195.4	196.9	530.8	487.7	141.5
29	2.07	1.00	60.0	10.50	91.21	1.0286	195.9	196.8	492.7	484.3	144.0
30	1.14	0.50	48.8	12.43	88.16	1.0390	194.4	195.1	506.1	526.6	452.2

¹NovaSil™ percentage calculated as wt./wt. of dispersion

²CaCl₂ percentage calculated as wt./wt. of peanut meal

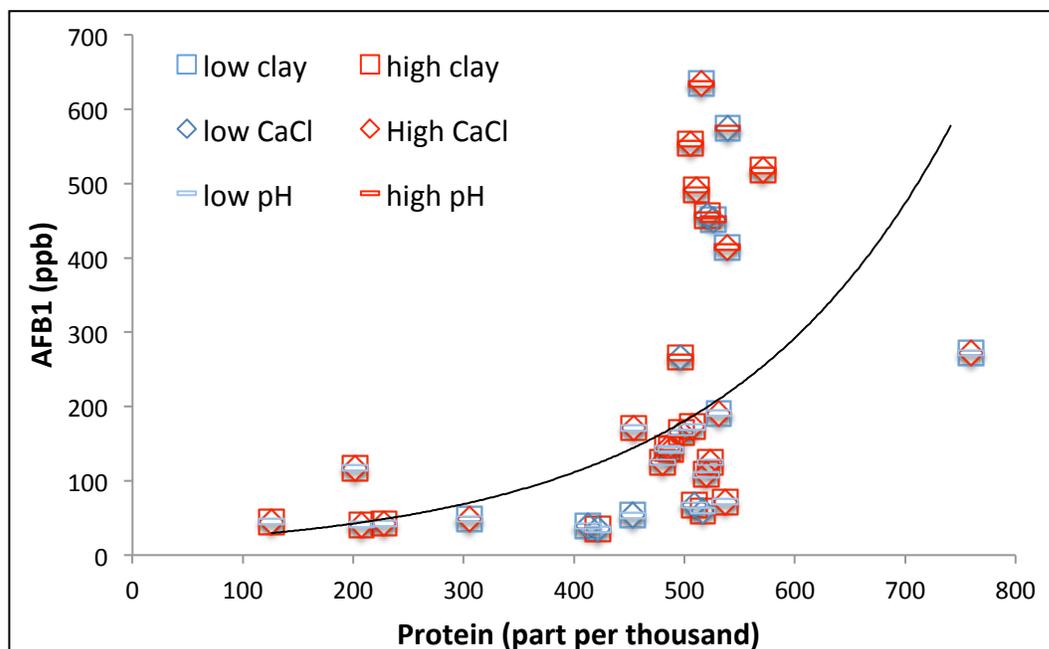


Figure 3.6. Protein vs. aflatoxin of central composite design runs. Samples with $\leq 1.40\%$ NovaSil™ or $\geq 2.06\%$ NovaSil™ are labeled “low clay” or “high clay”, $\leq 2\%$ CaCl₂ or $\geq 2.05\%$ CaCl₂ are labeled “low CaCl” or “high CaCl,” and $\text{pH} \leq 11.9$ or ≥ 12.0 NovaSil™ are labeled “low pH” or “high pH,” respectively.

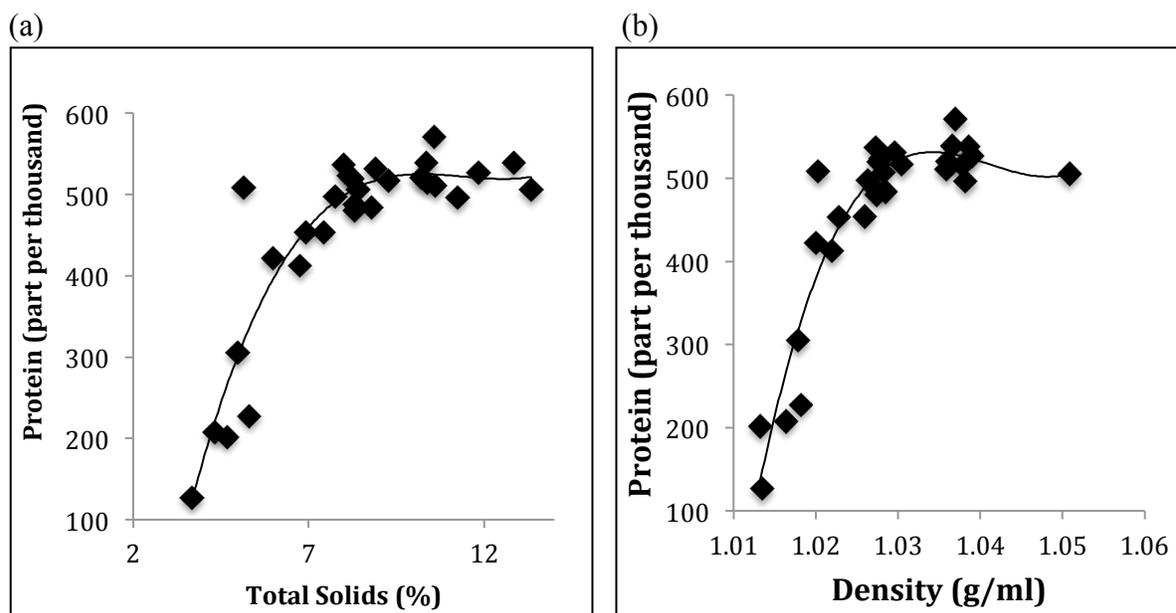


Figure 3.7. Plots of total soluble protein vs. (a) total solids and (b) density. Sample number 14 was removed as an outlier for both plots.

3.3.4 Response surface modeling of aflatoxin

AFB1 raw data showed a left-skewed distribution (Figure 3.8a) that was corrected for by transforming it according to the equation: $\log_{10}(\text{AFB1}+1)$. The left-skewed trend is attributed to samples with very high pH values that generate very large aflatoxin values that sit were to the far right of the distribution. No outliers were detected in the log transformed data using the leverage or Cook's distance test. The $\log_{10}(\text{AFB1}+1)$ was modeled by a linear (no interaction, main effects), 4-way interaction, and interaction/quadratic equation (Table 3.4). All terms within each model, regardless of statistical significance, were maintained after consultation with the Statistics Department at North Carolina State University. The full quadratic equation was chosen for evaluation as it had the best fit ($R^2 = .97$, adj. $R^2 = .90$). All diagnostic plots, including the residuals (Figure 3.8b) and a normal plot of the residuals (Figure 3.8c) showed appropriate random scatter. The predicted versus actual responses had good agreement (Figure 3.8d). The final equation coefficients may be found in Table 3.5.

Table 3.4. ANOVA and summary statistics for the linear, 4-way interaction, and fully crossed with quadratic effects model for $\text{Log}_{10}(\text{AFB1}+1)$.

Fit	Model Degree of Freedom	Sum of Squares	Mean Square	F value	P value	R^2	Adj. R^2
Linear	4	3.90	.97	26.46	<.0001	.81	.78
Interaction	15	4.37	.29	9.09	<.0001	.91	.81
Quadratic	19	4.65	.24	14.59	<.0001	.97	.90

Table 3.5. Coefficients for the $\text{Log}_{10}(\text{AFB1}+1)$ predictive quadratic equation and their associated p -values ($\alpha = .05$) based on the student's t -test.

Term	Coefficient	Prob > t
pH	0.271	<.0001
Time	0.010	0.0016
CaCl ₂ %	0.212	0.0025
(pH-10.8824)*(Time-37.5)	-0.008	0.0032
(pH-10.8824)*(pH-10.8824)	0.040	0.0179
(Clay %-2.05544)*(Clay %-2.05544)	0.069	0.0406
(CaCl ₂ %-1.00015)*(Time-37.5)	0.010	0.1217
(Time-37.5)*(Time-37.5)	-0.000	0.1605
(Clay %-2.05544)*(CaCl ₂ %-1.00015)*(pH-10.8824)	0.071	0.1760
(Clay %-2.05544)*(CaCl ₂ %-1.00015)*(pH-10.8824)*(Time-37.5)	0.006	0.1828
(CaCl ₂ %-1.00015)*(pH-10.8824)	-0.063	0.1845
(CaCl ₂ %-1.00015)*(pH-10.8824)*(Time-37.5)	-0.005	0.2657
Clay %	-0.017	0.5818
(Clay %-2.05544)*(pH-10.8824)	0.010	0.6798
(Clay %-2.05544)*(Time-37.5)	-0.001	0.6955
(Clay %-2.05544)*(pH-10.8824)*(Time-37.5)	0.001	0.7385
(Clay %-2.05544)*(CaCl ₂ %-1.00015)*(Time-37.5)	-0.002	0.7806
(Clay %-2.05544)*(CaCl ₂ %-1.00015)	-0.016	0.8285
(CaCl ₂ %-1.00015)*(CaCl ₂ %-1.00015)	-0.007	0.9484

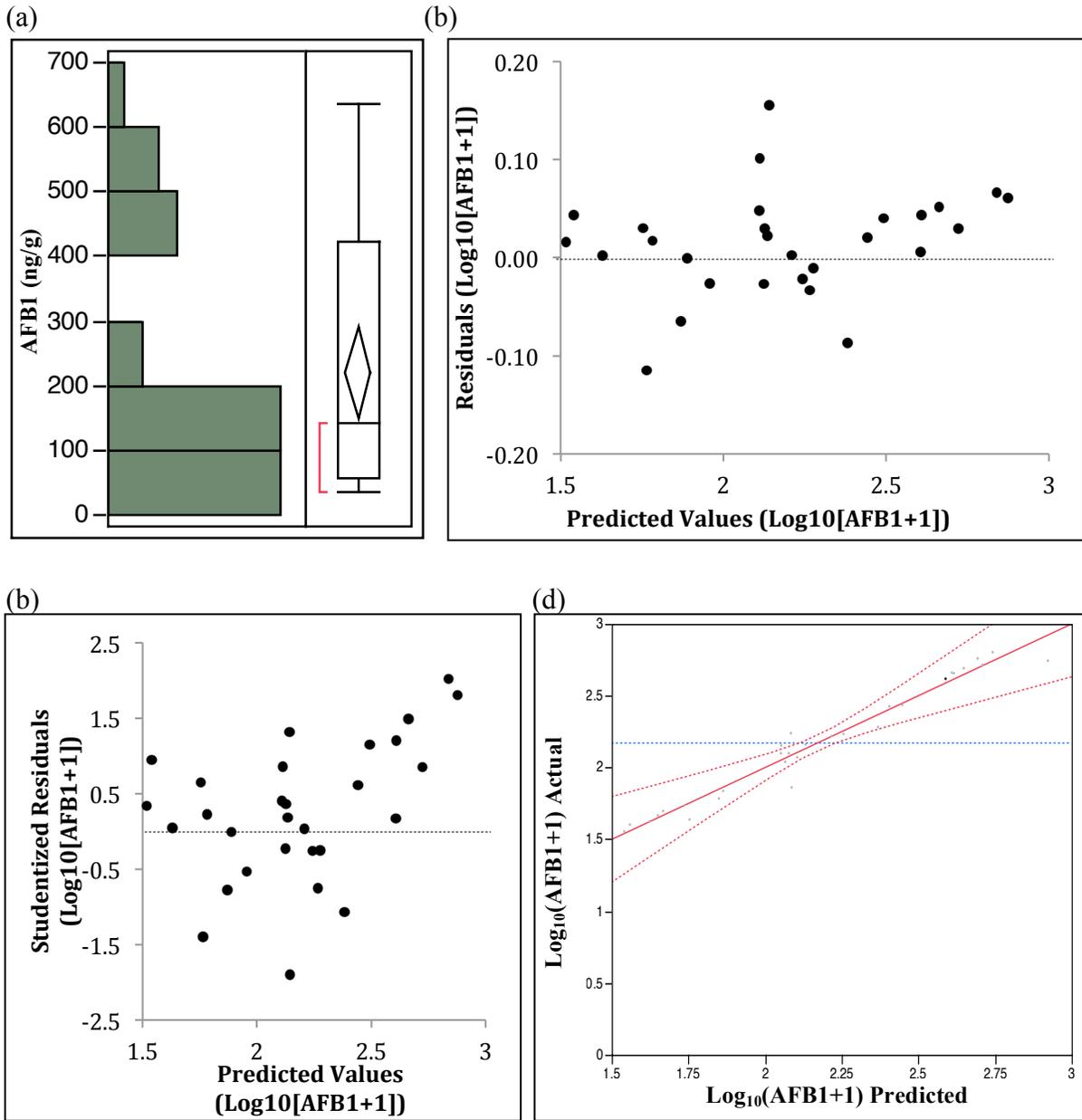


Figure 3.8. (a) AFB1 distribution for central composite design data points, and diagnostic plots for AFB1 logarithmically transformed data: (b) residuals plot, (c) studentized residuals plot, and (d) actual versus predicted response variables by the quadratic equation including all interaction terms, dotted lines show the 95% confidence interval for predicted values.

3.3.5 Response surface modeling of protein

Protein data generated via the BCA assay and soluble N-analysis showed approximately a Gaussian fit and were not transformed (Table 3.3). Though BCA data and N-analysis data were similar, modeling was completed with nitrogen data, as the BCA assay is known to have varying error when quantifying small peptides (generated at a high pH) versus whole protein. The protein data was not transformed and was modeled by a linear (no interaction, main effects), 4-way interaction, and interaction/quadratic equations (Table 3.6). The quadratic equation was chosen for final evaluation and had an R^2 of .94 and adjusted R^2 of .83. All diagnostic plots, including the residuals (Figure 3.9a) and a studentized plot of the residuals (Figure 3.9b) showed appropriate random scatter. The predicted versus actual responses had excellent agreement (Figure 3.9c). The final equation coefficients may be found in Table 3.9.

Table 3.6. ANOVA and summary statistics for the linear, 4-way interaction, and fully crossed with quadratic effects model for protein.

Fit	Model Degree of Freedom	Sum of Squares	Mean Square	F value	P value	R^2	Adj. R^2
Linear	4	203890	50973	4.54	.0068	.42	.33
Interaction	15	378747	25250	3.34	.0148	.78	.55
Quadratic	14	456389	24021	8.55	.0007	.94	.83

Table 3.7. Final coefficients for the protein predictive quadratic equation with their associated *p*-values ($\alpha = .05$) based on the student's t-test.

Term	Coefficient	Prob > t
Intercept	-145.41	0.1863
Clay %	-27.21	0.0447
CaCl ₂ %	-26.16	0.2546
(Clay %-2.05544)*(CaCl ₂ %-1.00015)	-80.26	0.0201
pH	51.75	<.0001
(Clay %-2.05544)*(pH-10.8824)	23.83	0.0381
(CaCl ₂ %-1.00015)*(pH-10.8824)	28.10	0.1541
(Clay %-2.05544)*(CaCl ₂ %-1.00015)*(pH-10.8824)	69.89	0.0057
Time	4.71	0.0006
Intercept	-145.41	0.1863
(Clay %-2.05544)*(Time-37.5)	-2.56	0.0763
(CaCl ₂ %-1.00015)*(Time-37.5)	5.49	0.0422
(Clay %-2.05544)*(CaCl ₂ %-1.00015)*(Time-37.5)	-5.38	0.0638
(pH-10.8824)*(Time-37.5)	-2.37	0.0153
(Clay %-2.05544)*(pH-10.8824)*(Time-37.5)	1.91	0.0576
(CaCl ₂ %-1.00015)*(pH-10.8824)*(Time-37.5)	-1.60	0.3477
(Clay %-2.05544)*(CaCl ₂ %-1.00015)*(pH-10.8824)*(Time-37.5)	4.39	0.0327
(Clay %-2.05544)*(Clay %-2.05544)	13.58	0.2833
(CaCl ₂ %-1.00015)*(CaCl ₂ %-1.00015)	14.86	0.7194
(pH-10.8824)*(pH-10.8824)	-23.08	0.0027
(Time-37.5)*(Time-37.5)	-0.25	0.0109

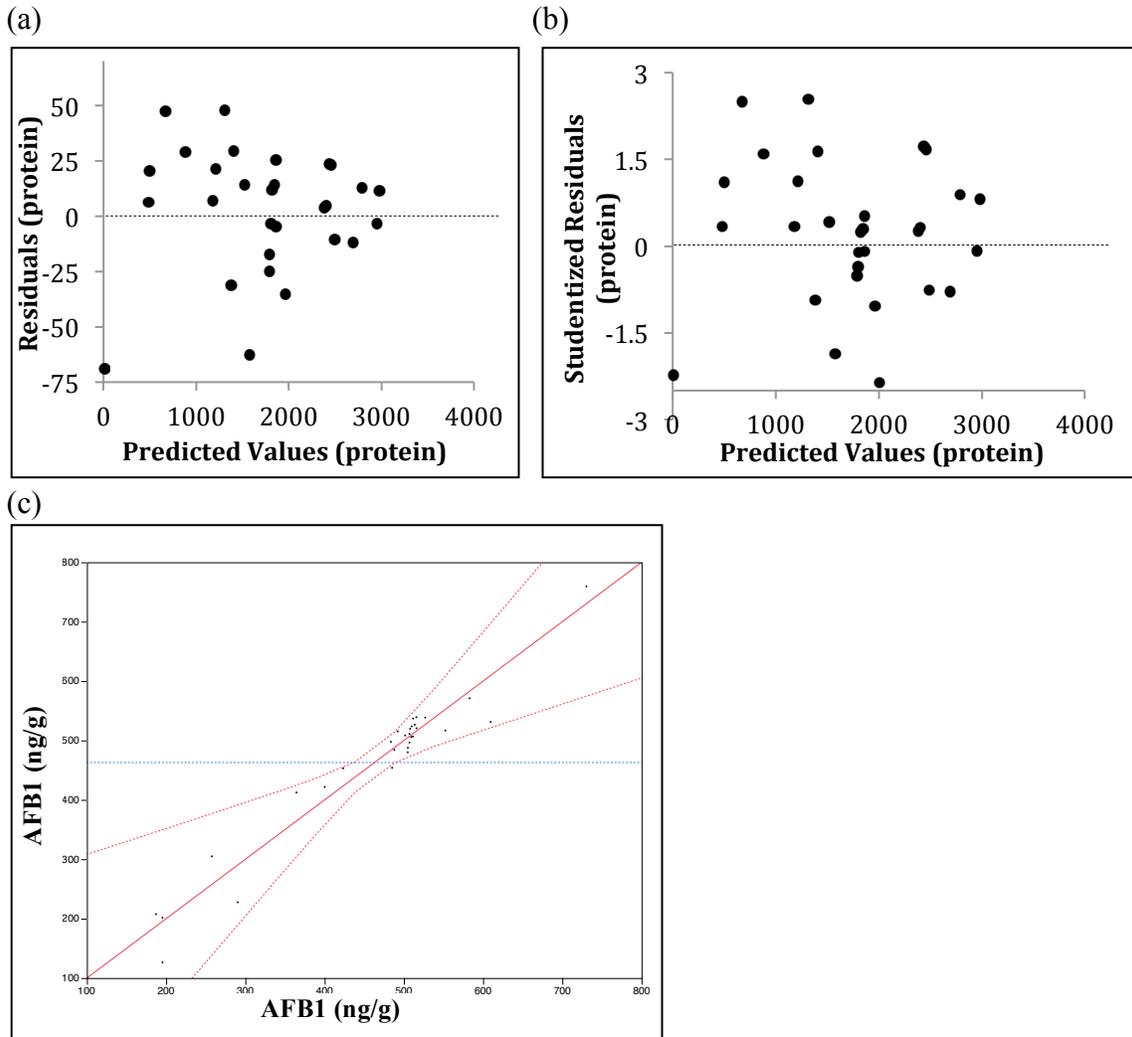


Figure 3.9. Quadratic equation diagnostic plots for protein: (a) residuals plot, (b) studentized residuals plot, and (c) actual versus predicted response variables, dotted lines show the 95% confidence interval for predicted values.

3.3.6 Desirability functions for protein and aflatoxin

Desirability functions were generated as a means of quantitatively optimizing aflatoxin and soluble nitrogen protein predictive quadratic equations. For $\log_{10}(\text{AFB1}+1)$ functions, a desirability of 1 and 0 were set for a response of 0 ng/g and 3.25 ng/g, respectively. For protein, a desirability of 1 and 0 were set for a response of 1100 mg/g and 50 mg/g, respectively. The desirability functions were performed with extended ranges from the original experimental design, with the pH range from 6-14 and the clay range from 0-4%. Maximized desirability to minimize aflatoxin or maximize protein, was achieved by running desirability functions with 20 trips, 250 iterations, and a convergence tolerance of .000001. The parameters' trends and associated desirability values are shown in Figure 3.10.

For aflatoxin, the greatest desirability (.99) was predicted for a dispersion comprised of 1.11% clay and 2% CaCl_2 at a pH of 6 and extracted for 15 min. pH and time have the greatest affect on aflatoxin extracted; increasing the value of either parameter results in very large penalties to the final desirability score, as they are correlated with large increases in aflatoxin (Figure 3.10a). With these conditions, AFB1 is predicted with 95% confidence to be between -1 ng/g and 96.7 ng/g. It is therefore important to address the limitations of this model and the potential causes for a large range. As is reflected in the 95% confidence interval bounds in Figure 3.10, error increases as the parameters approach the extremes of their ranges (or go outside). In the case of time headed to the extreme of 60 min, for example, the 95% confidence interval expands in range, though the same upward trend in aflatoxin is predicted. However, in the case of clay, for example, the model shows poor confidence in its ability to predict if an increase or decrease in aflatoxin will occur toward the extremes.

Studies from this lab and the literature clearly state that increasing clay results in decreasing aflatoxin in a linear fashion, the model is not refined enough to make this determination at the conditions shown in Figure 3.10a.

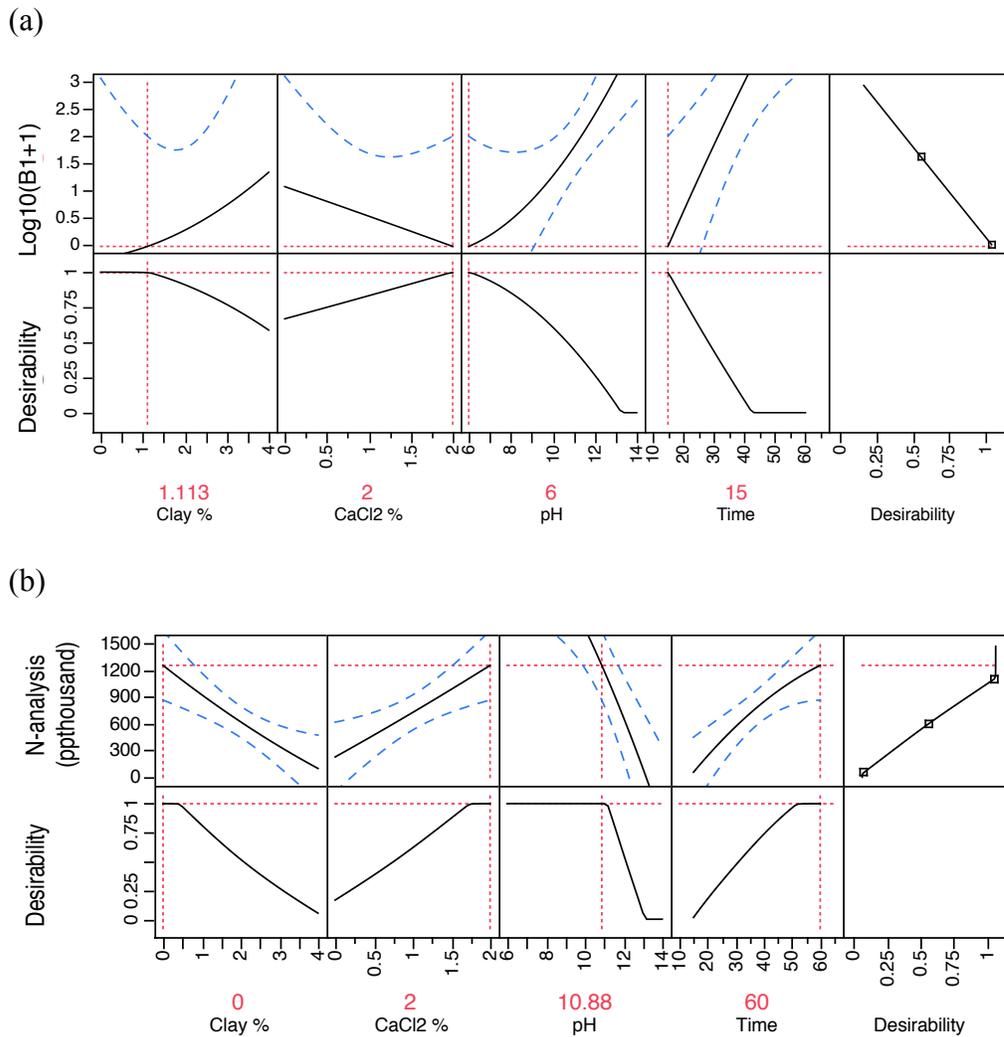


Figure 3.10. Desirability functions for (a) $\text{Log}_{10}(\text{AFB1}+1)$ minimized and (b) protein maximized. Each plot uses a red crosshair to indicate the point (specified value under each plot) at which a parameter is maximized for desirability. 95% confidence intervals are plotted by the blue dashed lines. The desirability plot shows the desirability value predicted by its function at a given $\text{Log}_{10}(\text{AFB1}+1)$ or protein concentration.

The maximized desirability (1.0) for protein was formulated as a dispersion of 0% clay and 2% CaCl₂ at a pH of 10.88 extracted for 60 min. This set of desirability functions (Figure 3.10b) highlighted different trends in the data from the aflatoxin functions, particularly for pH. At a short time frame (15 min), maximum protein was predicted to occur at the highest pH (14) and at a high level of calcium, which presumably helps solubilize hydrolysates produced at alkaline conditions. A similar value was predicted for protein extracted for a longer time period (60 min) and at a pH pre-hydrolysis (10.88). Unfortunately, the desirability plots reveal certain limitations and intrinsic errors of this model. In particular, the sample space of high calcium, low pH (particularly outside the factor bounds) needs to be further refined. The level of clay also appears to have a large effect on the amount of protein extracted. At low pH values, increasing levels of clay correlate to decreasing levels of protein.

3.3.7 Predictive functions for protein and aflatoxin

Two dispersions were made to test the accuracy of the prediction equations and to produce soluble fractions with low aflatoxin and low protein (Test 1) and low aflatoxin and high protein (Test 2) (Table 3.8). While the functions did a reasonable job in generating fractions with the desired qualities, there was significant disagreement between the predicted and experimental data set particularly for the aflatoxin values. However, for the aflatoxin model, a good agreement between the predicted and actual values would not necessarily be expected, as the parameters are either at the extreme of their ranges, or outside of the original tested ranges. To accurately predict aflatoxin in this region, it is likely that a smaller, but new, experimental design would have to be completed with refined ranges. Though this

model is an excellent fit for aflatoxin, its predictive abilities are limited by its quadratic equation restraint (versus another nonlinear fit) and the tested ranges. In both test dispersions, aflatoxin values would be considered relatively low compared to the starting peanut meal (around 100 ng/g), and the low aflatoxin/high protein dispersion contained approximately six times more protein than that of the low aflatoxin/low protein dispersion. In terms of protein, the high protein dispersion agreed well with the predicted values and was within the 95% confidence interval, while the low protein dispersion had significant error. Isoelectric precipitation of protein in test point 2 resulted in a ten-fold reduction in the solubilized protein (28.3 mg/ml to 2.6 mg/ml, n = 3).

Table 3.8. Analysis of optimized dispersion for aflatoxin and aflatoxin/protein according to the desirability functions in Figure 10a and 10b, respectively.

Test	Clay (%) ¹	CaCl ₂ (%) ²	Time (min)	pH	Moisture (%)	Density (g/ml)	Experimental Mass (g)	Theoretical Mass (g)	BCA Protein (mg/g)	Protein Predicted Desirability (0-1)	Predicted Protein (mg/g)	AFB1 (ng/g)	Predicted AFB1 (ng/g)	AFB1 Predicted Desirability (0-1)
1	1.49	0.0	15	6.18	96.52	1.0118	194.89	195.41	80.1	0.0	0.0	112.9	7.0	0.72
2	2.11	0.0	15	9.61	94.16	1.0191	196.55	196.65	475.4	0.28	353.9	132.1	17.1	0.61

¹NovaSil™ percentage calculated as wt./wt. of dispersion

²CaCl₂ percentage calculated as wt./wt. of peanut meal

3.3.8 Characterization of extracted peanut protein by SDS-PAGE

Distinct trends in the protein profiles could be identified from the SDS-PAGE gels of the CCD runs, primarily corresponding to changes in pH (Figure 3.11). Generally, dispersions extracted at a pH > 12 showed a significant quantitative increase in small

peptides. Dispersion 17, at a pH of 13.2, was hydrolyzed to the point where peptides ran off the gel. With the increase in pH, the first bands to disappear are at 35 kDa and 63 kDa, which correspond to seed storage/allergenic proteins Ara h3 and Ara h1, respectively. This is supported by studies that have demonstrated the susceptibility of these allergens to alkaline hydrolysis (Koppelman SJ and others 2010).

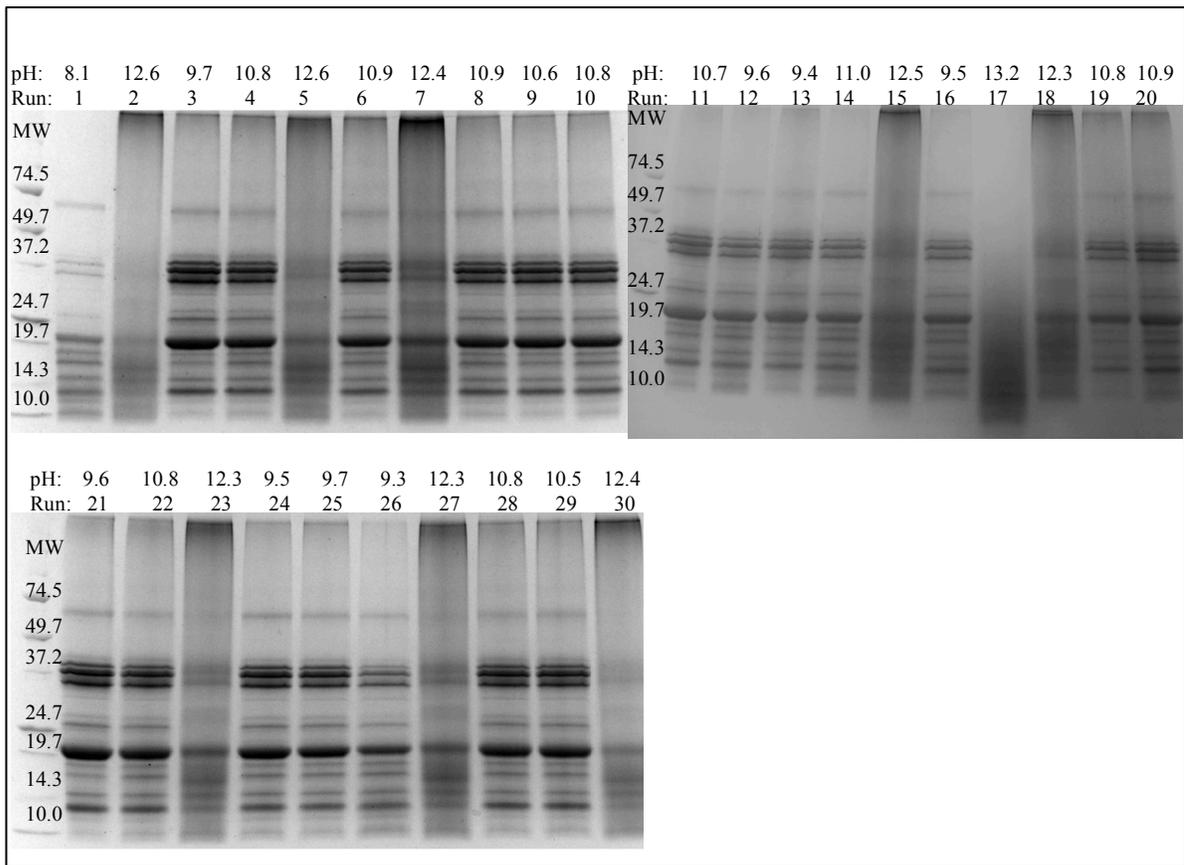


Figure 3.11. SDS-PAGE gels of central composite design runs loaded with 20 μg of protein per well.

SDS-PAGE results supported changes in visual observations between samples. The color of the samples, ranging from a cloudy, light yellow to opaque, near black changed with pH (Figure 3.12). Furthermore, there was a noticeable decrease in the viscosity of the high pH samples. On storage at -10 °C, samples at a lower pH (< 9.5) had a tendency to precipitate large quantities of protein, while those at a high pH remained homogenous. On adjustment of the pH to 6 -7 for aflatoxin quantification, samples containing alkaline-hydrolyzed protein emitted a sulfur-like odor and displayed significant levels of precipitation. The protein also had excellent foaming ability at this pH, with foams replacing over 50% of the volume at times. Foams took over an hour to collapse even with centrifugation. Do to the interesting physical changes observed, peanut meal extracted protein could serve as an interesting sample to investigate foaming and surface active abilities.

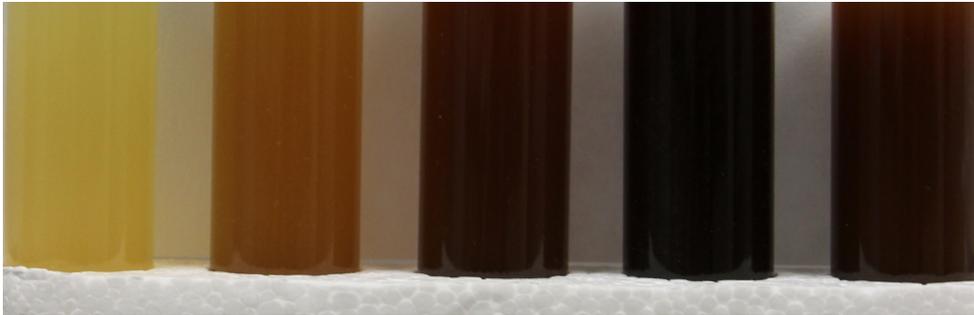


Figure 3.12. The effect of pH on the color of samples. From left to right, test sample 1 (pH 6.18), run 1 (pH 8.14), run 11 (pH 10.71), run 30 (pH 12.43), and run 17 (pH 13.18).

3.3.9 Enzymatic degradation of aflatoxin in protein-rich peanut meal extract solutions

Enzymatic experiments were carried out on sample 1, representative of a low protein/low aflatoxin and on sample 2, representative of a high protein/high aflatoxin sample

(Table 3.9). Degradation of sample 1 or 2 was not significantly dependent on the pH of the treatment (pH 8 or 6). Though samples were collected over an extended time period, no changes in the percent aflatoxin degraded were detected between 10 min and 3 hours (data not shown). At pH 8 and pH 6, respectively, aflatoxin was reduced $96 \% \pm 0.01 \%$ and $95 \% \pm 0.01 \%$ for sample 1 (n=3) (Table 3.10). Sample 2, however, demonstrated extremely variable behavior in the amount of aflatoxin degraded at both pH values tested, with reductions ranging from 32 to 99 % or 40 to 98 % for pH 6 and pH 8, respectively (n = 5 and n = 3, respectively) (Table 3.10). A control sample was prepared containing only the cofactors of MnP (MnSO₄, H₂O₂, and malic acid). Under these conditions, aflatoxin was also reduced to $99 \% \pm 0.00 \%$ (n = 3) in sample 2, pH 8 (Table 3.10).

SDS-PAGE was performed on samples to observe if there were changes in band densities. No differences in band densities between MnP treated and the control were detected for sample 1 or sample 2 (Figure 3.13).

Table 3.9 Soluble phase properties of samples used for enzymatic treatment.

Sample Number ²	Starting Soluble Phase Conditions						
	NovaSil TM (%)	CaCl ₂ (%)	Time (min)	pH	Density	Total Solids	Protein ² (mg/g)
1a	2.06	1.01	37.5	8.14	1.01352	0.0367	126.39
1b	2.06	1.01	37.5	8.99	1.01584	0.0447	-
2a	1.14	0.49	26.3	12.62	1.03659	0.1035	539.11
2b	1.14	0.49	26.3	12.63	1.01365	0.1056	-

¹Soluble phases extracted from the original central composite design run (a) or an identically prepared run (b). Despite keeping the parameters constant, differences are expected due to the variability associated with peanut meal.

²Soluble nitrogen could not be obtained for samples with “-.”

Table 3.10. Effect of MnP treatment of aflatoxin in sample 1 and sample 2¹.

Sample Number ²	Initial Aflatoxin (ng/g)	Final Aflatoxin Post-treatment (ng/g) ^{3,4}								
		.001 U/ml MnP pH 6			.001 U/ml MnP pH 8			No MnP, Cofactors present, pH 8		
		Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3
1a	45.5	ND			ND					
1b	35.9	1.6		1.6	1.3		1.7			
2a	574.5				9.4			6.9	9.5	7.4
2b	963.1	532.8	650.1	8.0	536.4	579.4				

¹3 trials were conducted per sample number. They are reported per sample batch “a” or “b.” A blank space indicates no experiment completed.

²Soluble phases extracted from the original central composite design run (a) or an identically prepared run (b). Despite keeping the parameters constant, differences are expected due to increased aflatoxin in stored peanut meal with time.

³ND = not detectable, with the LOD of the HPLC at .01 ng/ml

⁴Each column represents the value from a single run

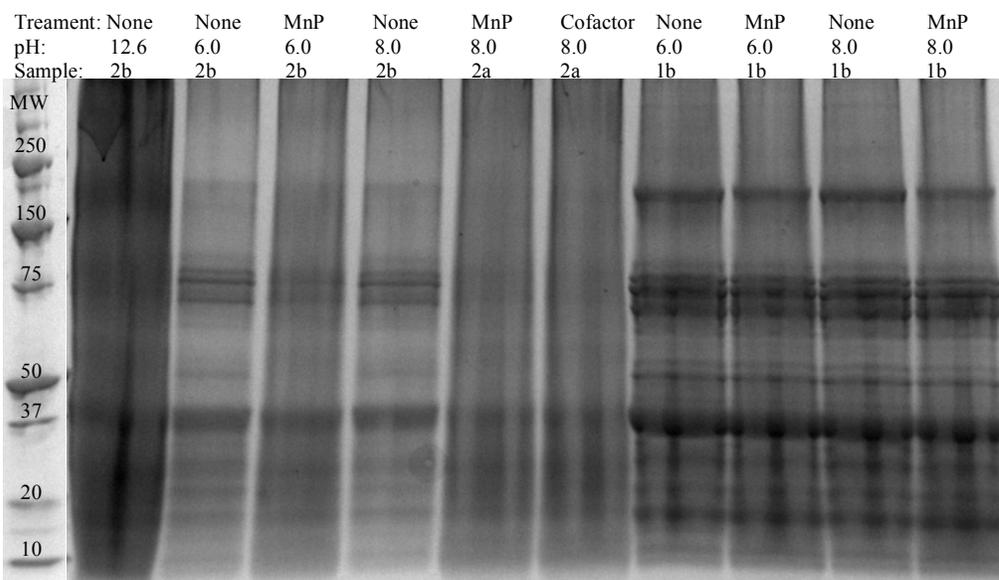


Figure 3.13. SDS PAGE gels of samples 1 and 2 with no treatment, enzymatic treatment, and cofactor treatment. Gel distortion is attributable to use of an expired gel by 29 days.

3.4.0 Discussion

Response surface modeling highlighted a number of key effects by the four variables experimentally tested, including the strong effect of pH on aflatoxin and protein extracted, the inverse correlation of calcium chloride with aflatoxin levels, and the effect of clay on decreasing both protein and aflatoxin in the system. Subtle effects can be best analyzed in an interaction plot (Figure 3.14), where significant interactions are visually identified by two curves on an interaction plot that are non-parallel. The interaction of pH and time is one such interesting example. It is predicted that at the longest extraction time, aflatoxin or protein extracted will remain relatively constant with only a slight increase with increasing pH, whereas for short extractions, there is an upward trend for both response variables with increasing pH. One explanation for the different slopes may be that longer extraction times promotes more irreversible degradation of aflatoxin at high pH values (Figure 3.5), whereas this does not occur sufficiently on a shorter time scale. pH is also the most important variable driving protein extraction within the system. Protein solubility drastically increases as the pH is moved away from the isoelectric point. Aflatoxin may be pulled out with protein in the system, and thus is highly correlated to pH. Furthermore, this demonstrates that either time or pH will be the driving force in the system for aflatoxin or protein extraction, as high pH levels effectively make extraction time an a relatively unimportant variable, and, vice versa, with long extraction times, pH does not have as large effects.

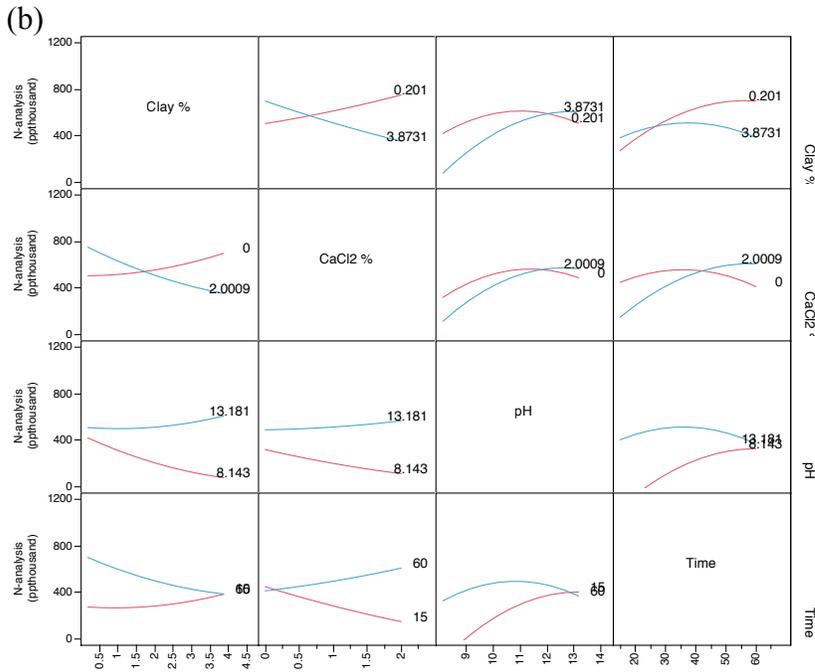
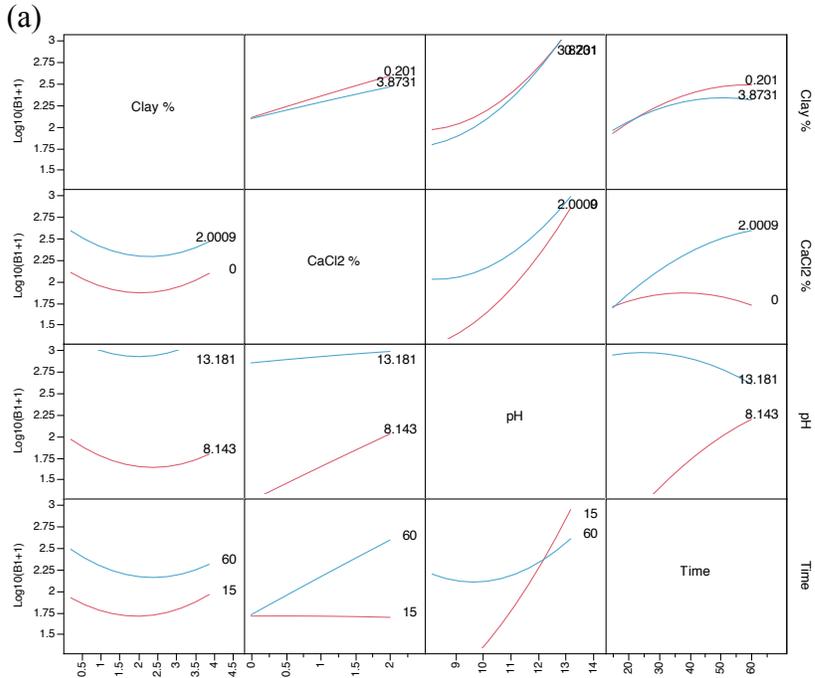


Figure 3.14. Interaction plots for (a) aflatoxin and (b) protein over the factor ranges: NovaSil™ (0.2-3.9%), CaCl₂ (0-2%), pH (8.1-13.1), and Time (15-60 min). The blue line on each plot is the maximized value for a given parameter, 3.9%, 2%, 13.1, and 60 min for NovaSil™, CaCl₂, pH, and time, respectively. The red line on each plot is the minimized value for a given parameter, .2%, 0%, 8.1, and 15 min for NovaSil™, CaCl₂, pH, and time, respectively.

Another interesting factor in this system is the effect of calcium chloride. At a given level of clay and pH (below the point for hydrolysis), increasing calcium chloride increases aflatoxin detected in the system. This may show that calcium is negatively interfering with the electrostatics of the clay, perhaps by replacing ions that had a higher aflatoxin-binding affinity (Deng Y and Szczerba M 2011). Attempts to examine this effect by FTIR (Arvide MGT and others 2008) were insufficient (data not shown). Calcium may also affect the amount of aflatoxin in the system by interfering with aflatoxin protein associations and therefore increasing the amount of free and available aflatoxin (Saalia FK and Phillips RD 2011). This theory is supported by the fact that these effects are only seen at extended times (60 min vs. 15 min) and this implies that there is an equilibration period. Furthermore, calcium chloride does not affect aflatoxin levels at a high pH value (13.1). This shows that calcium chloride is most important in systems with whole versus hydrolyzed protein, since, in the former, aflatoxin-protein interactions are stronger (Harter RD and Stotzky G 1973). There is also evidence that calcium chloride causes protein precipitation in the system. At a low pH where the protein is maintained in unhydrolyzed form, an increase in calcium leads to a decrease to decreased soluble nitrogen.

Unfortunately, the desirability functions only partially succeeded in mathematically modeling trends for protein and aflatoxin extraction, and quantifying the optimal conditions for maximization and minimization, respectively. To maximize protein, the function determined that zero clay should be added, which is in good agreement with results that document that adding clay under unhydrolyzed and high calcium conditions results in large decreases in soluble nitrogen. This presents an interesting problem potentially for designing

animal feed rations, as excess clay could bind protein and therefore reduce the nutritional value of the feed. Mechanistically, both whole protein and hydrolyzed protein can bind to clay, and it is unclear which structure confers more efficient binding (Harter RD and Stotzky G 1973). The predicted pH to maximize protein represents an interesting point, as it is just below the pH where extensive hydrolysis of the protein occurs (see Section 3.3.8). However, the equation predicts that for long extractions, protein is maximized at a pH <11 and at high levels of calcium. Experimentally, this is not possible as that combination of factors will cause the calcium to precipitate protein. It is likely that a follow-up study with refined pH and calcium chloride ranges could address this problem. The error associated with this predicted function is reflected in the disagreement between the predicted protein and experimentally determined protein in Test Point 1. The prediction of zero protein for the low protein model indicates that the decrease in protein is driven primarily by time and pH. On refinement, this prediction curve would likely taper off faster toward low protein values.

While the protein model has some mathematical error, generally, all trends made good experimental sense. With the aflatoxin data, the analysis of the response surface showed certain intrinsic problems. Under any time, pH, or calcium chloride conditions, increasing the level of clay up to 2% correlated to a decrease aflatoxin, and then increasing it from 2% - 4% correlated to an increase in aflatoxin. Previously published studies clearly refute this result and have explicitly shown that increasing clay is correlated to decreasing aflatoxin linearly (Phillips TD and others 1988; Seifert LE and others 2010). It is important to note that the 95% confidence interval for clay and calcium chloride on aflatoxin are extremely broad, and do not indicate an upward or downward curve. Furthermore, it is

important to emphasize here the limited nature of quadratic fits. Due to its parabolic nature, after an inflection point, the curve will trend in the other direction. While 2% clay may be an accurate inflection point, in reality, the curve may simply plateau or change slope numerically, not in sign. Thus, though this model is quite accurate with an R^2 of .97, it has mathematical limitations that could only be corrected for by modeling with a unique nonlinear fit. These methods are not traditionally employed in response surface analysis due to the complexity of the data.

One reason more error may be seen in the aflatoxin versus protein data is that the quantity of aflatoxin detected is not solely dependent on the amount extracted, but also on the amount reversibly or irreversibly degraded. Alkaline degradation of aflatoxin has been observed in corn processes during cooking (Dearriola MD and others 1988), but this is a rare and poorly understood phenomenon. It is hypothesized that degradation is only irreversible on covalent bonding of aflatoxin to nucleophiles, facilitated by the alkaline conditions opening the lactone ring (Kiermeie F and Ruffer L 1974; Dearriola MD and others 1988; Perez-Flores GC and others 2011). Work is ongoing to understand this phenomenon of interest in a simple aqueous system.

It is important to reconcile the high aflatoxin values reported throughout the central composite design study (section 3.3.3) with the irreversible degradation of aflatoxin under alkaline conditions (pH 11.5) reported in section 3.3.1. While the aflatoxin levels contained in these alkaline dispersions are high (maximum 634 ng/g), it is useful to put this number in perspective. With the starting peanut meal containing an average aflatoxin content of 373 ng/g, there is a maximum of ~13,000 ng of aflatoxin being extracted from 35 g of peanut

meal into 157.5 ml of water. Assuming a density of 1.02 g/ml, and a total solids of 4%, a sample with 100% of the peanut meal aflatoxin extracted would contain 2,031 ng/g on a dry weight basis. A proportionate amount of the aflatoxin in the meal is expected to remain in the insoluble fraction bound to peanut proteins, and in protein-optimized systems, only a portion of the protein is soluble. However, in the high aflatoxin/high protein system, the aflatoxin value on a dry weight basis is still approximately 38% less than that of a conservative estimate assuming 50% soluble aflatoxin. Therefore, levels are in fact likely reduced due to alkaline conditions as demonstrated in Figure 3.5b. This could be explained by the conjugation of aflatoxin to peanut meal protein and sugars. When this pH study is completed in a pure, isolated system (without nucleophiles), the irreversible loss of aflatoxin on pH adjustment is not seen (data not shown).

Subsequent enzymatic treatment of the low aflatoxin (< 50 ng/g) extracts (sample 1, Table 3.10) reduced aflatoxin to well below 20 ng/g (the US legal limit). However, the mechanism by which treatment with MnP and cofactors does so is not clear. As evidenced by sample 2a (Table 3.10), there is reasonable support that the cofactors could account for the entirety of the aflatoxin degradation reported. This is not necessarily surprising, as hydrogen peroxide is known for its ability to conjugate the lactone ring of aflatoxin. These findings are in good agreement with Rhee and others (1977) who reported that a solution of 0.3 % (0.1 mM) H₂O₂ for 30 min achieved nearly 100 % degradation of aflatoxin in a 900 ng/g peanut concentrate solution (Rhee KC and others 1977).

It is also important to consider the variability in aflatoxin degradation in sample 2. What differentiates this sample from sample 1 is that 1) it has much higher protein, 2) it was

prepared at a much higher pH (12.6) and thus is overall more hydrolyzed (Figure 3.13), 3) it has much higher aflatoxin, and 4) it showed significant, large variation in the aflatoxin, protein, moisture, and total solid values between dispersion replications. These factors likely influenced enzymatic treatments in this experiment as adjustment of the pH to more acidic values (6 and 8) caused varying degrees of protein precipitation. Future studies will need to control for these problems by working with the soluble phase prepared from one single dispersion and by adjusting the pH within each enzymatic preparation individually. Though this work holds promise, more studies certainly need to be conducted to address these problems.

As a secondary step to protein extraction and aflatoxin sequestration with clay, washing with hydrogen peroxide may have excellent promise as a cheap and highly effective aflatoxin-removal step. However, peroxidase enzymes still warrant evaluation in peanut meal soluble extracts for their potential protein allergen effects and cross-linking behavior, though changes in polymerization were not observed in SDS-PAGE samples.

3.5.0 Conclusion

The use of response surface modeling to explore aflatoxin and protein extractions from peanut meal highlighted many previously unreported trends regarding alkaline degradation of aflatoxin, alkaline hydrolysis of protein and calcium interactions with clay and protein. pH was the most significant variable influencing the quantity of aflatoxin and protein extracted and was highly positively correlated with both response variables. It was also shown that high pH conditions could cause aflatoxin to irreversibly bind to other co-solutes in a peanut meal extract. From a processing standpoint, it is extremely difficult to

balance the goal of minimizing aflatoxin and maximizing protein in dispersions. Optimal pH should be < 12 (before alkaline hydrolysis, amino acid racemization (Liardon R and Ledermann S 1986), or degradation), time should be minimized, clay level should be at least 2%, and calcium should not be added. It was found that isoelectric precipitation of protein could achieve a ten-fold reduction in protein remaining in the soluble phase, suggesting that this could be an appropriate processing step for protein isolation. Based on the preliminary secondary processing experiments, it is likely that hydrogen peroxide is responsible for the degradation of aflatoxin in treated samples. This conclusion is supported by the degradation observed in a control treatment with only enzyme cofactors, and the lack of polymerized protein generated in the enzyme-treated system.

The interaction effects characterized in this thesis highlight the complexity of the relationship between aflatoxin and protein. It is challenging to balance and optimize the extraction parameters that influence the clay's electrostatics, the solubility of protein, and the amount of both soluble and free aflatoxin in the system. From a scientific exploration perspective, the nuances of aflatoxin and protein interactions in the peanut have remained relatively uncharacterized in the literature. This work laid forth result-grounded hypotheses regarding the alkaline degradation of aflatoxin and protein-clay interactions and provided accurate and insightful models to characterize the extraction of aflatoxin and protein from peanut meal.

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