CHARACTERIZATION AND SAFETY OF CLAYS AS POTENTIAL DIETARY SUPPLEMENTS TO PREVENT AFLATOXICOSIS

A Dissertation

by

ALICIA GUADALUPE MARROQUIN-CARDONA

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2011

Major Subject: Toxicology

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Committee Members,	Youjun Deng
	Charles Thomas Hallmark
	Roger Harvey
Chair of Toxicology Faculty,	Weston W. Porter

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ABSTRACT

Characterization and Safety of Clays as Potential Dietary Supplements to Prevent Aflatoxicosis. (May 2011)

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Chair of Advisory Committee: Dr. Timothy D. Phillips

Aflatoxins are toxic metabolites produced by *Aspergillus flavus* and *A. parasiticus* fungi. Aflatoxin B_1 (AFB_1) is the most toxic and is a potent carcinogen with antinutritional and immunosuppressive effects. Several natural outbreaks of poisoning have been reported in both animals and humans, with fatal consequences in some of the cases. Inclusion of clay minerals in the diet is a promising strategy to reduce the bioavailability of aflatoxins from contaminated foods. Several clay-based products are currently sold as "mycotoxin binders" for addition in feeds, many of them lacking of detailed efficacy and safety data. Similarly, clays intended for human consumption in different countries also lack of safety studies, and for most of them the mineral composition is unknown. Earlier studies in our laboratory have shown that NovaSil clay (NS), a Ca-bentonite, is able to reduce the adverse effects associated with aflatoxin exposure in different animal species and recent human trials have confirmed its efficacy and safety. Most clays are derived from naturally-occurring deposits and batch-to-batch variations in composition, particle size, non-framework trace metal content and dioxin

levels are expected. Therefore, objectives in this research were to determine the mineral composition, aflatoxin binding capacity and potential safety of "mycotoxin binders" and edible clays for humans, and to investigate the mineral characteristics and safety of two potential aflatoxin adsorbents, a refined clay with more uniform particle size (UPSN) and a sodium bentonite (Na-BENT). Both clays have low dioxin/furans and heavy metals levels. According to mineralogical analysis, most of the "binders" contained montmorillonite but the sorption capacities for AF varied. Most of the edible clays for humans contained kaolinite, mica and quartz, and they had low sorption capacities for aflatoxin. UPSN and Na-BENT had similar mineral characteristics and high sorption capacities for aflatoxin. After a 3-month rodent study using Sprague Dawley rats, no overall toxicity of was observed for either clay. No changes were observed for most of the blood and serum biochemical parameters. Important findings included the increased serum Na, Ca, vitamin E and Na/K ratio and the reduction of serum K and Zn (in males) due to ingestion of the bentonites. Nonetheless, all parameters fell within the normal ranges reported for rats less than 6 months old and no trends of dose dependency were observed. We conclude that ingestion of low levels of bentonites does not present a health risk.

ACKNOWLEDGEMENTS

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I. INTRODUCTION

1.1 Aflatoxins

Aflatoxins (AFs) are harmful by-products of mold growth that are potentially fatal to humans and animals (CAST 2003). AFs are produced by the common fungi *Aspergillus flavus* and *A. parasiticus*; they are heat stable and resistant to a wide variety of food processing methods (CAST 2003). AFs were first identified in the United Kingdom in 1961, where highly contaminated animal feed was responsible for the deaths of 100 000 turkeys (Sargeant et al. 1961). Staple foods that are highly susceptible to aflatoxin (AF) contamination include maize, peanuts (Phillips et al. 2006), cottonseed and tree nuts (IARC 2002). There are four major AFs: aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1) and aflatoxin G_2 (AFG_2) that occur as direct contaminants of foods and feeds (Figure 1). Additionally, an important metabolic product, aflatoxin M_1 (AFM_1), is commonly found in milk of animals consuming AFB_1-contaminated diets (Figure 1). This metabolite is considered a group 2B carcinogen and is of particular importance due to the high consumption of milk and milk derived products by infants.

1.1.1 Chemical and physical properties of aflatoxins

AFs are colorless to pale-yellow crystals that intensely fluoresce under ultraviolet light. AFB_1 and AFB_2 emit blue fluorescence and AFG_1 and AFG_2 emit green or green–blue fluorescence, while AFM_1 emits blue–violet fluorescence (Castegnaro et al. 1980;

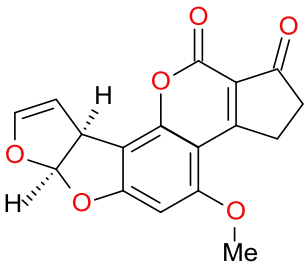
This dissertation follows the style of *Food Additives and Contaminants.*

Hartley et al. 1963; O'Neil et al. 2001). Melting points of AFs range from 237 to 299 °C. They are slightly soluble in water (10–30 μg mL^{-1}); insoluble in non-polar solvents; soluble in moderately polar organic solvents, e.g., chloroform and methanol, and especially dimethyl sulfoxide (Cole and Cox 1981). Commonly, AFs are unstable to ultraviolet light in the presence of oxygen, and are particularly susceptible to extreme pH values (< 3 and > 10) and to oxidizing agents. AFs can be degraded by reaction with ammonia or sodium hypochlorite, with the lactone ring being particularly susceptible to alkaline hydrolysis (Castegnaro et al. 1980; 1991; O'Neil et al. 2001).

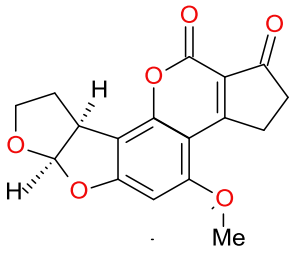
1.1.2 Occurrence of aflatoxins

AFs can be found in a variety of agricultural commodities, but is more frequently found in maize, peanuts, cottonseed, and tree nuts. Their occurrence is worldwide, but is common to find higher prevalence in tropical regions. This correlates well with the climate conditions that promote *Aspergillus* growth (Figure 2), which include drought, excessive rainfall and flooding. Recently, global climate change may be responsible for the emergence of AF problems in crops from regions that used to have low incidence of AF outbreaks. Under favorable pre-harvest conditions, *A. flavus* and *A. parasiticus* can produce AFs in developing seeds of corn, peanuts, cotton, almond, pistachio, and other tree nuts (Bhatnagar et al. 2002; CAST 2003). Contamination with the mold is not always coupled with toxin production. The production of AFs depends on many environmental and nutritional factors including moisture, temperature, substrate, aeration, pH levels, quantity of carbon and nitrogen sources, lipid content,

concentrations of different metal salts and specific nutrient requirements (Cary et al. 2000; CAST 2003; Ominski et al. 1994).



AFB_1: $C_{17}H_{12}O$ MW: 312.3



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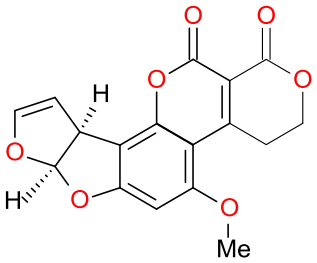
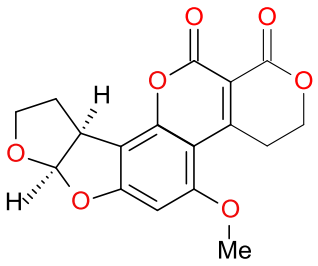
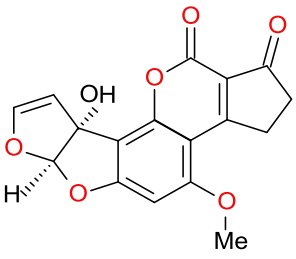


AFG_1: $C_{17}H_{12}O_7$ MW: 328.3



AFG_2: $C_{17}H_{14}O_7$ MW: 330.3



AFM_1: $C_{17}H_{12}O_7$ MW: 328.3

Figure 1. Molecular structures of the four major aflatoxins. AFB_1, AFB_2, AFG_1, and AFG_2, plus the important metabolic product AFM_1.

Figure 2. Corn artificially contaminated with *Aspergillus flavus*. Courtesy of Dr. Youjun Deng from the Soil and Crop Sciences Department at Texas A&M University.

Contamination with AFs can occur in preharvest as well as during storage depending on environmental conditions. Temperature ranges from 25-34 °C (Wilson and Payne 1994) and moisture levels exceeding 7% (Williams et al. 2004) are the main factors that influence preharvest and storage contamination. Water activity (a_w) is a particularly important factor for contamination during storage. Values of $a_w \geq 0.85$ have been shown to result in increased contamination of susceptible crops (Wilson and Payne 1994).

On the other hand, AFM_1, the hydroxylated metabolite of AFB_1, mainly occurs in milk and milk derived products. As a biomarker of exposure to AFB_1, the quantification of this metabolite in urine is well documented in humans and animals (Jolly et al. 2006).

From the cytochrome P450 enzymes, CYP1A2 is particularly responsible for oxidizing AFB_1 into the AFM_1 metabolite (Eaton et al. 1994; Wang et al. 1999) (Figure 3). Even though AFM_1 is less toxic than the parent compound, it is still considered a public health concern. Different regulatory agencies like the Food and Drug Administration (FDA) and the European Food Safety Authority (EFSA) have established maximum permitted levels of 0.5 $\mu g\ L^{-1}$ (FDA 2011) and 0.05 $\mu g\ L^{-1}$ (EFSA 2004) in milk, respectively. EFSA has a more rigorous regulation for infant formulas, with a permitted level of 0.025 $\mu g\ L^{-1}$ (EFSA 2004). To comply with these requirements strict maximum allowance levels have been established for AFs in feed ingredients used for dairy cows; for instance the level of AF allowable in feed has been set to 20 $\mu g\ kg^{-1}$ in the U.S.A.

AFs can also co-occur with other mycotoxins. The most common and potentially dangerous case of co-occurrence is with fumonisin B_1 (FB_1). Several authors have documented the presence of both mycotoxins in samples of corn used for human consumption (Kimanya et al. 2008; Ueno et al. 1997). In a short-term carcinogenesis model using rat livers, Gelderblom (2002) demonstrated the cancer promoting activity of FB_1 in AFB_1-initiated hepatocytes. These synergistic toxicological and carcinogenic effects are of particular importance since populations at high risk of aflatoxicosis can also be co-exposed to fumonisin (Ueno et al. 1997).

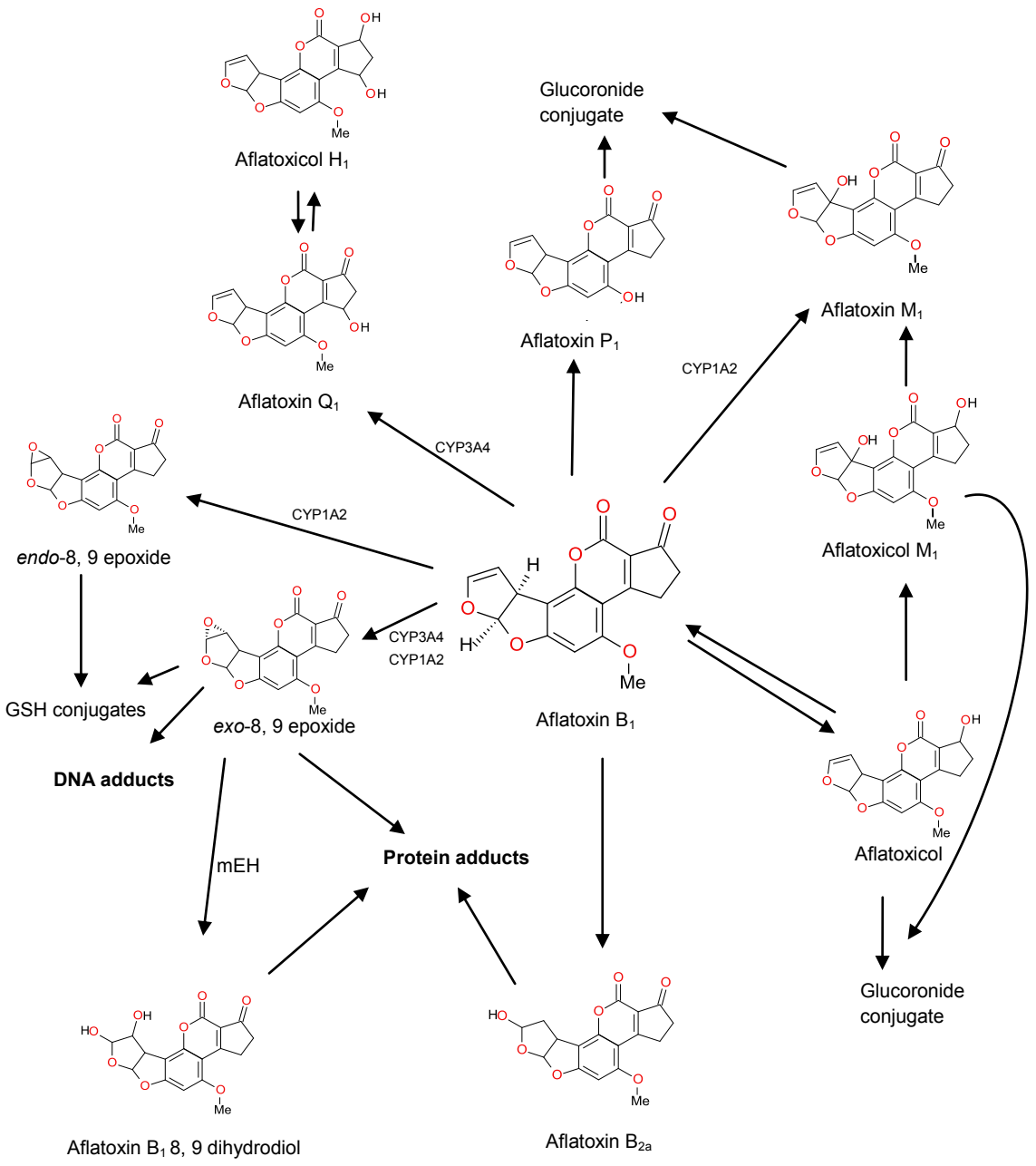


Figure 3. Biotransformation pathways for AFB_1. The AFB_1 *exo*-8,9-epoxide is the metabolite responsible for protein and DNA adduct formation. Adapted from Eaton et al. 1994.

1.1.3 Exposure to aflatoxins

Human exposure to AFs occurs predominantly through consumption of contaminated foods. In order to create a global report of exposure to this toxin, different countries have submitted information on the levels of exposure to AFs through foods to the Joint FAO/WHO Expert Committee on Food Additives (JECFA). Australia estimated an average intake of 0.15 ng AF per kg body weight (bw) per day (JECFA 1998). The estimate for China was reported to range from 0 to 91 μg AFB_1 kg-bw^{-1} per day (Chen 1997). The European Union (9 countries) provided estimates of AF intake ranging from 2 to 77 ng per person per day for AFB_1 and from 0.4 to 6 ng per person per day for AFM_1 (JECFA 1998). In the U.S.A., the FDA estimated intakes by using data from the National Compliance Program for maize, peanut and milk products. The results revealed an intake of 18 ng per person per day for AFB_1 and 44 ng per person per day for AFM_1. The AFM_1 intakes estimated for developed countries differ markedly from African countries like Zimbabwe, for which theoretical maximum intake of AFM_1 for a child was estimated to be 1.05 μg per day (JECFA 1998). In most places, AFM_1 exposure, at levels of nanograms per day, occurs mainly through consumption of AF-contaminated milk, including mother's milk.

AFs can also be absorbed through the lungs and skin (Coulombe 1994; Leeson et al. 1995). Respiratory tract exposure is mainly due to inhalation of AF-contaminated dust (Coulombe 1994). Recently, in utero exposure to AFs has been documented. Studies in The Gambia have shown AF-albumin adducts in maternal, cord, and infant

(<1 year old) blood, demonstrating the importance of this source of exposure and its association with growth faltering (Turner et al. 2007).

1.1.4 Metabolism of aflatoxins

AFs must be bioactivated in order to exert their toxic and carcinogenic effects. Extensive research has focused on the biotransformation pathways for AFB_1, since it is the most prevalent and carcinogenic of the AFs (Figure 3). AFB_1 metabolism includes major biochemical processes like oxidation (Essigmann et al. 1977; Groopman 1994), reduction (Wong and Hsieh 1978), hydroxylation (Moss and Neal 1985), and conjugation (Holeski et al. 1987). Before systemic distribution, AFB_1 can be metabolized in the cell barrier of the small intestine by cytochrome P450 enzymes (CYP). Enterocytes are known to express high levels of CYP3A isozymes and AFB_1-adduct formation has been documented in intestinal cells from rats and humans (Kolars et al. 1994). After absorption through the gastrointestinal tract, AFB_1 enters the liver through the portal vein and then distribution to most soft tissues and fat deposits can occur. Nevertheless, the majority of the toxins accumulate in the liver and kidneys, where most biotransformation occurs (Leeson et al. 1995). In the human liver, AFB_1 is mainly metabolized by CYP3A4 and CYP1A2 isozymes, leading to the formation of AFB_1 *exo*-8,9-epoxide and *endo*-8,9-epoxide (Gallagher et al. 1994; Guengerich et al. 1998). The formation of the *exo*-8,9-epoxide has been reported to be the more dominated form. Because of its instability, this *exo*-epoxide can covalently bind to DNA, commonly at the N-7 position of guanine (Essigman et al. 1983; Gopalakrishnan et al. 1990). This adduct can trigger a mutation in codon 249 of the p53 protein, specifically a G-T transversion,

which has been observed in hepatocarcinoma (HCC) cases from regions with high AF exposure (Smela et al. 2001). AFB_1 can also undergo hydroxylation and demethylation, resulting in the formation of AFM_1, AFQ_1 (Ramsdell and Eaton 1990), and AFP_1 (Eaton et al. 1994; Wong and Hsieh 1980), respectively. All these metabolites are excreted in urine (Coulombe 1993; Groopman et al. 1985). Additionally, lipoxygenases and prostaglandin H synthase may also play a significant role on AF metabolism in extra-hepatic organs (Battista and Marnett 1985; Liu and Massey 1992). Metabolic enzymes in the phase I group, such as epoxide hydrolases (EH), are responsible for the conversion of the epoxide to dihydrodiols, while enzymes in the phase II, such as glutathione S-transferase (GST), convert the epoxide into a more soluble form by addition of GSH conjugates. These products are eventually excreted in urine (Hayes et al. 1993; Wild and Turner 2002), although a small portion may remain in body fluids and tissues (Leeson et al. 1995).

There are animal species differences in metabolisms of AFs, these differences account for the variations in toxicity and carcinogenic effects exerted by AFs in animals and humans. As an example, chickens and mice are very resistant species, while rabbits and ducklings are highly susceptible (Roebuck and Maxuitenko 1994) and this is likely due to their differences in metabolism.

1.1.5 Toxic effects of aflatoxins

Children and the young of all animal species are the most susceptible to the toxic effects of AFs. The International Agency for Research in Cancer (IARC) considers AFB_1 and mixtures of AFs as group 1 carcinogens, while AFM_1 is listed as a group 2B.

Signs and symptoms like jaundice, fever, ascites, vomiting and edema of the feet have been observed in documented cases of aflatoxicosis in India (Krishnamachari et al. 1975). Fatalities occurred in people that may have consumed 2-6 mg AF daily over a month. One important observation noted in these cases, was the possible tolerance of women to AFs, since men appeared to be more susceptible. In Kenya, centrolobular necrosis of the liver was associated with the consumption of maize contaminated with AFB_1 levels up to 12 mg kg^{-1} (Ngindu et al. 1982). AFs have also been associated with the development of cirrhosis (Amla et al. 1971), kwashiorkor (Hendrickse and Maxwell 1989), and encephalitis and fatty degeneration of the viscera (Shank et al. 1971).

Outbreaks of aflatoxicosis have been reported in different species of farm animals. Some important outbreaks in poultry have occurred in India (Choudary and Rao 1982; ICRISAT 2002) where high mortality occurred. The swine industry has also been affected, for instance, a recent outbreak was reported in Sentinela do Sul, RS, Brazil, in 2004. Apathy, anorexia, jaundice, dark urine, hematuria, and photosensitization were among clinical signs observed. Necropsy revealed generalized jaundice, an orange-yellow liver, edema of the gall bladder wall and yellowish effusion in the abdominal and pericardial cavities (Zlotowski et al. 2004). Although bovines are more resistant to the AF effects than other species, they can also be affected by AFs. There is a report from 1985, where more than 200 head of feedlot cattle died of aflatoxicosis after eating contaminated feed with levels of AFs from 96 to 1700 μg kg^{-1} (Osweler and Trampel 1985). In that study, diagnosis was based on macroscopic findings on liver, AF levels in cottonseed feed products and detection of AFB_1 and AFM_1 in urine and liver from

affected calves. Cases of aflatoxicosis have also been documented in companion animals, such as dogs. Earlier reports include the natural outbreak of 1987 in the Republic of South Africa (Bastianello et al. 1987), in which 10 cases were analyzed and classified as acute (1 case), subacute (7 cases), and chronic (2 cases), according to the severity of the lesions in liver and bile ducts. Another example of aflatoxicosis in dogs includes the 1998 episode in Texas, with 55 confirmed deaths of dogs. The food implicated in that outbreak contained approximately 150 ppb AFs (Garland and Reagor 2001). A more recent outbreak in dogs in the United States started in December 2005, in which 2 dogs died of acute hepatic failure after consuming a commercially available dog food (Stenske et al. 2006). After those cases, more dogs were reported to develop clinical signs of aflatoxicosis. The FDA was notified and testing confirmed contamination with AF. In spite of the prompt response of the FDA and veterinarians, approximately 100 dogs died due to the consumption of the contaminated diets. The amount of AF in two of the commercial diets obtained from affected owners had levels of AFs of 60 and 223 $\mu g\ kg^{-1}$, respectively (Stenske et al. 2006).

Besides the macroscopic lesions caused by AFs in different organs, AFs are also known to cause DNA injury (Cullen and Newberne 1994). Chromosomal aberrations and strand breaks, micronuclei, sister chromatid exchange, unscheduled DNA synthesis, and DNA-adduct formation are examples of modifications that AFB_1 has been reported to produce (IARC 1987). Even though, the liver is the predominant target organ for toxicity and carcinogenicity (Miller and Wilson 1994), other organs and complete systems can be affected (Coulombe 1994; Kolars et al. 1994; Larsson and Tjalve 1995).

This appears to be the case of the immune system, for which AFs have been shown to have suppressive effects (Bondy and Pestka et al. 2000). Induction of thymic aplasia (Pier 1986), reduction of T-lymphocyte function and number, suppression of phagocytic activity, reduction of complement activity (Pier 1986; Reedy et al. 1987; Richard et al. 1978) and modifications in cytokine secretion and interleukin gene expression (Han et al. 1999; Moon et al. 1999) are among the suppressive effects exerted by AFs in the immune system of different animal species. Data for the immunosuppressive effects of AFs in humans is scarce. However, studies by Turner et al. (2003) revealed important changes on immunity in Gambian children as a function of AF-albumin adducts. Recently, in a Ghanaian population highly exposed to AFs, Jiang et al. (2005) found significantly lower percentages of CD3+ and CD19+ cells expressing the CD69+ activation marker, than in participants with low exposure. In the same study, the percentages of CD8+ T cells that contained perforin or perforin and granzyme A, were significantly lower in participants with high AF levels. The changes in cell counts were significantly correlated with AF, further supporting its potential immunosuppression effects in humans.

1.1.6 Carcinogenic effects of aflatoxins

The most severe carcinogenic effect associated to AFB_1 exposure is the increased incidence of HCC (Shupe and Sell 2004), which is one of the four most common cancers and the third leading cause of cancer worldwide (National Cancer Institute 2010). About 80% of the global HCC cases occur in developing countries (Wild and Hall 2000). Internationally, there are geographic variations with age-specific incidence estimated to

be 3 and 80 per 100,000 people in North America and China, respectively (Dyer et al. 2005), the highest rate being found in Sub-Saharan Africa, as well as East and Southeast Asia (Dyer et al. 2005; El-Serag 2003). The risk of hepatocarcinoma from AF consumption is significantly higher in individuals chronically infected with hepatitis B virus (Henry et al. 2002; Sun et al. 1999). Besides liver carcinogenicity, AFB_1 has been implicated as a cause of cancer development in other organs, such as the colon (Wogan and Newberne et al. 1967), glandular stomach (Butler and Barnes et al. 1966) and kidney (Epstein et al. 1969). AFB_1 is a procarcinogen that has to be metabolically activated to the 8,9-epoxide, which is the ultimate carcinogen (Lin et al. 1977). This epoxide can form the nucleic acid adduct, N7-guanine derivative 2,3-dihydro-2-N7-guanyl-3-hydroxyaflatoxin B_1 (Muench et al. 1983) (Figure 4), resulting in a DNA mutation, specifically a GC-TA tranversion (Foster et al. 1983). These adducts are more frequently found in mitochondrial DNA than in nuclear DNA, specifically in the p53 gene. This adduct leads to a transversion in the base pair 3 of codon 249 (Hollstein et al. 1991) suggesting AF exposure can enhance carcinogenicity by structural alteration of a tumor suppressor gene (Dragan and Pitiot 1994).

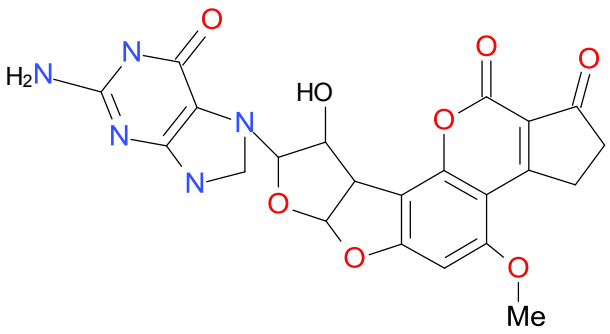


Figure 4. N7-guanine derivative 2,3-dihydro-2-N7-guanyl)-3-hydroxyaflatoxin B_1. This adduct is excreted in urine and has been used as a biomarker of exposure to AFs. Adapted from Groopman 1994.

1.2 Strategies to control aflatoxins

Some strategies for the reduction of AFs include; thermal inactivation, irradiation, solvent extraction, mechanical separation, density segregation, adsorption from solution, microbial inactivation, chemical inactivation, chemoprevention and clay therapy (Kensler et al. 1994; Phillips et al. 1994). Among the different methods, the last three are the most practical for feeds and foods.

1.2.1 Chemical inactivation

Ammoniation is the most viable chemical approach to detoxify AFs and under the right conditions, it can result in a significant reduction of AFs in some staple foods (Dollear et al. 1968). The ammoniation process, using ammonium hydroxide or gaseous ammonia is successfully used to reduce the AFB_1 levels by more than 99% in corn,

peanut-meal cakes and whole cotton seed products. The mechanism of detoxification by ammoniation appears to involve the hydrolysis of the lactone ring and chemical conversion of the parent compound AFB_1 to products with lower toxicity (Figure 5). The first step in the reaction is reversible if the ammoniation process is not correctly done, however if the reaction is allowed to proceed sufficiently, the process is irreversible and degradation is achieved. Several countries like Mexico, France, South Africa, USA, Senegal, and Brazil, are already applying this method to effectively decontaminate AFs (Phillips et al. 1994).

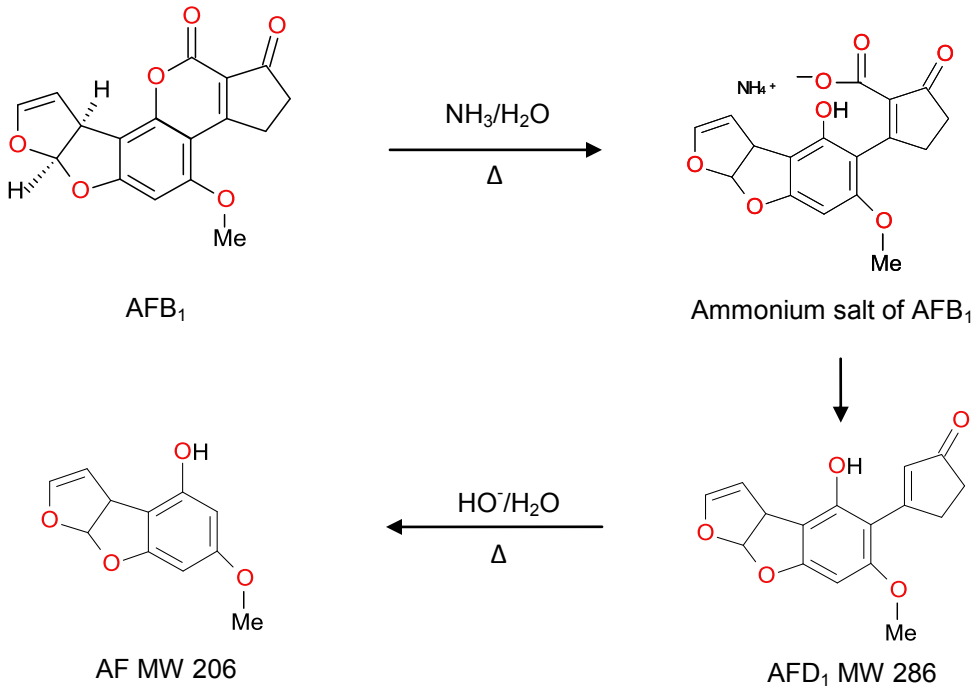


Figure 5. Ammoniation reaction of AFB_1. The ammonium salt of the lactone is formed when AFB_1 is reacted with ammonia, then it is decarboxylated by heat to form aflatoxin D_1 and a product with molecular weight of 206 g mol^{-1}. Adapted from Phillips et al. 1994.

Another method that has been used to inactivate AFs is ozonation. This method is been successfully tested *in vitro* (McKenzie et al. 1997) and *in vivo* (McKenzie et al. 1998). In the latter study, a reduction of AFs > 95% was achieved in naturally contaminated corn using electrochemically generated ozone (O_3) at 200 mg min^{-1}. One day old turkeys were fed this treated corn for three weeks. Parameters measured included body weight, relative organs weights, serum enzyme activities, hematological parameters and blood chemistry. After the study period, there was no significant difference between the control group and the group receiving O_3 treated corn, while significant differences were observed in the animals ingesting AF contaminated corn. Importantly, O_3 treatment resulted in no negative effects and appeared to be safe for the poults while protecting them from the toxic effects of AFs. The mechanism of AFB_1 degradation by O_3 involves the disruption of the C8 and C9 double bond (Figure 6), which is the site responsible for its carcinogenic and mutagenic effects. Contrarily to ammoniation, which opens the lactone ring but does not disrupt the C8 and C9 double bond, ozonation is a more desirable method to chemically degrade AFs that contain this double bond.

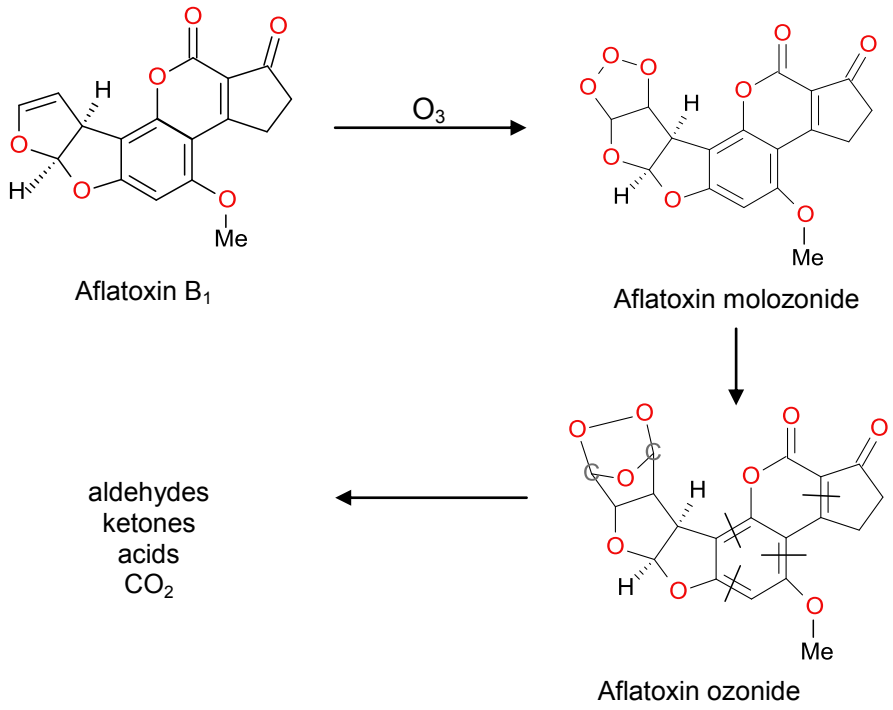


Figure 6. Degradation of AFB_1 by ozononation. The primary site of attack of ozone is at the C8–C9 double bond on the terminal furan. This site has been shown to be responsible for aflatoxin's mutagenicity and carcinogenicity. Adapted from McKenzie et al. 1998.

1.2.2 Chemoprotection

Chemoprotection is known to be an effective approach for cancer prevention in humans (Hong et al. 1990). Possible mechanisms of action of some chemoprotectants used for AFs toxicity are: 1) induction of certain phase I enzymes (enhancement of detoxification); 2) inhibition of phase I enzymes (delay of activation); 3) induction of phase II enzymes (e.g. glutathione-S-transferase, GST), for enhancement of

detoxification and elimination; and 4) nucleophilic trapping of reactive intermediates (Kensler et al. 1994). Several chemoprotectants have been shown to confer protection against deleterious effects of AFs *in vitro* and in animal studies. A few of them have been successfully translated to human trials.

Oltipraz is a dithiolethione that has been shown to protect against AFB_1 induced hepatocarcinogenesis in rodents (Kensler et al. 1992) and has also been used in human interventions. In an intervention trial in Qidong, People's Republic of China, the ingestion of oltipraz at high levels (500 mg per week), resulted in inhibition of phase I activation of AFs, while low levels (125 mg per week) increased phase II conjugation mechanisms, yielding higher excretion of AF-mercapturic acid (Wang et al. 1999). Clearly, both phenomena observed in this study can contribute to protection against the toxic effects of AFs in humans. In a recent double-blind, randomized, placebo-controlled trial performed among healthy individuals, oltipraz had no major effect on oxidative DNA damage, which suggests that prevention of oxidative DNA damage is not an important mode of action for its chemopreventive effects (Glintborg et al. 2006). This further supports the evidence that remediation of AF exposure through oltipraz relies on the chemoprotectant's ability to modulate phase I and II enzymes.

1.2.3 Clay therapy in animals and humans

The ingestion of clay by animals and humans is not a new practice. Geophagy, defined as the ingestion of earth or clay, has been practiced in many regions and is used as in traditional medicine in many African countries and China (Diamond 1999; Johns and Duquette 1991).

The use of clay-based products as enterosorbents for AFs is a frequently used strategy to reduce AF exposure in animals. Natural bentonites are the common sorbents used for this purpose. Earlier studies showed that inclusion of a calcium bentonite clay (NovaSil, NS) in animal feed, is able to reduce the adverse effects associated with AF exposure in different animal species (Phillips 1999; Phillips et al. 1988) and to decrease the level of an AFB_1 metabolite (AFM_1) in milk from lactating dairy cows and goats (Harvey et al. 1991; Smith et al. 1994). Equilibrium adsorption isotherms, molecular modeling, and *in vivo* studies, have been used to demonstrate that NS preferentially binds AFB_1 in the gastrointestinal tract, thereby reducing AF systemic bioavailability (Phillips 1999; Phillips et al. 2002). Importantly, findings from a recent clinical intervention study showed that NS was effective in reducing AF protein adduct levels in serum, and urinary excretion of the AFM_1 metabolite, in humans naturally exposed to AFs via contaminated foods (Afriyie-Gyawu et al. 2008b; Wang et al. 2008). Importantly, in this study, no nutrient interactions were observed due to NS consumption. Clay intervention for AFs appears to be the most culturally and economically plausible solution for populations that are at high risk for exposure. However, more research needs to be conducted in this area to characterize those smectites that are most favorable for AF sorption and those that are safe for prolonged human consumption.

1.3 Smectites as adsorbents for aflatoxins

1.3.1 Smectite structure

Bentonite is defined as a relatively impure clay rock mainly consisting of montmorillonite, the most abundant mineral from the smectite group, which also includes beidellite, nontronite, hectorite, saponite and sauconite. Smectites are 2:1 layered minerals of high importance in soils because of their expansive properties and net negative interlayer charge (Figure 7). The principal source of charge in smectites is isomorphic substitution, but some pH dependent charge is also present. For instance, in montmorillonite, the substitution of Mg^{+2} for Al^{+3} in the octahedral layer is what produces the layer charge. Smectites are mainly found in the clay fraction of soils developed in temperate regions, sediments and poorly drained environments. These minerals are well known for their small particle size of <2μm, and their high internal surface area of 600-800 $m^2 g^{-1}$. Smectites are classified according to the type of structure (i.e. dioctahedral or trioctahedral), the predominant octahedral cation (i.e. Mg^{+2}, Al^{3+} and Fe^{3+}) and the location of isomorphic substitution (Reid-Soukup and Ulery 2002) (Table 1). For industrial purposes, bentonites can be classified as sodium (Na) or calcium (Ca) depending on the cation found in greater amounts. Both cations have important effects on the behavior of smectites.

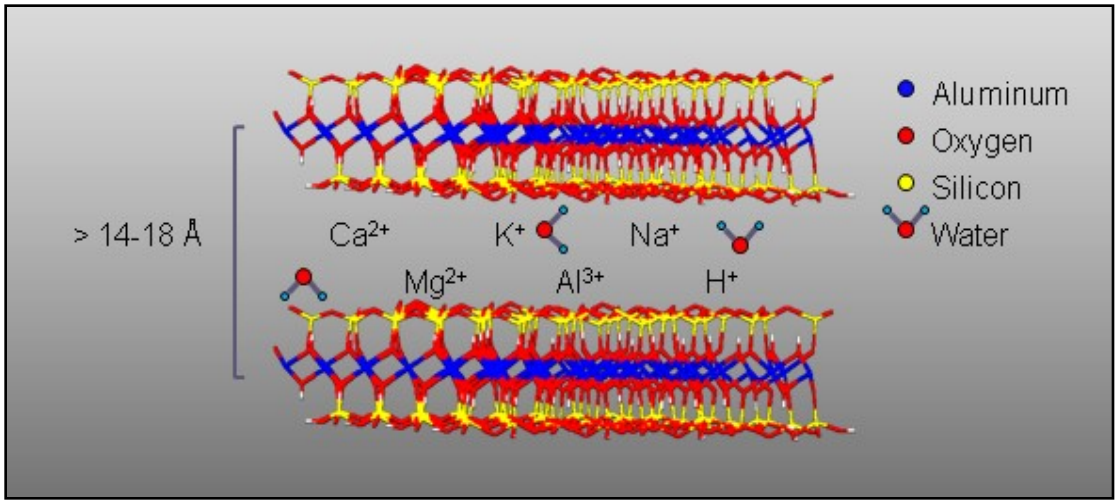


Figure 7. Smectite structure. The octahedral aluminum layer is depicted between the two silicon tetrahedral layers. Isomorphic substitution with Mg^{2+}, Al^{3+} and Fe^{3+} may occur in the octahedral layers. Water molecules and cations are shown in the interlayer zone.

Table 1. Smectite classification and ideal chemical structures

Substitution	Dioctahedral	Trioctahedral
Octahedral	Montmorillonite $M^{+}_{0.33}(Si_4)^{IV}(Al_{1.67}Mg_{0.33})^{VI}O_{10}(OH)_2$	Hectorite $M^{+}_{0.33}(Si_4)^{IV}(Mg_{2.67}Li_{0.33})^{VI}O_{10}(OH)_2$
Tetrahedral	Beidellite $M^{+}_{0.33}(Si_{3.67}Al_{0.33})^{VI}(Al_2)^{VI}O_{10}(OH)_2$	Saponite $M^{+}_{0.33}(Si_{3.67}Al_{0.33})^{VI}(Mg_3)^{VI}O_{10}(OH)_2$
	Nontronite $M^{+}_{0.33}(Si_{3.67}Al_{0.33})^{IV}(Fe^{+3})_2{}^{VI}O_{10}(OH)_2$	Sauconite $M^{+}_{0.33}(Si_{3.35}Al_{0.65})^{VI}(Zn_3)^{VI}O_{10}(OH)_2$

M represents one or more exchangeable metal cations and may have a value ranging from 0.2 to 0.6. Adapted from Reid-Soukup and Ulery 2002.

1.3.2 Sorption patterns and mechanisms of aflatoxin adsorption

Four main classes of adsorption have been described for organic solutes; this classification is based on the nature and slope of the initial portion of the curve. According to the progression of the curve, these major classes are divided into subgroups (Figure 8). The main classes are: H, L, C and S curves (Giles et al. 1960). The H class is depicted by molecules that show "high affinity" for a surface, hence the name. This pattern is characterized by a commencement at a positive value on the "concentration in solid" axis. The L or "Langmuir" patterns are usually indicative of molecules adsorbed in a planar arrangement on the surface. C patterns are represented by linear curves and usually describe a "constant partition" phenomenon, in which the availability of adsorption sites remains constant at different solute concentrations. The S pattern is indicative of a cooperative adsorption effect, in which molecules that have been initially adsorbed (monolayer) facilitate the adsorption of more molecules (multilayer).

According to isothermal analysis, the adsorption of AFB_1 onto smectites can be classified as a Langmuir 1 or 2 curve. In this pattern, the sorbent is reaching saturation or saturation has been reached as the concentration of the solute is increased. It has been reported that L type curves are commonly depicted by chemisorption mechanisms between adsorbates and adsorbents (Adamson 1982). An initial strong adsorption followed by a plateau in the isothermal plot, suggests a specific type of binding with subsequent saturation of that type of binding site (Grant and Phillips et al. 1998).

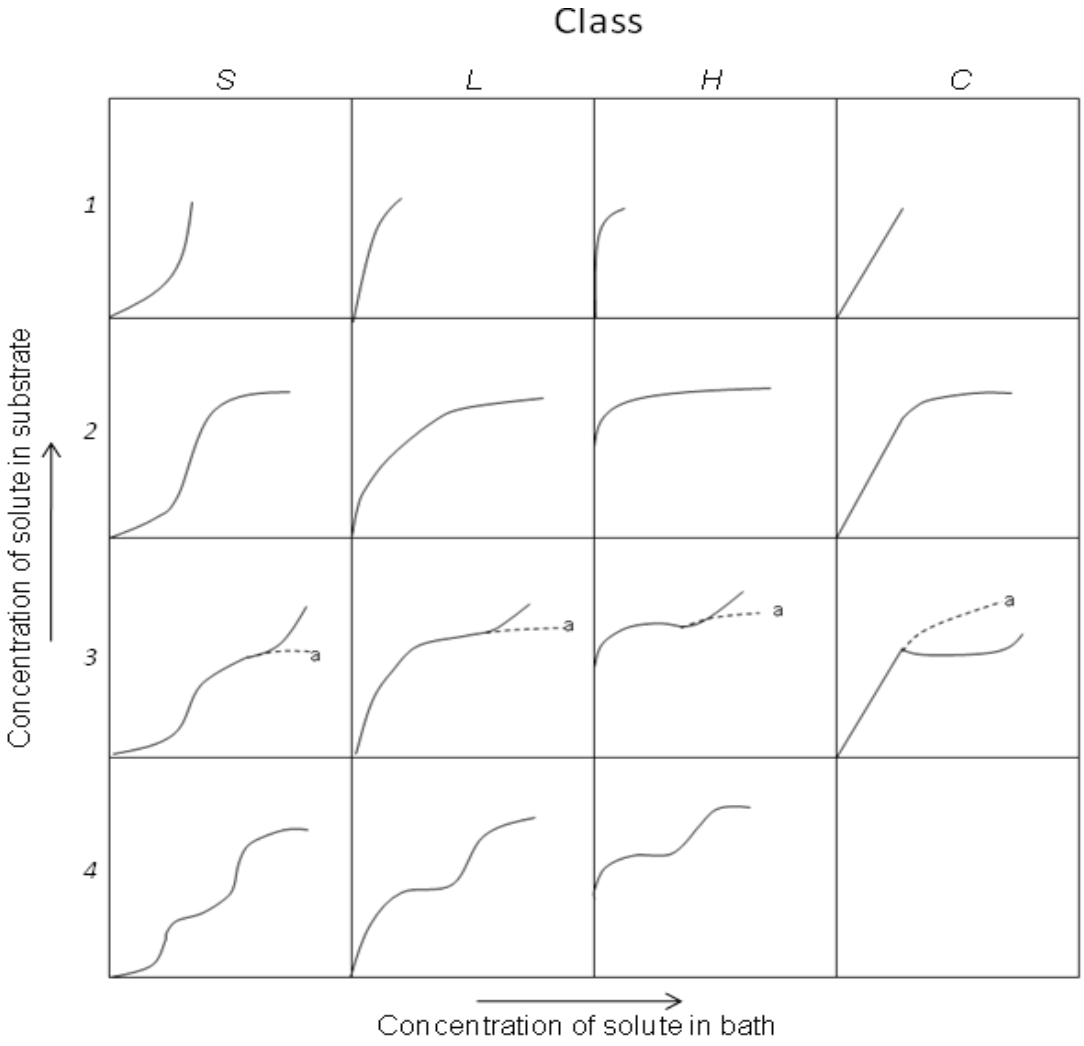


Figure 8. Classification of adsorption patterns. The patterns represent adsorption of organic molecules onto surfaces of adsorbents. Adapted from Giles et al. 1960.

Different sites of adsorption of AFB_1 onto the smectite surfaces have been proposed and include the external surfaces, edge surfaces (Desheng et al. 2005) and interlayer spaces (Kannewischer et al. 2006; Phillips et al. 2002). Data supporting the interlayer adsorption were first presented by Grant and Phillips (1998). In that study, a hydrated sodium calcium aluminosilicate (HSCAS) was heated at 800° C for 1 hr to collapse the interlayer area, after that, isothermal analysis of AFB_1 adsorption resulted in significant reduction of binding capacity, demonstrating the importance of the interlayer as an adsorption site (Figure 9). Recently, more evidence of the interlayer adsorption of AFB_1 was offered by Kannewischer et al. (2006); by using XRD, the authors documented the resistance of smectite clay to collapse when previously saturated with AF. In this study, smectite clay was mixed with AFB_1-benzene-acetonitrile solution for posterior heating at different temperatures, ranging from 100°C to 245°C. XRD patterns of these AF-saturated heated clays were recorded along with controls and the major finding was the AF-reacted smectites resistance to collapse at 245°C when compared to controls. More recently, Deng et al. (2010) verified the interlayer absorption of AFB_1 and had similar conclusions as Kannewischer. Other authors (Desheng et al. 2005) have shown opposite XRD evidence for interlayer absorption of AF onto montmorillonite clay when using different solvents such as methanol, acetone, chloroform and water. Nevertheless, in the Desheng's study, no heating treatments were done previous to recording the XRD patterns, and this may account for the differences observed among the studies.

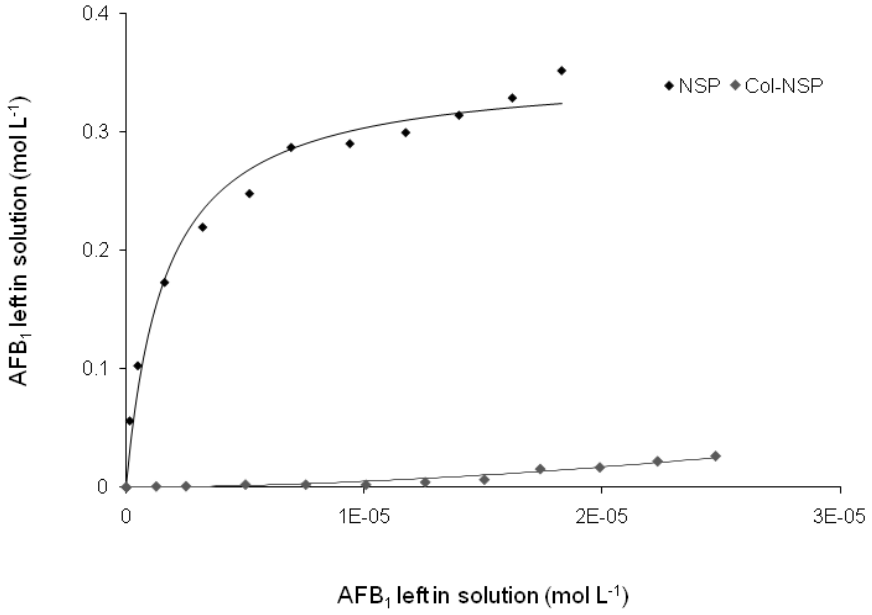


Figure 9. Aflatoxin adsorption isotherms on intact and collapsed NovaSil clay (Ca-montmorillonite). Collapse was achieved after heating the clay at 800°C for 1 hr.

There are not only various binding sites for AFB_1 sorption on montmorillonites, but there are also numerous proposed binding mechanisms that could be occurring at these respective sites. Previously, the following six mechanisms have been proposed: selective chemisorption, electron donor acceptor (EDA) mechanism, hydrogen bonding, bonding through a furan ring, ion dipole interactions and coordination between the exchange cations and the carbonyl groups.

Using simulated molecular models, Phillips et al. (1995) proposed that AFB_1 can be bound at the edges, the interlayer surfaces, and the basal surfaces of smectite by selective chemisorption. Evidence of selective chemisorption was collected through a

series of experiments which concluded that the carbonyls from the lactone and cyclopentenone rings of AF would form chelates with transition metals in the clay surfaces (Phillips et al. 1995). They specifically suggested the formation of a mononuclear (bidentate) chelate with the transition metals contained in the clay.

The electron donor mechanism was proposed later, based on experiments that calculated the enthalpy of adsorption. Phillips et al. (1999) documented an enthalpy value of -40 kJ mol^{-1} for AFB_1 bound to the surfaces of montmorillonite clay. In this study, the authors proposed that the partially positive carbon atoms of the two carbonyl groups on the AF molecule shared electrons from the negative surface of the clay. Additionally, they observed a positive correlation between the magnitude of partial positive charges on the carbons C11 and C1 of the *β*-dicarbonyl system of planar analogs and derivatives of AFB_1 and the strength of adsorption. The hydrogen bonding mechanism for AF binding to the clay, has been reported by Desheng et al. (2005). They proposed that formation of double hydrogen bonds between AF and the edge sites of montmorillonite is the main reaction mechanism of sorption. Additionally, Tenorio et al. (2008) proposed that epoxidation of the dihydrofuran ring may occur due to smectite-AFB_1 interactions and further suggested that this epoxidation may contribute to the bonding of the toxin to smectites. The chelation of AFB_1 with Ca^{2+} and other cations or edge-site metals was also proposed by Phillips et al. (1999) as potential mechanisms for binding. Observations made by Tenorio-Arvide et al. (2008) using infrared analyses, offered important support to the idea that exchangeable cations in the interlayer can coordinate with the two carbons of the coumarin moiety. Recently, more specific reports

on AFB_1 binding to smectites suggests that, according to the hydration conditions, the mechanisms involved can vary (Deng et al. 2010). For instance, ion-dipole interactions and coordination between exchangeable cations and carbonyl groups are proposed to be of major importance under dry conditions. However, under high moisture conditions, H-bonding between carbonyl groups and exchangeable-cation hydration-shell water are the predominant forces involved.

Clearly, some of the above mentioned mechanisms have more experimental evidence than others, and there might be other mechanisms that have not yet been investigated. Nonetheless, all of them may be occurring simultaneously which may account for the high capacity of smectite clays to bind AF.

1.4 Research objectives

The AF contamination of foods and feeds is a worldwide problem. Because of its practicality and acceptance, the addition of clay in the diets to prevent AF exposure represents a highly desirable strategy. There are many types of clays and confusion about how to select those which are most effective is an ongoing problem. More importantly, due to the natural origin of these clay minerals, some may contain more contaminants than others (e.g. heavy metals, dioxins and furans). For instance, kaolinite clays (which are consumed in many parts of Africa) have been associated with high levels of dioxins. Hence, clays intended for AF control in animals and humans must have sufficient studies that prove safety and efficacy. Studies in this dissertation focus

on the mineral characterization and safety assessments of clays with potential use as AF enterosorbents, and include the following specific objectives:

1) to elucidate the mineral characteristics, AFB_1 adsorption capacities and potential safety of edible clays available and marketed as AF binders for inclusion in animal feeds;

2) to investigate the mineral composition, AF sorption capacities and potential safety of edible clays intended for human consumption by using mineralogical probes, adsorption isotherms and a hydra toxicity bioassay;

3) to investigate the mineral composition and *in vitro* AFB_1 sorption capacity of Uniform Particle Size NovaSil (UPSN) and parent NovaSil clays and to assess the safety/toxicity of UPSN in a three month rodent study by evaluating growth rates and other biochemical parameters in serum and blood;

4) to investigate the mineral composition and *in vitro* AFB_1 sorption capacity of a sodium bentonite and to assess its safety/toxicity in a three month rodent study by evaluating growth rates and other biochemical parameters in serum and blood.

II. IN VITRO AND IN VIVO CHARACTERIZATION OF MYCOTOXIN BINDING ADDITIVES USED FOR ANIMAL FEEDS IN MEXICO*

2. 1 Introduction

AFB_1 is the most toxic of four naturally occurring AFs (i.e., B_1, B_2, G_1 and G_2). It is classified as a Class A carcinogen by the US Environmental Protection Agency (USEPA) and a Group 1 human carcinogen by the International Agency for Research on Cancer (IARC 2002). Because of its common occurrence in animal feed and its potent hepatotoxicity and carcinogenicity, considerable research has been directed at protecting animals against this toxin (Grant and Phillips 1998). The use of clay-based products as enterosorbents for AFs is a frequently used strategy to reduce AF exposure in animals. Natural bentonites (e.g. montmorillonite) are the common sorbents used for this purpose.

In pioneering studies in Texas, inclusion of calcium montmorillonite clay (NovaSil, NS) in animal feed has been shown to notably reduce the adverse effects associated with AF exposure in different animal species (Phillips et al. 1988; Phillips 1999) and to decrease the level of an AFB_1 metabolite (AFM_1) in milk from lactating dairy cows and goats (Harvey et al. 1991; Smith et al. 1994). Equilibrium adsorption isotherms, molecular modeling and *in vivo* studies have been used to demonstrate that

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NS preferentially binds AFB_1 in the gastrointestinal tract, thereby reducing its bioavailability to blood, liver, and other organs (Phillips 1999; Phillips et al.2002). Sodium bentonites have also been shown to be effective against AFs *in vitro* (Diaz et al. 2002) and *in vivo* (Pasha et al. 2007), although earlier investigations revealed that a 1% inclusion rate in the diet was not enough to protect rats against liver lesions due to AF (Voss et al. 1993). Zeolites (Mayura et al. 1998; Lemke et al. 2001b) and organozeolites (Dakovic et al. 2005) have been used as AF adsorbents as well, but some studies have reported that these minerals do not bind AFB_1 as effectively as the bentonites (Harvey et al. 1993). Importantly, there is evidence that clinoptilolite (a commonly used zeolite) was ineffective in preventing maternal and developmental toxicity of AFB_1 in rats (Mayura et al. 1998). This could be due to the natural conformational properties of clinoptilolite that prevent large molecules like AFs from entering the zeolitic pores. The size of AFB_1 is estimated to be 12.78Å, (from the hydrogen at C2 to the hydrogen at C8), and 10.38 Å, from oxygen at C11 to the exocyclic carbon in the O-methyl group (Phillips et al. 1995). The size of the pores on natural clinoptilolite can range from 4 to 7Å (Li et al. 2005); hence, the adsorption of AFB_1 could be limited to the external surface only. Decreased AF-binding capacities for organozeolites could also be due to the exchange of these materials with synthetic surfactants like octadecyldimethylbenzylammonium (ODMBA) (Dakovic et al. 2005) which may interfere with toxin/surface interactions. Low inclusion rates and easy management of mycotoxin-binding additives has led to the widespread acceptance of these products by the farm animal industry and the introduction of a variety of materials labeled as

mycotoxin binders for use in feed. Thus, it is important to determine the effectiveness and safety of these products. The objective of this research was to characterize and compare twelve different products that are routinely distributed in Mexico as mycotoxin-binding additives, by means of: (1) equilibrium isothermal analyses to delineate sorbent/toxin surface interactions; (2) a variety of mineralogical probes; and (3) a hydra toxicity bioassay.

2.2 Material and methods

2.2.1 Chemicals and reagents

AFB_1 from *Aspergillus flavus* was purchased from Sigma Chemical Co. (St Louis, MO, USA); CAS No. 1162-65-8. Acetonitrile, HPLC-grade was obtained from Fisher Scientific; CAS No. 75-05-8. Na_2CO_3 (ACS grade) was acquired from Fisher Scientific. $MgCl_2$ (ACS grade) was purchased from Mallinckrodt Chemicals. All of the adsorption experiments were performed using high-purity deionized water (18.2MΩcm). Tested additives included Milbond-TX® (MLB) from Milwhite, Inc., Mycoad® (MCA) from Avimex SA, Volclay FD181® (VOL) from Volclay de Mexico, Fixat® (FXT) from Süd Chemie, Toxinor® (TOX) from Norel Nature, Klinsil® (KLS) from HELM de Mexico, Mycofix Plus 3.0® (MIX) from Biomin, Mexsil® (MEX) from Mexsil, Mycosil® (MYS) from Dresen, Zeotek® (ZEO) and Duotek® (DUO) from Nutek S.A. and Mycosorb™ (MSB) from Alltech. All these additives are marketed in Mexico and claim to bind AFs to some extent, and some further claim to have multiple mycotoxin-binding and/or inactivation potential. These additives were compared with NovaSil™

Plus (NSP) (Engelhard, Inc.), an effective AF binder that has been extensively studied (Mayura et al. 1998; Phillips et al. 1998; 2006). The composition of matter for each additive based on information provided by individual websites and/or other publications was reported as follows: NSP (HSCAS, Ca-montmorillonite), VOL (Na-montmorillonite), FXT and KLS (aluminosilicates), MEX, TOX, MLB, MYC, MCA (Hydrated sodium calcium aluminosilicates, HSCAS), ZEO, DUO (organo-aluminosilicates), MSB (glucomannan/ß-D-glucan containing yeast product) and MIX (a synergistic mixture of minerals, biological constituents, including enzymes and BBSH microbe, plant-derived extracts such as flavonolignans, saponins and terpenoids, and some algae materials). Since kaolinite, mica, silica, and clinoptilolite can be present in some of these products, adsorption isotherms of AFB_1 were also conducted on these reference minerals at pH 6.5. The abovementioned reference minerals were obtained from the Clay Mineral Repository (Purdue University, IN, USA) and were used as negative controls (i.e., materials that do not effectively sorb AFB_1) (Masimango et al. 1978; Phillips et al. 1988, Mayura et al. 1998; Huwig et al. 2001).

2.2.2 Sample characterization

An initial physical, mineralogical, and chemical characterization was performed to investigate the properties of all the additives. The pre-screening consisted of the following procedures: (1) the determination of the swelling volume (SV); (2) powder mount X-ray diffraction analysis (XRD); (3) fractionation in aqueous suspension; and (4) measurement of pH. The determination of SV was used as a general indicator of the swelling properties of the additives. Swelling (to various extents) is a required

characteristic of smectites. The SV, an adaptation of the coefficient of linear extensibility (COLE) (US Department of Agriculture, Natural Resources Conservation Service 2007), was examined by adding 5 cm^3 of each additive to a 25 ml graduated cylinder followed by adding distilled water to a total volume of 25 ml. Each mixture was shaken vigorously to ensure thorough wetting, and the suspension was left to stand for 24 h at room temperature. After 24 h, the expanded volume of settled product was measured, and SV was calculated based on the ratio of (volume after hydration) to (volume before hydration) minus one. Since the swelling properties of some organic materials can mimic the swelling properties of smectites, the SV values for MSB and MIX were not determined due to the biological composition of MSB (i.e. yeast cell walls) and the mixed nature of MIX (i.e. algae, plants, and bacteria).

XRD powder mount analysis was made to evaluate the presence of smectite (montmorillonite) in the additives. Random powder mounts were prepared for the whole product of each additive. A random sample (approximately 300 mg) for each additive was placed in a standard rectangular aluminum plate holder (about 2 mm thick) with a rectangular cavity in which the sample was gently packed. All the additives were passed through a 140 mesh (105 mm) sieve to prevent interference due to differences in particle size. The presence of organic matter in the additives can interfere with the detection of mineral clays in the powder mounts due to aggregation effects or dilution. For that reason, the definite evidence of smectite presence was determined in the clay fraction following Mg^{2+} saturation in most of the additives, except MSB. Carbonate and organic matter in MEX and MIX were destroyed in order to minimize the aggregation problems.

The X-ray diffractograms were recorded with CuKα-radiation on a Phillips X-ray diffractometer equipped with a graphite monochromator and theta-compensation slit. The pattern was measured in 0.05°2θ intervals from 2 to 32°2θ. The particle size distribution in the additives was determined using an automatic fractionator. The percentage of particles with diameter > 0.05 mm (sand), 0.05–0.002 mm (silt), and ≤ 0.002mm (clay), as reported by the U.S. Department of Agriculture, Natural Resources Conservation Service (2007), was calculated based upon the weight of each fraction from an initial amount of 30 g of air dried sample. The samples were then dried in an oven at 105°C to adjust for moisture content. Briefly, the procedure consisted of placing the sample into one 400 ml glass bottle filled with 30 ml of 5% $NaCO_3$ and 250 ml of deionized water. The bottles were placed in a reciprocating shaker and shaken at 120 cycles per min overnight at room temperature. Once the shaking process was complete, the samples were passed through a 300 mesh (53 μm) sieve to separate the sand particles. The sieved suspension containing the silt and clay fraction was collected into a polypropylene cylinder and placed on the fractionator to run the following program consisting of seven cycles: distilled water addition; 5 min for mixing and stirring; and 8 h for settling. After each settling step, the suspension containing the clay fraction was siphoned automatically and saved in plastic containers. The sediment in the cylinders (silt fraction) was collected at the end of the fractionation process. In order to remove possible remaining clay and silt particles, the sand and silt fractions were rinsed with distilled water several times until the water was clear and then dried in a 105°C oven overnight. The clay fraction collected in 20 liter containers was flocculated by additions

of 1N $MgCl_2$. Following flocculation, the supernatant was siphoned off and clays were concentrated by centrifugation. The amount of clay was calculated by subtraction of the sand and silt weights after drying from the total sample weight. The pH of each additive was measured by adding 4 g per sample into a beaker with 200 ml of deionized water. The suspension was stirred for 10 min, and the pH was measured with a pH meter according to the procedure described in the US Pharmacopeia for pH measurement of bentonite samples (U.S. Pharmacopoeia 2005).

2.2.3 Aflatoxin adsorption analyses

Isotherms were performed as previously described (Grant and Phillips 1998). A stock solution of AFB_1 was prepared by dissolving pure crystals (5 mg) in acetonitrile (1 ml) and then injecting a volume from the previous dissolved AFB_1 (approximately 0.16 ml) into 100 ml of purified water to yield a solution concentration of 8 mg ml^{-1}. The concentration was further verified by measuring the absorbance at 362 nm on a Shimadzu scanning UV-visible spectrophotometer, Model 1601PC. Isothermal analyses for each additive were conducted at pH values of 2 and 6.5 to simulate stomach and small intestine pH conditions, respectively. The pH of the solutions used was adjusted with concentrated hydrochloric acid (HCl) and 1M sodium hydroxide (NaOH). Briefly, an isotherm consisted of eleven concentrations of AFB_1 (0.4, 0.8, 1.2, 2.4, 3.6, 4, 4.8, 5.6, 6.4, 7.2 and 8 mg ml^{-1}) mixed with 0.1 mg of each additive in a total volume of 5 ml in 16 x 125 mm borosilicate glass tubes. Three replicates were used for each solute concentration. A suspension of each additive was prepared by weighing 100 mg into a 50 ml Erlenmeyer flask and adding 50 ml of water to yield a concentration of 2 mg ml^{-1}.

The suspension was mixed to ensure slurry homogeneity. From this suspension, 50 ml (0.1 mg of additive) were added to each of the dilution tubes and the suspensions were vigorously stirred during the addition of the additive. Along with the dilution tubes, there were three controls consisting of 5 ml of water, 5 ml of AFB_1 stock solution and 5 ml of water with 0.1 mg of additive. The samples and controls were capped and placed on a shaker at 1000 rpm for 2 h in an incubator at 25°C. After shaking, the samples were centrifuged at 2000 rpm for 20 min. The supernatant was measured for absorbance at 362 nm. Isotherms for negative controls (kaolinite, mica, colloidal silica and clinoptilolite) were run at pH 6.5 following the same procedure as for the additives.

In order to compare the binding properties of the clay fraction with sand and silt fractions, an additional single-concentration adsorption analysis of AFB_1 at pH 6.5 was performed with the sand, silt and clay fractions from the additives that exhibited the highest AFB_1 sorption capacities based on the isotherms. The analysis consisted of triplicate aliquots of AFB_1 (8 μg ml^{-1}) in 5 ml reaction vials plus controls. The addition of sorbent, incubation time and the UV absorbance measurements were the same as in the isotherm procedures. The presence of smectite in the sand and silt fractions was also evaluated by XRD.

2.2.4 Data calculation, curve fitting and statistics

The UV adsorption data were used to calculate the amount of AFB_1 left in solution at equilibrium (C_w) and the amount adsorbed to the additive (q) using the software Table Curve 2D v.2 and an Excel program developed in our laboratory to fit the data to the standard Langmuir-derived isotherm equation: $q = Q_{max} [K_d C_w/(1 + K_d C_w)]$.

The binding capacity (Q_{max}) and the distribution constant (K_d) were determined for each additive as previously described (Grant and Phillips 1998). SPSS 14.0 software was used to calculate the Pearson's correlation coefficient for the data obtained from the parameters measured in the pre-screening analysis and from the isotherms. Data were first verified for normal distribution and the absence of outliers as required for this model.

2.2.5 X-ray diffractograms of Mg^{2+}saturated samples

In order to confirm the presence of smectite in the additives, three ceramic tile slides of Mg^{2+} saturated clay fractions were prepared by vacuum for XRD analyses. Three treatments were performed with the slides: (1) clay treated with 10% ethylene glycol (EG), (2) heating at 350°C, and (3) heating at 550°C. For the second and third treatments, the tiles were allowed to air dry for 24 h before heating. When treated with EG, smectite d-space increased from approximately 14Å to 18Å. When heated at 300 and 550°C, the smectite 14Å d-space collapses to ~10Å. X-ray diffractograms were taken for each treatment in the same way as for the powder mounts. Additionally, a pattern was taken with the air-dry clays without any treatment.

2.2.6 Hydra toxicity bioassay

Adult *Hydra vulgaris* have been reported to be a sensitive *in vivo* indicator of toxicity for environmental and food-borne chemicals (Mayura et al. 1991; McKenzie et al. 1997; Ottinger et al. 1999; Huebner et al. 2000). Previous work in our laboratory has reported the use of hydra as a very sensitive method to evaluate toxicity of clay minerals and other sorbents (i.e. organoclays) (Afriyie-Gyawu et al. 2005); and contamination

with various priority toxic metals (other than the framework metals) have been shown to be toxic to adult hydra (Karntanut and Pascoe 2002), thus, we have utilized the hydra bioassay as an initial indicator of safety for these materials. Maintenance and feeding of hydra were carried out according to methods previously described (Mayura et al. 1991; Huebner et al. 2000; Ake et al. 2001). Hydra were not fed for 24 h before initiating the experiments. The assay was performed by exposing the hydra to three different concentrations of each additive. The concentrations (0.1%, 0.3% and 0.5%) were based on the inclusion rates for animal feed recommended by the additive's manufacturers. Each Pyrex® 60 x 15 mm test dish contained three normal healthy adult hydra in 4 ml of medium containing 1mM $CaCl_2$ dihydrate, and 0.458mM TES (N-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid, sodium salt) buffer (adjusted to pH 7). The dishes were maintained at 18°C. Hydra were examined for signs of toxicity at 0, 4, 20, 28, 44, 68 and 92 h. The toxic endpoint was determined by the 'tulip' or 'disintegration' stage of the hydra. In each test, experimental treatments were compared with untreated and solvent controls.

2.3 Results

2.3.1 Sample characterization

The twelve additives showed a large range of SV from zero to greater than 4 (Table 2). MEX showed a value of zero. In contrast, VOL had a value slightly greater than 4 due to its strong swelling nature upon hydration. The remaining additives had SV values between 0.28 and 2.92. According to the X-ray diffractograms of the powder

mounts, most of the additives except for MSB, DUO, MEX and MIX showed evidence of possible smectite presence (Figure 10). After carbonate and organic matter removal, powder mounts of MIX showed evidence of poorly crystallized aluminosilicates and quartz, while MEX confirmed previous results (no smectite evidence). Most of the additives except MSB showed quartz peaks, and interestingly, the additive DUO showed a peak compatible for clinoptilolite. The pH values of the additives ranged from 5.4 to 9.6. In the fractionation analysis, ZEO and DUO (known as organoclays) registered the highest silt and the lowest clay-sized particle content, even though they had SV values of 0.80 and 0.68, respectively. MEX registered the highest percentage of sand-sized particles (46%). The lowest percentage of sand-sized particles was obtained for MCA (6%), and this additive showed favorable sorption characteristics based on isothermal analysis (see below). The same trend was observed for VOL which had the highest clay percentage (51%) and a favorable sorption pattern. The rest of the additives ranged from 4 to 47% clay content. According to the XRD the high percentage of clay-sized particles observed for MSB (60%) was mainly composed of organic matter. However, some XRD peaks observed for this additive were compatible with calcite mineral (Figure 10A). A similar phenomenon of organic matter in the clay fraction was also observed for MIX.

Table 2. Physical and chemical characteristics of clay additives used in animal feeds

Test additives	SV	pH	Q_{max} (mol AFB_1/ kg additive)	K_d	r^2	Smectite	Particle size (%)			Color
							>0.05 mm	0.05–0.002 mm	<0.002 mm	
VOL	>4.00	9.6	0.212	4.87E+05	0.97	+	8	41	51	5Y 8/1
KSL	2.92	8.8	0.130	0.95E+05	0.99	+	18	55	27	2.5Y 7/1
MCA	2.64	8.8	0.267	17.3E+05	0.97	+	6	47	47	2.5Y 8/3
TOX	1.92	9.6	0.146	0.92E+05	0.99	+	18	47	35	10Y 8/1
FXT	1.64	9.0	0.236	0.71E+05	0.99	+	21	35	44	10Y 8/2
MIX	NA	5.4	0.086	1.71E+05	0.88	−	25	40	35	NA
MSB	NA	5.6	0.015	2.51E+05	0.76	−	28	12	60	NA
MLB	1.36	6.7	0.312	4.11E+05	0.96	+	15	47	38	2.5Y 83
NSP	1.28	8.9	0.396	4.90E+05	0.99	+	20	53	27	5Y 7/2
ZEO	0.80	8.9	0.119	0.37E+05	0.88	+	26	70	4	NA
DUO	0.68	8.9	0.044	0.60E+05	0.92	−	35	58	7	A
MYC	0.28	7.4	0.069	1.30E+05	0.98	+	42	40	18	5GY 8/1
MEX	0.00	7.5	0.070	2.11E+05	0.94	−	46	37	17	2.5Y 8/2

Notes: Smectite presence was determined by both X-ray diffractograms with Mg^{2+} (air dry, heated at 300°C and 550°C) and ethylene glycol treatments. Q_{max}, K_d and r^2 values were from pH 6.5 isotherms. Color classification from Munsell® soil color charts. SV: Swelling Volume; NA: not applicable; +: Positive for smectite; -: Negative for smectite; G: Gray; Y: Yellow.

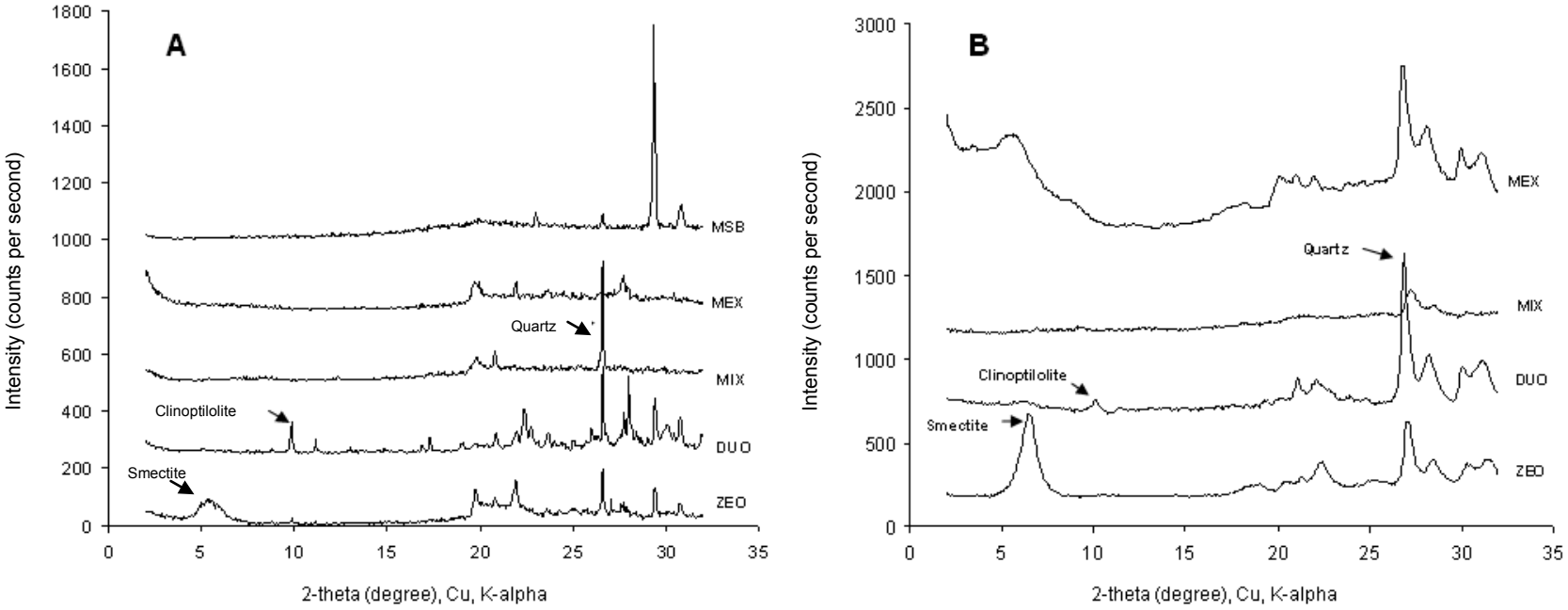

Figure 10. A) Powder mounts X-ray diffractograms of representative additives from Mexico. Specific patterns of a smectite containing additive (ZEO) and 4 potential additives without smectite (DUO, MIX, MEX and MSB) are shown. B) Mg^{2+} saturated clay samples confirmed the smectite containing additive (ZEO) and the 3 potential samples without smectite (DUO, MIX, MEX). MSB was not included in Figure B due to difficulties preparing an oriented sample because of its mainly organic composition. Arrows display the mineral identification of the peaks

2.3.2 Isothermal and single-concentration AFB_1 adsorption analyses

Computer-generated equilibrium isotherms were extrapolated from additives data and fit to the Langmuir model (based on r^2 values and randomness of the residuals). The parameters of Q_{max} (mol AFB_1 kg^{-1} additive) and K_d were estimated to delineate the maximum sorption to the surface and the affinity of the sorption interaction. Major differences in AFB_1 binding were observed among the additives at pH 2.0, with just five additives fitting the Langmuir model, i.e., NSP, MCA, VOL, MLB and MIX. The remaining products did not fit the Langmuir model; instead, they showed evidence of a constant (non-saturable) partition trend (Figure 2). In contrast, at pH 6.5, L-shaped curve characteristics of the isotherms were observed for many of the additives (Figure 11) with some variation in the r^2 values. MSB and ZEO did not fit the Langmuir model at pH values of 2.0 or 6.5. The most effective binding additives for AF at both pH values were NSP, MLB, MCA and VOL. The highest Q_{max} value was obtained for NSP (Q_{max}= 0.4 mol AFB_1 kg^{-1}) at pH values of 2.0 and 6.5. In order to directly compare the additives, all adsorption isotherms were force-fitted to the Langmuir equation obtaining Q_{max} and K_d values regardless of the shape or fit of the curve (Table 2). By doing so, the lowest value was obtained for MSB (Q_{max} = 0.009 and 0.015 mol AFB_1 kg^{-1}) at pH values of 2.0 and 6.5, respectively. Both organoclays (ZEO and DUO) showed low AF-binding capacities. In studies comparing NSP (Ca-montmorillonite) with the negative controls, it was confirmed that the active ingredient for AFB_1 binding is smectite clay (Figure 12). In the single-concentration adsorption analysis, NSP showed the highest percentage of AFB_1 binding in the bulk material, the sand, silt and the clay fractions followed by MLB

and MCA. Other additives showed variable sorption percentages in the different fractions (Figure 13). All of the additives were processed under the same standard protocols that are used in mineralogy studies to separate the fractions of soil samples. XRD methodology is capable of detecting very small amounts of clay minerals, and while the sand fraction is composed mainly of quartz, in NSP, the sand fraction contained considerable amounts of smectite clay (XRD data not shown). The silt fraction of NSP was also found to contain smectite. This suggests that smectite aggregates with the size of sand or silt are prevalent in this material and in some of the other additives (e.g. VOL and MLB). Smectite presence in the sand and silt fractions of additives may be attributed to a variety of causes including, lack of complete dispersion of the sample, or inadequate fractionation.

2.3.3 Pearson's correlation coefficient

The data from these studies were shown to be normally distributed and without the presence of outliers before calculating Pearson's correlation coefficient. Significant correlation values at the 0.01 level were found for SV with sand [-0.859 correlation coefficient (CC) ($p = 0.001$)] and clay [0.747 CC ($p = 0.008$)]. Significant correlation values at the 0.05 level were found for Q_{max} with sand [-0.660 CC ($p = 0.027$)] and clay [0.606 CC ($p = 0.048$)]. The pH values were not included in the correlation analysis because the data for this parameter was not normally distributed. Q_{max} values from isotherms at pH 6.5 were used in the correlation analyses.

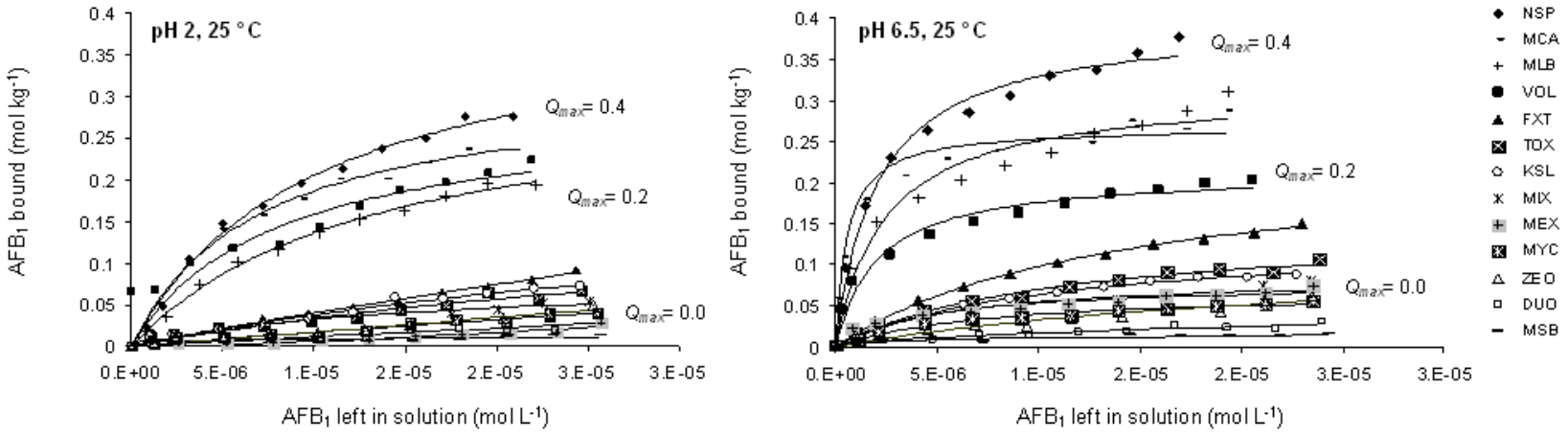

Figure 11. AFB_1 sorption isotherms on binding additives at pH 2 and pH 6.5. Each point represents the values calculated for AFB_1 bound and left in solution for the corresponding 11 dilutions. Isotherms were performed at 25°C. Q_{max} is expressed as mol AFB_1/kg of additive.

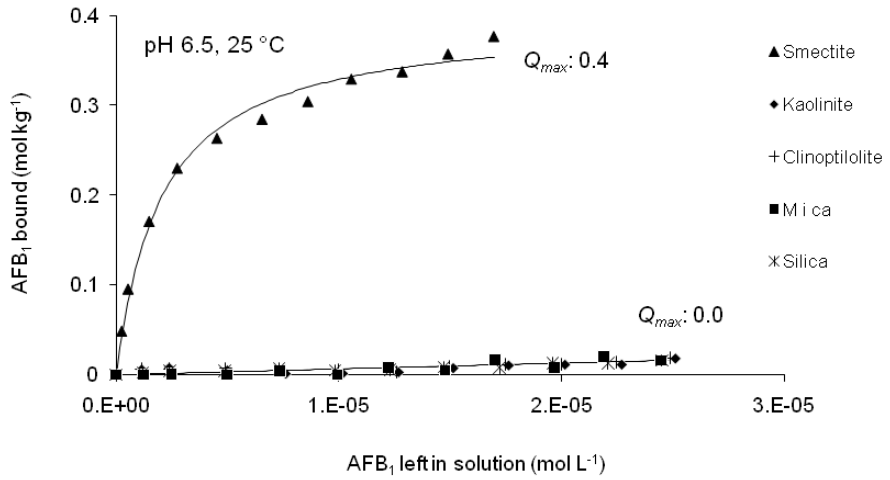

Figure 12. AFB_1 sorption on smectite clay versus non-binding materials. NSP clay was used as positive control for binding. Non-binding minerals included kaolinite, clinoptilolite, mica and silica. Isotherms were performed at pH 6.5. The highest capacity (Q_{max}) for AFB_1 sorption was obtained for smectite (0.4 mol AFB_1 kg^{-1} sorbent). Q_{max} values of 0.0 obtained for the other minerals demonstrate their negligible sorption

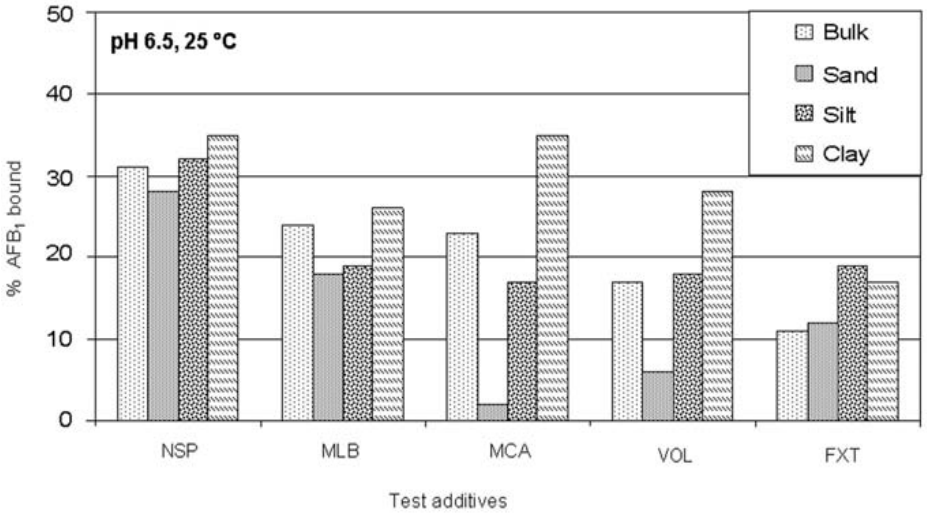

Figure 13. AFB_1 binding onto bulk versus fractionated additives. Single sorption assays performed at pH 6.5 (25°C temperature). Percentage of AFB_1 bound was calculated based on the amount adsorbed by the materials from a total concentration of 8 mg ml^{-1} AFB^1. NSP had the highest percentage of AFB_1 binding in the bulk, sand and silt fractions. MCA and NSP registered the highest AFB_1 sorption in the clay fraction.

2.3.4 XRD-pattern of Mg^{2+} saturated samples

Based on the XRD-pattern from Mg^{2+} treated samples, most of the additives except MSB, DUO, MEX and MIX showed evidence of smectite according to XRD analyses. However, the amount of smectite could not be accurately quantified without additional chemical analysis. Mg^{2+} saturated MSB ceramic tile could not be prepared due to difficulties forming an oriented sample with organic clay-size materials. The only XRD pattern for this sample was the powder mount. Previous degradation of carbonates and organic matter in the clay fraction (air dry), MEX showed a pattern suggestive of smectite (Figure 10), however the Mg^{2+} EG treatment did not confirm the smectite presence and MEX was classified as an additive without smectite. Interestingly, MIX additive is known to contain silicate minerals (bentonite according to a personal communication) but evidence of smectite was not found in this material according to our procedures; nevertheless quartz and apparently poorly crystallized aluminosilicates were present in this sample. A possible reason for non-detection of smectite in MIX could be a low inclusion (dilution) of bentonite.

2.3.5 Hydra toxicity bioassay

As a well-established and sensitive in vivo indicator of toxicity, hydra were used to estimate the potential toxicity of these additives. In the hydra toxicity bioassay, most of the additives were non-toxic to hydra except for ZEO, DUO and MSB at levels of 0.3 and 0.5% (equivalent to the common levels of inclusion in animal feed) (Figure 14). Importantly, organoclays were toxic to hydra (even at the 0.1% inclusion rate).

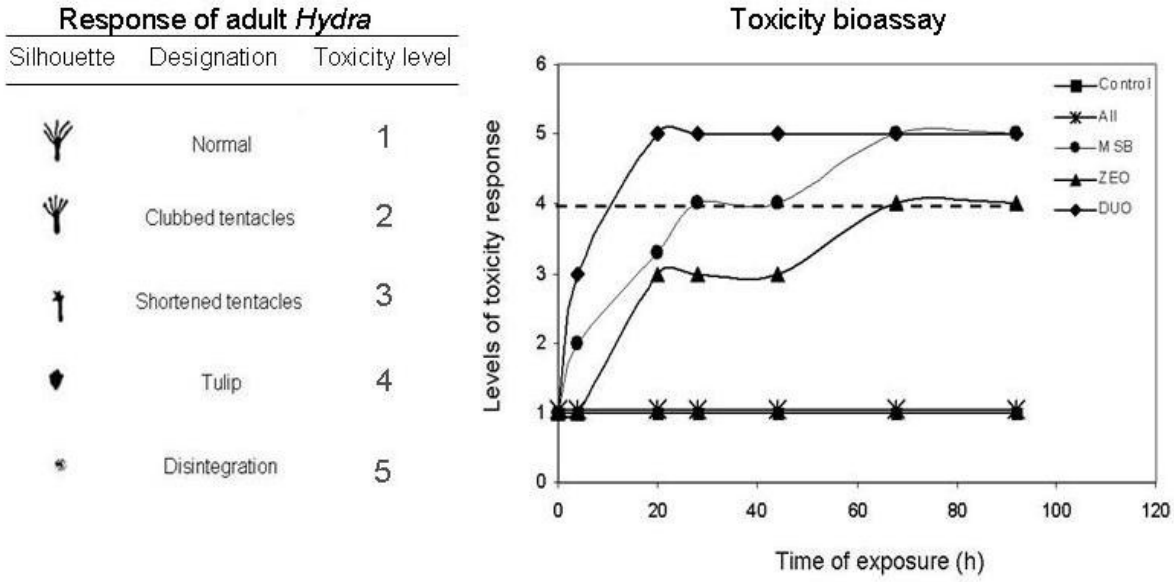

Figure 14. Hydra toxicity bioassay for binding additives. Dashed line represents the toxic endpoint (tulip stage). Each point represents the toxicity levels at different times of exposure.

2.4 Discussion

In the sample characterization, the COLE values of the additives had a wide range. According to traditional estimation of COLE values, samples with a value greater than 0.03 are considered to contain significant amounts of smectite/montmorillonite clays (Buol et al. 1980). In our study, we used the same parameter from the COLE value (0.03) to determine qualitatively if the binding additives had significant smectite content. The results indicated that all the additives analyzed, except MEX, possessed a detectable amount of smectite. It is important to mention that traditional COLE values were used to

describe bulk soils, not pure clays, raw materials and mixtures as seen in this study. For this reason, the presence of smectite was confirmed by means of XRD powder mounts and clay fraction analysis. According to diffraction analysis of the powder mounts, evidence of smectite was found in most of the additives except for MSB, DUO, MEX and MIX. Concerning the pH values, most of the additives had pH values greater than 6.7. It is not uncommon to find alkaline pH values with various aluminosilicates (Garcia-Morales et al. 2004), as well as in confirmed smectite-containing products (Kannewischer et al. 2006). The US Pharmacopeia has reported a pH of 9.5–10.5 for natural bentonites and 9.0–10.0 for pure bentonites (US Pharmacopoeia 2005). In our study, we found the presence of smectite in nine of the additives and (on average) they had pH values of 8.55; however, there were two additives that did not correlate with these pH values. MLB had a pH of 6.7 with confirmed smectite presence, and MEX had a pH of 7.5 without evidence of smectite. The low pH values observed for MYC and MSB could be due to the presence of biological materials other than clay minerals as reported by other authors in similar products (Kannewischer et al. 2006). For this reason, pH values cannot be definitively used as a characterization tool for smectite presence or for effectiveness of sorbents for AFB_1 binding.

In the fractionation analyses, samples ZEO and DUO had the lowest clay content; this effect can be due to the processing of these clays. Similar organoclays are exchanged with long chain surfactants (Lemke et al. 1998) that can aggregate clay minerals and prevent the action of dispersant agents used during the fractionation process. According to fractionation analysis, 46% of MEX was composed of sand-sized

particles and this additive did not effectively bind AFB_1. Most likely, the sand fraction of complete soils is composed of quartz (SiO_2) or other primary silicates (Brady and Weil 2002). However, sometimes clay aggregates can be found in the sand and silt fractions. According to XRD, most of the additives contained smectite except for MEX, DUO, MSB and MIX. MIX additive is known to contain mineral clays, but we did not find smectite evidence in this sample. This could be related to the nature of this product since it is been reported that, significant dilution of minerals with other materials (e.g. organic carbon) observed for similar additives, can have a negative influence on the quality of XRD patterns (Kannewischer et al. 2006).

In the sorption analyses, the 4 best binders had Q_{max} values above 0.2 mol AFB_1 kg^{-1} additive. Importantly, the results obtained for glucomannan-based products like MSB in our study, differ from the *in vitro* study of Diaz et al. (2002). In that study, MTB-100 (a yeast cell wall based-material) at an approximate level of 1% in solution was reported to sorb 96.6% of 5 mg of AF, although the sorption trend was not determined by isothermal analysis. In recent reports, the molecular mechanism of binding of AFB_1 to ß-D-glucans (ingredient of glucomannan products) was shown to be Van der Waals attractions and hydrogen bonds (Yiannikouris et al. 2006). It is well established that these bonding forces are reversible and depend largely on the orientation of the molecules. In contrast, the proposed mechanism of binding for AFB_1 on interlayer surfaces of smectites (e.g. NSP, Ca-montmorillonite) involves chemisorption bonding mechanisms (Grant and Phillips 1998) that are stronger than Van der Waals forces and hydrogen bonding interactions. Importantly, this interlayer interaction is

thermodynamically favored (Grant and Phillips 1998) and has been confirmed by XRD in another laboratory (Kannewischer et al. 2006).

As has been noted, the AF-binding capacities of the additives that contained smectite were considerably different. This disparity may be due to a variety of factors. For example, the smectite inclusion level in the sample may be too low for effective binding. In addition, a lack of purity of the materials and contamination in mixtures (organic materials) could be a risk for masking the effects of smectite due to clay aggregation problems or dilution. Finally, processing procedures to make the materials more lipophilic (Lemke et al. 1998) like the organoclays, may interfere with AFB_1 adsorption onto smectite. When comparing smectite with other common minerals potentially found in the additives, we verified that smectite is the active ingredient, and this conclusion has been supported by other studies (Phillips et al. 2002; Kannewischer et al. 2006).

As mentioned earlier, most of the additives were safe to the hydra except the organoclays and MSB, these data reinforce previous studies in mice that demonstrated the toxicity of organoclays (Lemke et al. 2001a; Afriyie-Gyawu et al. 2005) which suggested leaking of quaternary amines from the interlayer of the clays. As for MSB, the toxic effect exerted may be due to the growth of intact yeast and microorganisms that have been observed in these types of products (Kannewischer et al. 2006).

In accordance with isothermal and single-sorption analyses from these studies, NSP showed the best sorption characteristics for AFB_1, followed by MCA, MLB and VOL. Since considerable amounts of clay minerals were found in the sand and silt

fractions of some of the additives, further work is warranted to delineate this phenomenon. In order to determine the relationship of particle size to binding capacity, AF-binding additives need to be studied on a case-by-case basis, determining potential contaminants versus the primary clay mineral content. Even though most of the additives tested showed evidence of smectite presence, the binding capacities were significantly different, and only four of them showed high adsorption capacities for AFB_1. The best binding additives showed an L-shape pattern of sorption at both pH conditions, and a Q_{max} = 0.283 or 0.212 at pH values of 2.0 and 6.5, respectively. The particle size distribution in the additives was found to be significantly correlated with the binding capacity for AFB_1. Again, the presence of smectite clays in the additives was responsible for the capacity to bind AFB_1 when compared with other major clay minerals. Major differences in the ability of these additives to bind AFs verify the critical need for further research and screening of these types of products.

Based on our work, AF-binding additives intended for animal feed must contain smectite (i.e., montmorillonite) clay mineral as a primary component and should be rigorously evaluated *in vitro* and *in vivo*. Apart from favorable thermodynamic characteristics of sorption and other *in vitro* characterization analysis, these additives should be challenged *in vivo* to evaluate their safety and efficacy under realistic contamination levels of AFs and other mycotoxins. Before using these additives in animal feed, clear evidence of their purity (free from hazardous contaminants) and their negligible nutrient interactions must be considered. Further investigations in the area are clearly warranted. Studies should focus on claims of multiple mycotoxin binding, which

would suggest nonselectivity and potential interference with nutrients and other critical feed additives.

III. CHARACTERIZATION AND AFLATOXIN SORPTION EVALUATION OF GEOPHAGIC CLAYS FOR HUMANS

3.1 Introduction

Geophagy, defined as the ingestion of earth or clay, has been practiced in many regions and has been used as an acceptable treatment in traditional medicine in many African countries and China (Johns and Duquette 1991; Diamond 1999). Geophagy is usually reported in pregnant women due to a variety of reasons including treatment for morning sickness. Nevertheless, other uses of clays as antidiarrheal medication, (Vermeer and Ferrell, 1985), mineral supplement, detoxifying agent from food contaminants (Johns and Duquette 1991) and alleviators of hunger (Keay 1993), have been reported. Specifically, montmorillonite clay has been reported to adsorb AFs, toxic byproducts from *Aspergillus flavus* and *Aspergillus parasiticus* growth (Grant and Phillips 1998). AFB_1 is the most potent congener of AFs and it has been classified as a Group 1 carcinogen (IARC, 2002). The mechanism of binding of AFB_1 onto the interlayer surfaces of montmorillonites has been supported by several *in vitro* investigations (Phillips et al. 1988, 1995; Grant and Phillips 1998; Kannewischer et al. 2006). Recent human studies in Ghana have demonstrated the safety and efficacy of NovaSil (a montmorillonite rich Ca-bentonite) in reducing the bioavailability of AFs when given in capsules before each meal (Afriyie-Gywau et al. 2008b; Wang et al. 2008). Ghana is located in West Africa and is included in a group of developing countries that are at risk of chronic exposure to AFs based on temperature and humidity

conditions (between the latitudes 40°N and 40°S) that lead to vulnerability of staple commodities for mycotoxin contamination (Williams et al. 2004).

Edible clays for human consumption are readily available in rural and ethnic markets of several developing and developed countries. The mineral composition of these edible clays has not been completely documented; however, due to its intended purpose and texture, it is believed that many contain chalk (a form of limestone composed of calcite) and kaolinite. Although according to geological maps (Benham and Brown 2007) smectite is not commonly found in Ghana, some reports have shown that smectite can be present at low percentages (from 0-20%) (He et al. 2007). If some of the edible clays available in the Ghana markets have the potential to adsorb AFs, a new solution for the AF contamination present in regions of Ghana will be available and sustainability issues will be attenuated in these regions. Countries like Haiti, Kenya and Uganda share similarities with Ghana regarding the availability of edible clays and AF contamination. These countries are also located in the "risk zone'' for chronic AF exposure which makes their edible clays worth investigation as potential strategies to prevent AF toxicity. An effective enterosorbent for AF not only has to protect against AF bioavailability but also must be a pure compound with very low levels of heavy metals and other soil contaminants. Thus, it is important to investigate both the effectiveness and safety of these materials. By using equilibrium isothermal analyses, the AFB_1 adsorption properties of edible clays from Ghana, Uganda, Kenya and Haiti, were evaluated. X-ray diffraction (XRD) analysis and other mineralogical probes were used to

identify the mineral composition of the edible clays and a bioassay using *Hydra vulgaris* was performed to investigate their potential safety.

3.2 Materials and methods

3.2.1 Chemicals and reagents

For all experiments, ultrapure deionized water (UP-H_2O 18MΩ cm^{-1}) was used. NovaSil Plus (NSP) clay was obtained from Engelhard Chemical Corporation (Iselin, NJ, USA). AFB_1 from *Aspergillus flavus* was purchased from Sigma Aldrich. All other reagents used in the sample preparation steps for the mineralogical analyses were analytical grade.

3.2.2 Sample preparation and evaluation

Edible clay samples from Ghana (70), Uganda (2), Kenya (2), Texas (3) and Haiti (1), were ground, passed through a 45 μm sieve and then dried at 110 °C overnight. Samples from Ghana were collected in the Ejura-Sekyedumase district (ESD). The specific origin of the clays from Ghana was traced to the Ashanti (22), Brong/Ahafo (17), Central (4), Greater Accra (22), Northern (2) and Western (3) regions (Figure 14). Specific regions for the rest of the clays were not available.

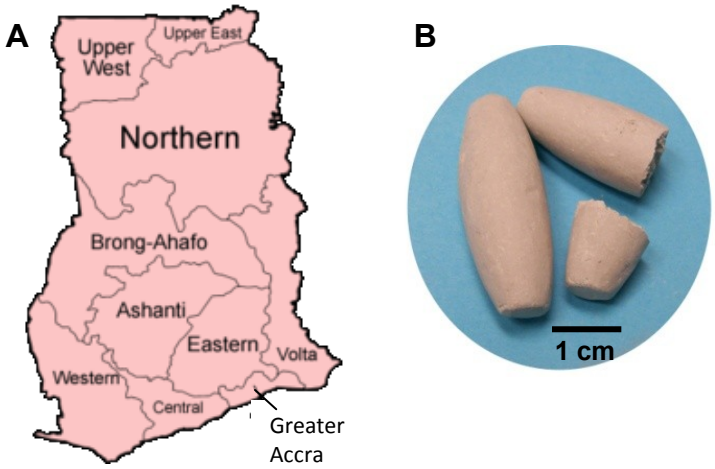

Figure 15. Map of Ghana and image of a representative edible clay. A) Regions from which samples were collected. B) Edible clay collected in one of these regions. Map was obtained from http://mapsof.net/ghana/static-maps/png/ghana-regions).

An initial sample evaluation was performed to assess the presence of carbonate minerals, sulfides, manganese oxides and evaporites. The presence of calcite ($CaCO_3$) in the samples was qualitatively determined by using 1M hydrochloric acid (HCl). The presence of oxidizing/reducing components was evaluated by adding 0.5 mL of 30% hydrogen peroxide (H_2O_2) to the samples (0.5 g). The observed reaction was qualitatively classified on a scale from 0 to 5, with 0 as no reaction and 5 as strong reaction. Occurrence of evaporite minerals was qualitatively estimated by measurement of electrical conductivity (EC) and acetone precipitation. The pH was measured according to the procedure described for bentonite samples (U.S. Pharmacopeia 2005) with slight adaptations. Briefly, 100 µg of each sample were placed in 15 mL glass

culture tubes and shaken for 10 min. After centrifugation at 2000 rpm, the supernatants were used to measure EC and pH sequentially.

3.2.3 Aflatoxin adsorption analyses

Isothermal analyses for AFB_1 adsorption were performed as previously reported (Marroquin-Cardona et.al, 2009). A stock solution of AFB_1 was prepared by dissolving pure crystals in acetonitrile and then injecting a volume from the previous dissolved AFB_1 into 100 ml of purified water to yield a solution concentration of 8 μg ml^{-1}. AFB_1 concentrations were verified by measuring the absorbance at 362 nm on a Shimadzu scanning UV-visible spectrophotometer, Model 1800. Isothermal analyses for each sample were conducted in UP-water with unadjusted pH. NSP clay was used as a positive control for AFB_1 adsorption. Data from the isothermal analyses were fitted to multiple isotherm equations (i.e., Langmuir, Freundlich, Langmuir-Freundlich combinations, Toth, and different transformations of these equations) (Giles et al. 1974ab; Grant et al. 1998). Data were used to calculate the sorption maximum capacity (Q_{max}) and affinity (K_d) of AFB_1 to the surfaces of the clays.

3.2.4 XRD analyses

XRD powder mounts were prepared to determine the preliminary mineral composition of the edible clays. The X-ray diffractograms were recorded with a Bruker D8 ADVANCE X-ray diffractometer using a Cu X-ray source and κα-radiation. Minerals in the clays were identified by using the computer software EVA (Bruker).

3.2.5 Hydra toxicity bioassay

As a preliminary safety/toxicity evaluation, a *Hydra attenuata* bioassay was used as previously reported by Marroquin-Cardona et al. (2009). Hydra were exposed to three different inclusion rates 0.25%, 1% and 2% w/v of 12 representative edible clays. NSP clay was used as positive control for safety, while a sample known to contain high levels of heavy metals was used as a negative control. For each sample, 10, 40 and 80 mg were weighed in Petri dishes and UV irradiated in a laminar flow hood for 1 hr to prevent bacterial growth during the study. Three healthy adult hydra were placed in a dish with 4.0 ml of media containing 1 mM $CaCl_2$ dihydrate and 0.458 mM TES [N-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid sodium salt] buffer (adjusted to pH 7) and were maintained at 18°C. Hydras were examined for signs of toxicity at 0, 4, 20, 28, 44, 68 and 92 h. The toxicity response was recorded according to a scoring procedure previously reported (Wilby 1988). The toxic endpoint was determined by the 'tulip' or 'disintegration' stage of the hydra.

3.3 Results

3.3.1 Sample evaluation

Most of the clays from Ghana did not react with HCl or H_2O_2, suggesting that the presence of calcite or oxidizing components and/or organic matter are negligible or minimally present in the samples. In contrast, the clays from Kenya showed a strong reaction to H_2O_2 (score of 5) which suggest that these clays contain high amounts of oxidizing/reducing components. The color or these clays was red implying high content

of iron oxides. Clays from Haiti, Texas and Uganda had a similar behavior as the Ghana clays, where no reaction to HCl and a slight reaction to H_2O_2 were observed. Regarding the pH and EC values, the clays from Ghana had pH values ranging from 4.9 to 8.2 (average value = 6.5), while the EC values ranged from 12.7 to 641 μS cm^{-1} (average value = 61.8 μS cm^{-1}). Similarly, the clays from Haiti, Kenya, Texas and Uganda had pH values ranging from 5.7 to 8.33 and EC values ranging from 10.8 to 1009 μS cm^{-1}. No gypsum precipitation with the acetone test was observed in any of the samples from Ghana or the other countries.

3.3.2 Aflatoxin adsorption analyses

The edible clays were grouped according to the isothermal AFB_1 adsorption pattern they portrayed. The classification included L-shape, H-shape, S-shape and C-shape patterns as reported by Giles (1974a). Another category termed NP (non pattern) was created to group the samples that displayed an unspecific sorption trend. In general, 55% of the 78 samples showed favorable patterns of adsorption for AFB_1; depicting L1, L2 and H shape curves. From the Ghana clays, 60% of the samples had favorable sorption patterns (i.e. L-shape pattern), 10% of the samples had non favorable sorption trends (S and C-shape patterns) and 30% did not show any specific pattern (Figure 15). Maximum adsorption (Q_{max}) and affinity (K_d) values were calculated for the samples that fitted the Langmuir equation with r^2 values greater than 0.80 (33 samples). Q_{max} values ranged from 0.010 to 0.08 mol kg^{-1}. A sample from the Brong Ahafo Region (sample 64) obtained the highest Q_{max} (0.08 mol kg^{-1}) value, while a sample from the Ashanti Region (sample 4) had the lowest value (0.001 mol kg^{-1}). When compared to NSP (0.40 mol kg^{-}

[1]), none of the Ghana clays showed similar capacity for AF binding (Figure 16). Similar observations were documented for the edibles clays from Kenya, Uganda, Haiti and Texas (Figure 17).

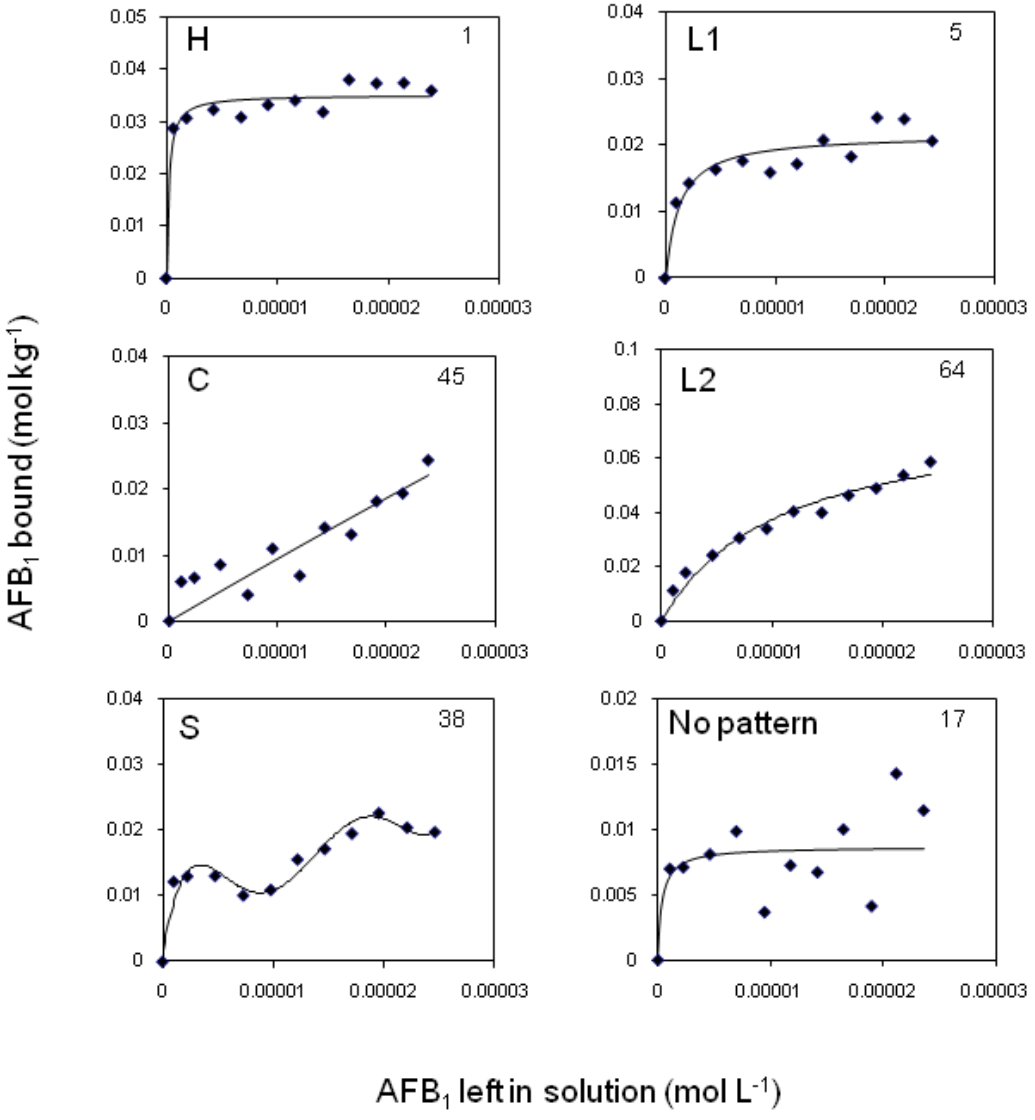

Figure 16. Representative adsorption patterns observed for clays from Ghana. The specific pattern type (letter) is shown in the upper left corner while the sample identification (number) is shown in the upper right corner.

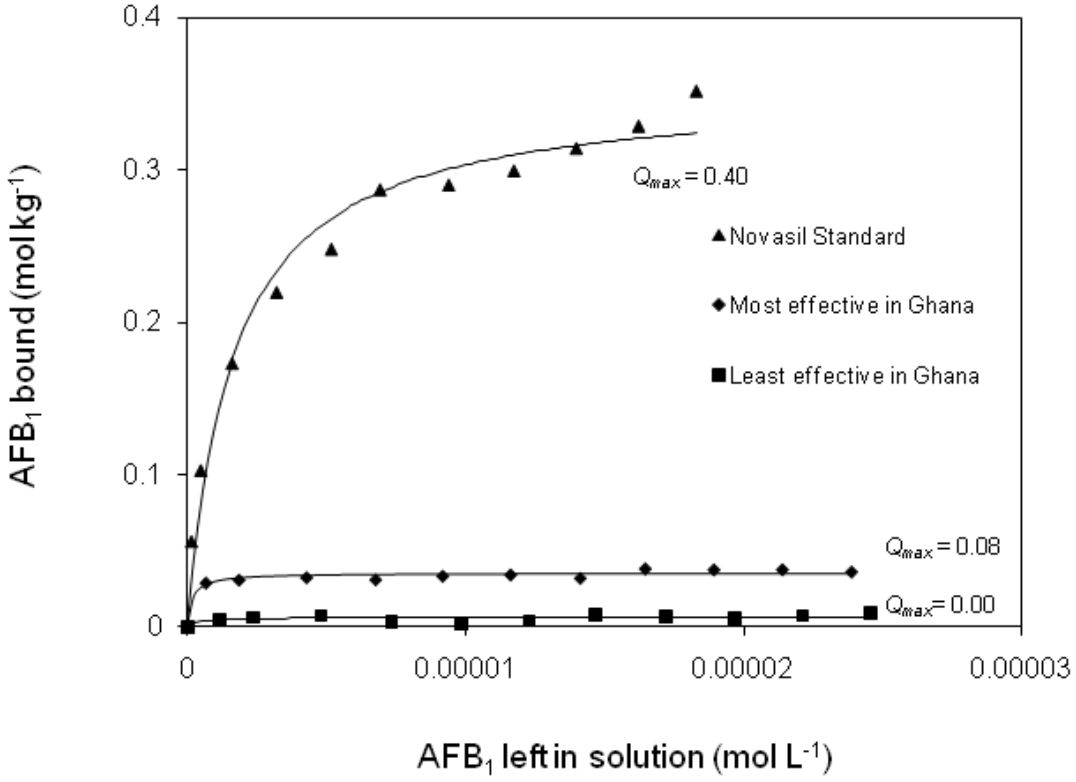

Figure 17. AFB_1 isothermal plots of the most effective and least effective samples from Ghana. These plots are compared with a standard isotherm for NovaSil. The most effective sample from Ghana was obtained from the Brong Ahafo Region (Q_{max} = 0.08 mol kg^{-1}), and the least effective sample was obtained from the Ashanti Region (Q_{max} = 0.00 mol kg^{-1}). None was comparable with NovaSil (Q_{max} = 0.40 mol kg^{-1}).

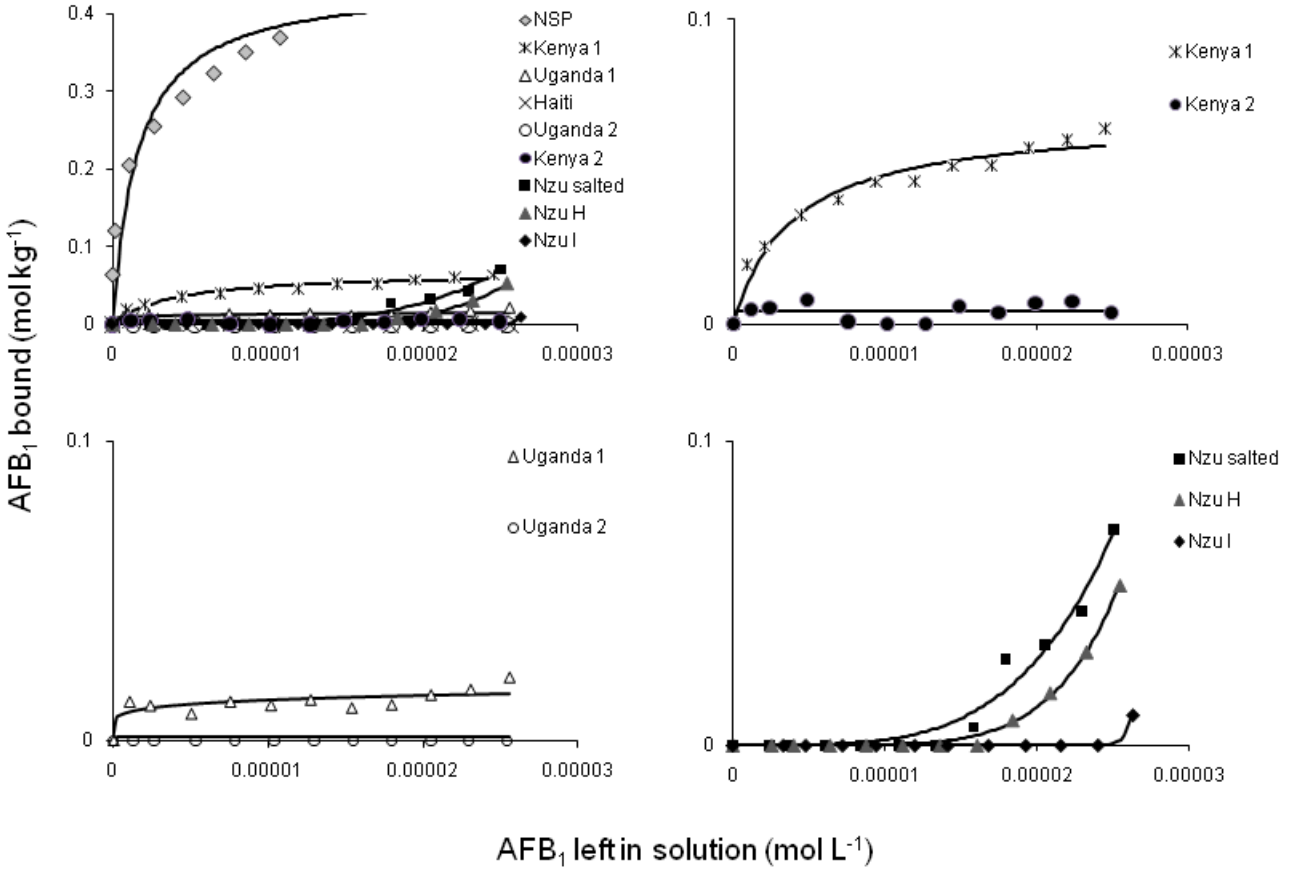

Figure 18. AFB_1 isothermal adsorption plots of NSP and clays from Haiti, Kenya, Texas ("Nzu" samples) and Uganda. Individual plots for groups of clays are shown in order to see the specific pattern of sorption. The most effective sample for AFB_1 sorption was a Kenya 1 (Q_{max} = 0.06 mol kg^{-1}). Nevertheless, none of the samples were comparable with NovaSil (Q_{max} = 0.40 mol kg^{-1}).

3.3.3 XRD analyses

Most of the edible clays from Ghana showed peaks of kaolinite, muscovite, feldspars and quartz minerals. The clays were grouped in four groups according to the minerals they contained. Some of them contained albite with chemical formula $NaAlSi_3O_8$ and feldspars from the orthoclase group with formula $KAlSi_3O_8$. A representative set of XRD patterns of the Ghanaian clays is presented in Figure 18. The Haiti sample showed peaks of muscovite 2M1 (10.01, 4.48, 2.56 Å), kaolinite (7.11, 2.56 Å), orthoclase feldspars (3.19, 4.02, 3.78 Å) and calcite (3.03, 2.28 Å). In this sample, a 14.3 Å peak was also observed suggesting that smectite, vermiculite or chlorite can be present (Figure 19). More research is warranted to determine the specific mineral identity of the 14.3 Å peak. Sample "Kenya 1" showed peaks of hematite (2.70 and 2.56 and1.69 Å), along with anatase (3.51, 2.37 and 1.84 Å), while "Kenya 2" revealed peaks of hematite (2.70 and 1.70 Å), magnetite (2.51, 1.61 and 1.48 Å) and quartz (3.34 Å), along with a 15.04 Å peak that is common for smectite, chlorite and vermiculite (Figure 20). "Uganda 1" sample showed peaks of kaolinite (7.21, 3.57 and 1.48 Å), quartz (3.34 Å) and albite high (6.47 and 3.24 Å). "Uganda 2" had peaks of kaolinite, quartz and alunite (2.99 and 2.28 Å) a sulfate mineral with formula $KAl_3(SO_4)_2(OH)_6$ (Figure 21). The Texas "Nzu" sample from Irving contained kaolinite (7.18, 4.34 and 3.57 Å), quartz, and anatase (3.51 and 2.37 Å), a titanium dioxide mineral, while "Nzu" from Houston and salted "Nzu" samples contained kaolinite, quartz and halite (NaCl) (2.82, 1.99 and 1.62 Å). XRD patterns of "Nzu" samples are presented in Figure 22.

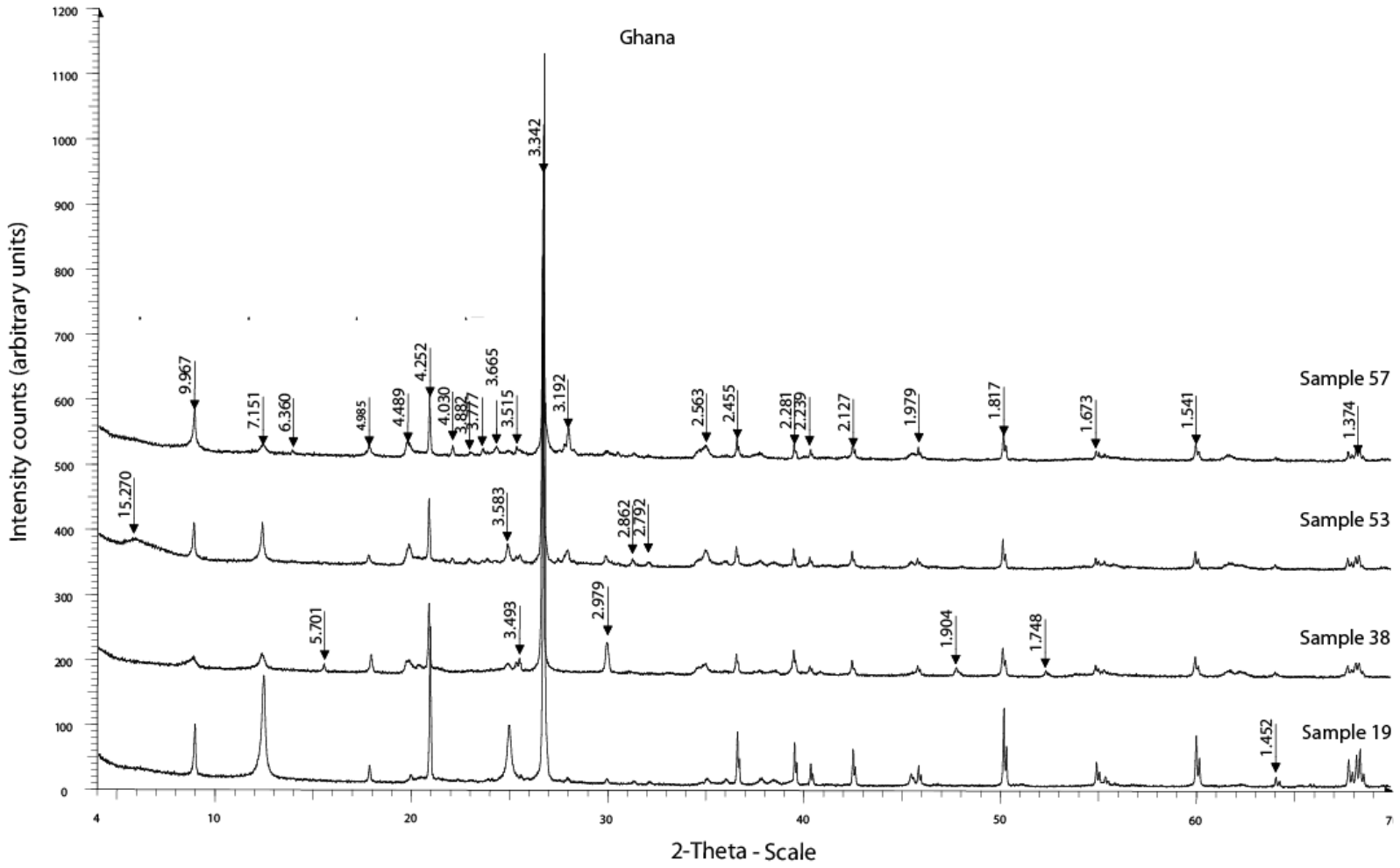

Figure 19. Representative XRD patterns of edible clays from Ghana. All samples depicted muscovite (9.9, 4.98 Å) and kaolinite (7.1, 3.5 Å) peaks as well as quartz (3.3 Å). Sample 38 showed peaks compatible with alunite (5.70, 2.97, 1.90, 1.74 Å). Sample 53 showed a broad 15.2 Å peak suggestive of vermiculite, chlorite or smectite, while sample 57 had albite peaks (6.3, 4.0, 3.6 Å).

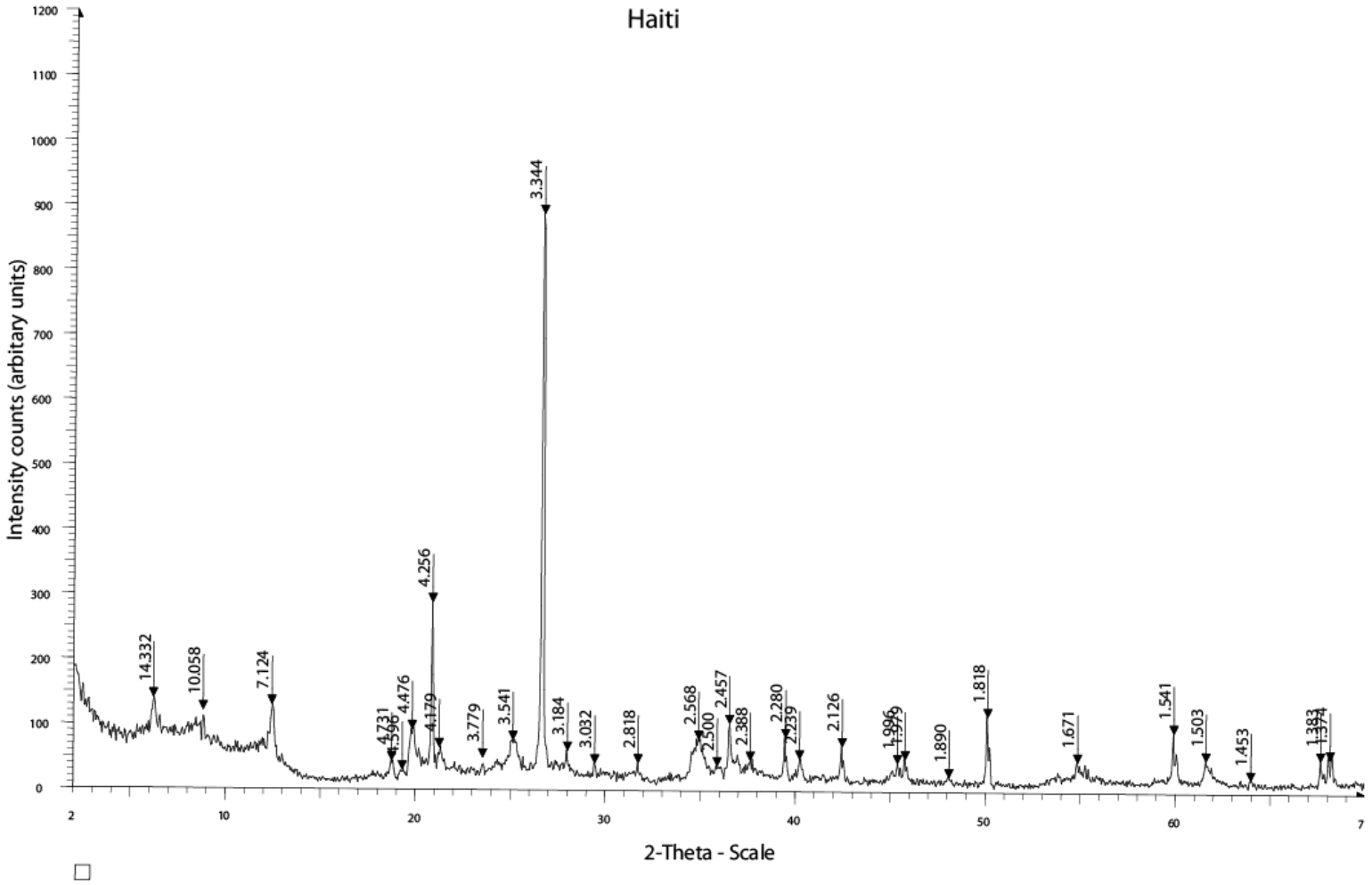

Figure 20. Powder mount XRD of clay sample from Haiti. XRD revealed evidence of vermiculite (14.33, 2.82 Å), muscovite 2M1 (10.01, 4.48, 2.56 Å), kaolinite (7.11, 2.56 Å), orthoclase feldspars (3.19, 4.02, 3.78 Å) and calcite (3.03, 2.28 Å).

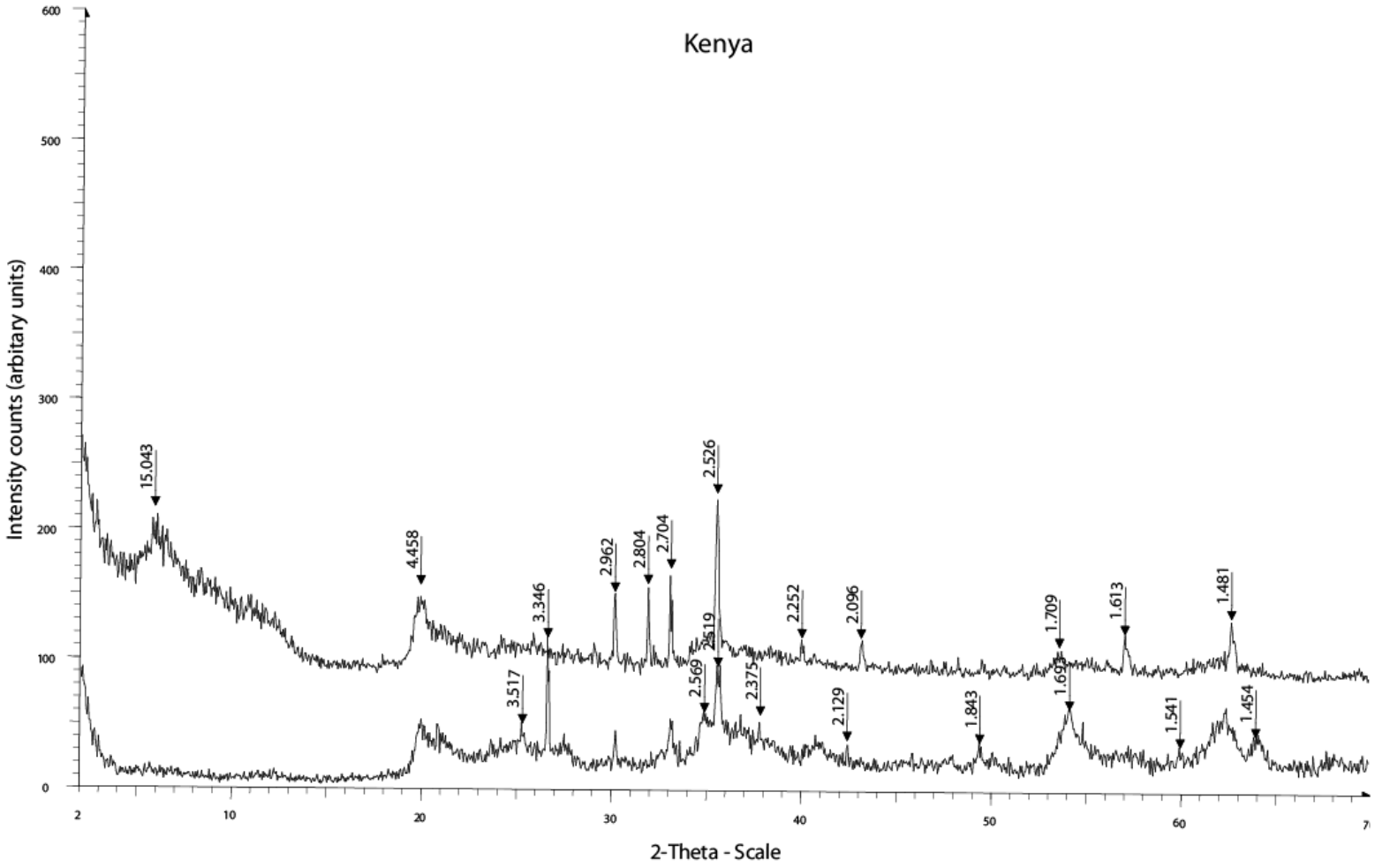

Figure 21. Powder mount XRD of samples from Kenya. “Kenya 1” showed peaks of hematite (2.69, 2.56 and 1.69 Å) and anatase (3.51, 2.37 and 1.84 Å), while “Kenya 2” revealed peaks of smectite, chlorite or vermiculite (15.04 Å), hematite (2.70 and 1.70 Å) and magnetite (2.51, 1.61 and 1.48 Å) along with quartz (3.34 Å).

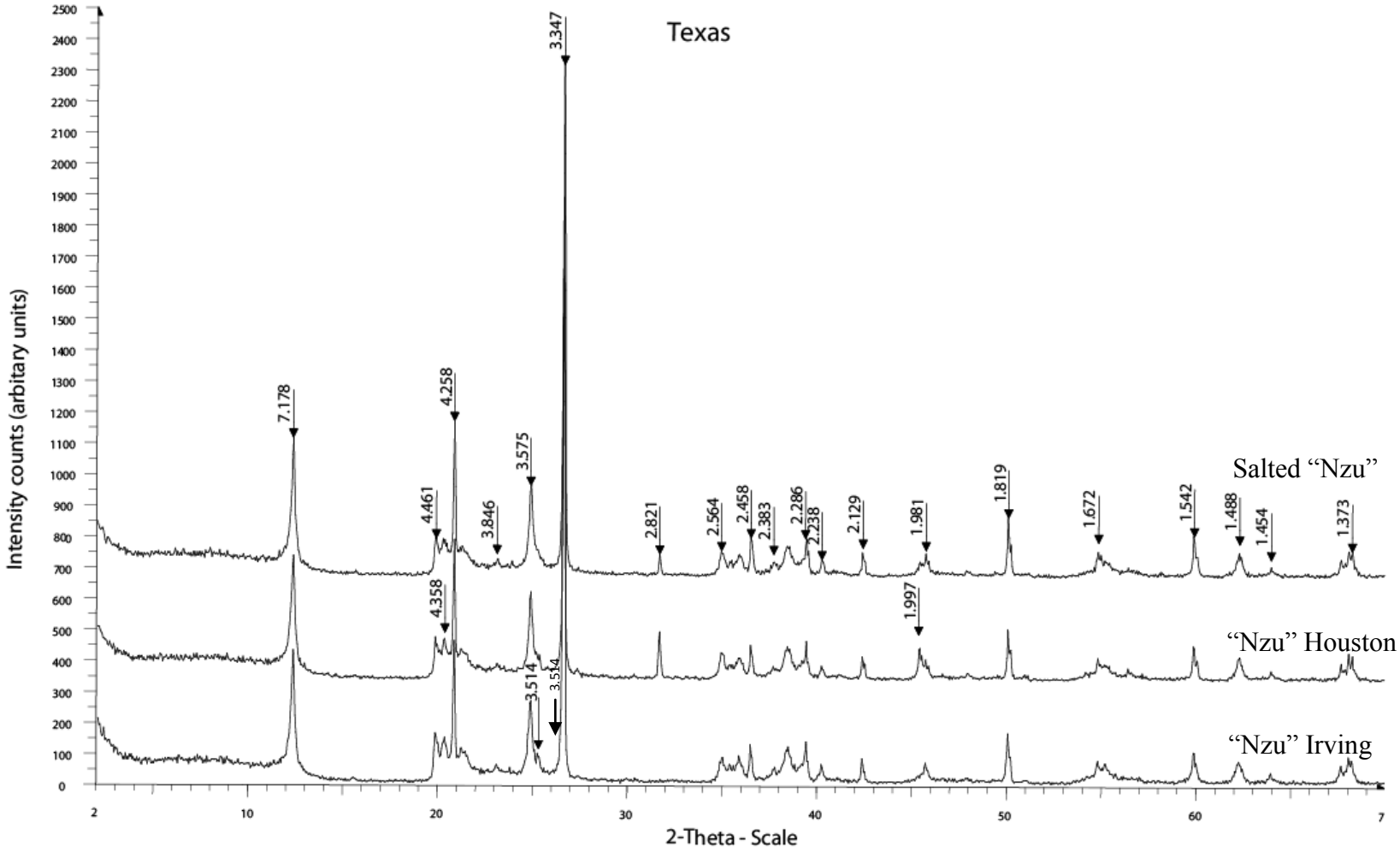

Figure 22. Powder mount XRD of clays from Texas. "Nzu" sample from Irving contained kaolinite 1A polytipe (7.18, 4.34 and 3.57 Å), quartz, and anatase (3.51 and 2.38 Å); a titanium dioxide mineral. "Nzu" from Houston and salted "Nzu" samples contained kaolinite, quartz and halite (NaCl) (2.82, 1.99 and 1.62 Å).

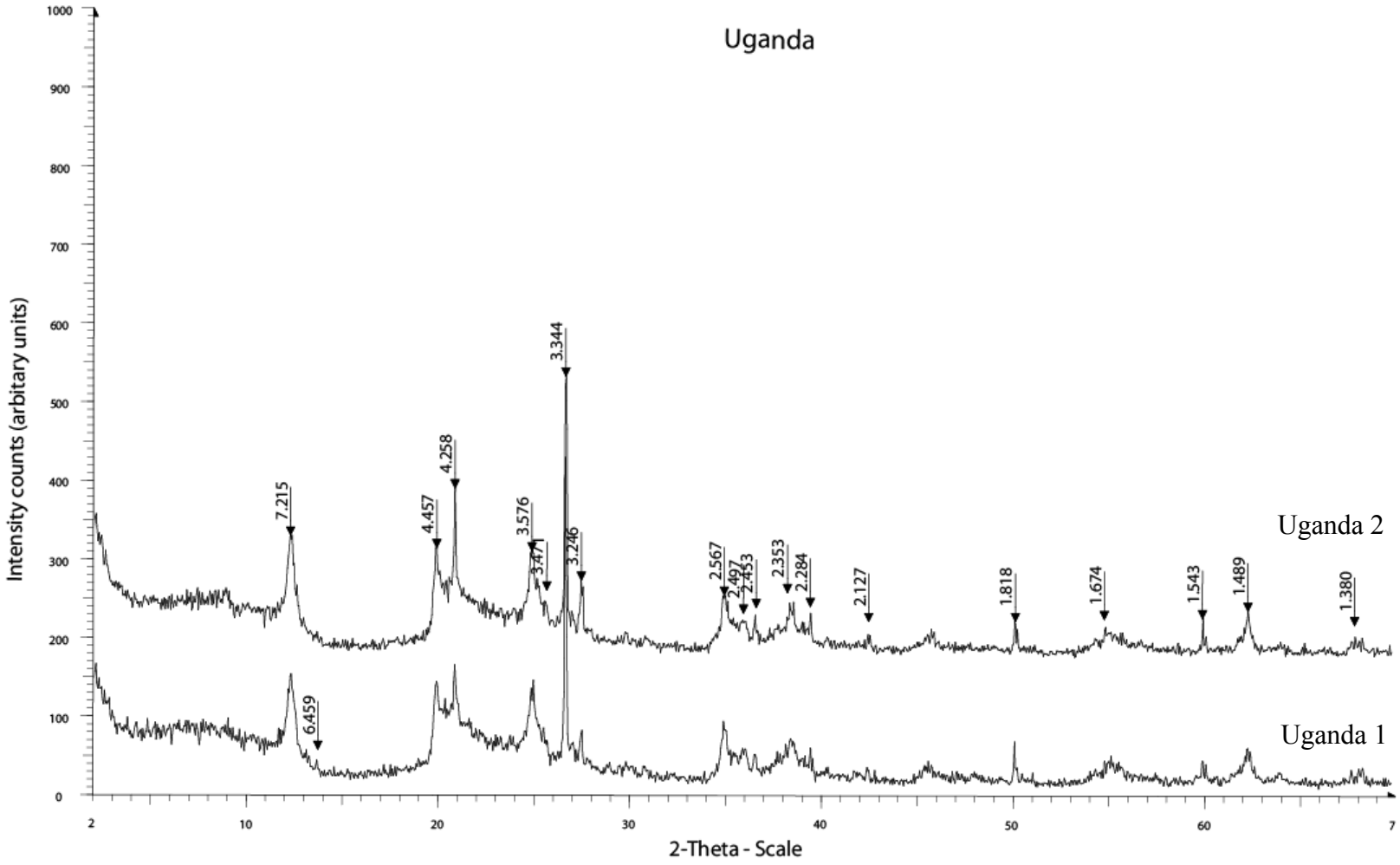

Figure 23. Powder mounts XRD of clays from Uganda. "Uganda 1" sample showed peaks of kaolinite 1Md polytipe (7.21, 3.57 and 1.48 Å), quartz (3.34 Å) and albite high (6.47 and 3.24 Å) while "Uganda 2" had peaks of kaolinite, quartz and alunite (2.99 and 2.28 Å) a sulfate mineral with formula $KAl_3(SO_4)_2(OH)_6$.

3.3.4 Hydra toxicity bioassay

In the Hydra bioassay, none of the 12 representative clays from Ghana or the rest of the edible clays from Haiti, Kenya, Texas and Uganda including the controls exerted evidence of toxicity at 0.25% w/v. Just a minor "clubbed tentacle" response was observed in hydra when exposed to "Kenya 2" but the tulip stage used as a toxic endpoint was not observed at any point throughout the study.

3.4 Discussion

From the total samples, 55% had a sorption pattern fitting either an L or H shaped curve. Nevertheless, none of the samples had comparable Q_{max} and K_d values to NSP. This was expected according to the mineral composition of the clays, with NSP containing high amounts of smectite as revealed by the XRD patterns.

Smectite is a group of minerals with 2:1 structure found in the < 2 μm fraction of bentonites. They are characterized by having layer charge ranging from 0.20 to 0.60 mol per formula unit and high CEC values commonly reported to range from 80 to 150 cmol kg^{-1} (Sparks 1995; Deer et al. 1978). The interlayer of smectites is expandable and contains water molecules and cations. This expansion makes internal surfaces available for ion exchange creating large surface areas that range from 600 to 800 m^2 g^{-1} (Reid-Soukup and Ulery 2002). Several reports showed that AF adsorption onto smectites mainly occurs in the interlayer surfaces (Grant and Phillips 1998; Kanewisher et al. 2006) and it seems to be related to the charge (net negative) (Phillips et al. 1999) and cations found in these internal surfaces (Deng et al. 2010). The effectiveness of smectites

has not just been demonstrated in several animal models but also in human studies that verified the safety and efficacy of ingested NS clay (a montmorillonite rich Ca-bentonite) in reducing the bioavailability of AFs (Afriyie-Gywau et al. 2008a; Wang et al. 2008). Bentonites have been used as anti-caking agents in feed and foods for many years and are considered GRAS additives by the FDA. In our study we found that those clays with a ~14-15 Å peak (suggestive of smectite, chlorite or vermiculite) had a higher percentage of L or H-shape sorption patterns for AF (79%). Further fractionation of the geophagic clays and analysis of the clay fraction is needed to determine the identity of the mineral depicting the 14-15 Å peak.

Kaolinite, muscovite and quartz were the most prevalent minerals present in most of the edible clays in the present study. From the total samples, kaolinite was found in 97% of the samples. Specifically for the Ghanaian clays, 100% of the samples had kaolinite. Johns and Duquette (1991b) previously reported geophagic clays for human use to have a mineral composition dominated by kaolinite. Kaolinite is a 1:1 dioctahedral phyllosilicate with the ideal chemical formula of $Al_2Si_2O_5(OH)_4$. This mineral is the most abundant phyllosilicate in highly weathered tropical regions, it has low surface area (Murray 2000) and CEC (1-5 $cmol_c\ kg^{-1}$), but it is an important source of anion exchange (White and Dixon et al. 2002). When compared to smectites, kaolinite showed unfavorable sorption pattern, and almost negligible Q_{max} affinity and capacity for AF adsorption *in vitro* (Marroquin-Cardona et al. 2009). The low CEC and low reactivity of kaolinite may explain its lack of effectiveness for AF. These characteristics may also appear to make its consumption safe. For instance, kaolinite is recognized as

GRAS additive when used as an indirect human food ingredient (food packaging) (FDA-CFR 2010) and in the past, one important use of kaolinite was as antidiarrheal medication or gastrointestinal adsorbent (Kaopectate) (WHO 2005). Nevertheless, its consumption has been implicated in exposure to dioxins. The FDA has banned the use of this mineral in animal feeds due to an episode that occurred in 1997, where high levels of dioxins were found in different animal products (FDA 1997). The source of contamination was traced to a kaolinite rich clay called "ball clay," which was used as an anti-caking agent for animal feeds. A similar incident occurred in Europe in 1999 (SCAN 2000). The abiogenic *in situ* formation of dioxins in kaolinitic clays is been recently proposed, suggesting that this mineral may promote synthesis of dioxins under high temperature and pressure (Horii et al. 2008). Because of the unavailable interlayer space and low CEC, charge and surface area, kaolinites are ineffective adsorbents for AFs. Additionally, because of the potential contamination with dioxins, the ingestion of kaolinitic clays is not recommended until safety is proven.

Muscovite is also a phyllosilicate; it is included in the mica group and has a 2:1 layer structure. It consists of two silica tetrahedral sheets on either side of the octahedral sheet that commonly contains Al, Mg or Fe. The layers of muscovite are held together by cations (especially K) that occur between the layers. Since this K is strongly held and not readily exchanged with other cations, the term “fixed K” is used. The general estimated CEC for micas at pH 7 is 30 cmol kg^{-1} (Brady and Weil 2002) and they have a layer charge of 1 mol per formula unit (Tompson and Ukrainczyk 2002), the highest one in the phyllosilicate group. Micas in the soils play an important role as K suppliers for

plants (Tompson and Ukrainczyk 2002). In spite of the large (negative) layer charge of micas, the AF sorption ability of micas has been shown to be more similar to kaolinites than that reported for smectites (Marroquin-Cardona et al. 2009). The low CEC and apparent inaccessibility of mica interlayer may explain the low AF sorption capacity of this mineral. Due to lack of laminar structure, charge differences and low CEC or pH dependent exchange capacity, minerals like iron oxides, magnesium oxides, carbonates (e.g. calcite), feldspars, alunite and quartz are also not expected to bind AF.

One important concern of the consumption of geophagic clays is the potential leaching of heavy metals from the different minerals that compose them. For instance, some authors (Carretero et al. 2006) have speculated the possibility of smectite decomposition under the acidic conditions of the stomach (pH 2), which implies bioavailability of the different metal components. In this regard, Alekseyev (2007) reported that dissolution rates of smectites under acidic conditions will depend on pH, temperature and degree of saturation of the solution. For instance, in mineral dissolution studies using FT-IR, a Wyoming montmorillonite incubated in 6M HCl at 95 °C showed no structural alteration after 4 hr, minimal structural alteration after 6 hr, and not complete dissolution even after 30 hr (Madejová et al. 1998). Under the conditions of acidity found in the human stomach, pH from 1.5 to 2.5 (Wildman and Medeiros 2000), not much dissolution of montmorillonite is expected. More importantly, the acid concentrations found in the stomach are low (0.1M HCl) and inconsistent, usually lasting only 2 or 3 hr, depending on the types of food ingested (Degen and Phillips 1996). Some authors have discussed the possibility of K bioavailability from weathered

micas and feldspars in geophagic clays (Wilson 2003). For example, plagioclase feldspars have been shown moderate dissolution in 0.01M HCl (Shotyk and Nesbit 1992) and in water (Huang and Kiang 1972). Importantly, leaching of Si, Al, Ca and Na from plagioclase feldspars in water was observed in the first 24 hr of incubation and it was assumed that the rapid increase of these elements was due to cation dissolution from broken bonds and highly strained and disordered surfaces caused by crushing (Huang and Kiang 1972). The gastric dissolution of other minerals like kaolinite and illite is not expected to occur since non swelling minerals are more resistant to HCl dissolution (Jozefaciuk and Bowanko 2002). Similarly, it has been reported that minerals like natural alunite do not dissolve in water or acids (Özdemir and Çetisli 2005).

Some components found in edible clays are expected to dissolve. For instance, the solubility of calcite, a carbonate mineral with formula $CaCO_3$, is described by an inverse correlation with pH levels (Doner and Grossl 2002). Hence, calcite is projected to act as a calcium supplement when ingested. With regard to iron oxides, the potential bioavailability of Fe is expected to be dependent on the nature and reactivity of the mineral (Wilson 2003). Usually iron oxide solubility in water follows the order ferrihydrate > maghemite > lepidocrocite > hematite > goethite (Schwertmann and Taylor 1989) and increases with decreasing pH levels (Bigham, Fitzpatrick et al. 2002). As a result, it is anticipated that hematite will be more stable than ferrihydrate in the acidic conditions of the stomach. Nevertheless, under reducing conditions, like the ones found in the large intestine, iron oxides may partially dissolve and Fe bioavailability could increase.

In relation to the potential safety/toxicity of these geophagic clays, no toxicity in the hydra bioassay was observed at 0.25% inclusion level. The apparent safety is likely to be associated to the mineral composition of the edible clays, since the most prevalent minerals (e.g. kaolinite and quartz) have low reactivities and others such as smectites have been used as mucosa protectors. Only a slight clubbed tentacle response was observed for one of the samples from Kenya. The mineralogy of this sample revealed the presence of iron oxides. Our observations of non-toxic effects in the hydra upon exposure to iron oxides are supported by other authors that used this bioassay to test the safety of ferrihydrite, a hydrous ferric oxyhydroxide mineral (Taylor et al. 2009).

In summary, most of the edible clays analyzed in the present study had limited capacity for AF adsorption. This was strongly related to the mineral composition of the clays and the specific structural characteristics of the minerals.

IV. CHARACTERIZATION AND SAFETY OF UNIFORM PARTICLE SIZE NOVASIL AS A POTENTIAL AFLATOXIN ENTEROSORBENT

4.1 Introduction

Ingestion of smectite clay is a promising approach among dietary intervention strategies to reduce AF exposure from food and feed. AFB_1 is a group 1 carcinogen (IARC 2002) mainly produced by *Aspergillus flavus* and *A. parasiticus* fungi that commonly contaminate maize and peanuts (CAST 2003). AFB_1 is a potent hepatocarcinogen (Busby and Wogan, 1984; Lopez et al., 2002) with genotoxic (Smela et al. 2001), immunotoxic (Hinton et al. 2003; Turner et al. 2003) and antinutritional (Williams et al. 2004; Pimpukdee et al. 2004) effects. In the U.S., the action level for AFs in foods intended for human consumption has been set at 20 $\mu g\ kg^{-1}$. The EU has set more conservative maximum values, ranging from 0.10 to 15 $\mu g\ kg^{-1}$. These include explicit values for single AFB_1 and total AFs, as well as specific values according to the commodities analyzed.

Montmorillonite, a mineral from the smectite group, has been shown to be the active ingredient for AF binding (Marroquin-Cardona et al. 2009; Kannewischer et al. 2006). The proposed mechanism of action is through the adsorption of AFs (mainly onto the interlayer surfaces of montmorillonite) in the gastrointestinal tract of animals and humans, thus reducing toxin bioavailability to the blood and organs (Phillips 1999). Bentonite clays, which are rich in montmorillonite, have been effectively used in dairy cows (Diaz et al. 2004; Harvey et al. 1991; Kutz et al. 2009), goats (Smith et al. 1994)

and several other animal species (Harvey et al. 1994; Ledoux et al. 1999; Marquez and Hernandez 1995; Phillips et al. 1990) to diminish the negative effects of AF exposure. Importantly, findings from a recent clinical intervention study showed that a montmorillonite rich Ca-bentonite (NovaSil-Plus, NS) was effective in reducing AF biomarkers in serum and urine with negligible nutrient interactions in humans naturally exposed to AFs via contaminated foods (Afriyie-Gyawu et al. 2008b; Wang et al. 2008).

Because of their natural origin, clays may contain numerous contaminants including heavy metals, dioxins and furans. For instance, in 1997, the FDA banned the use of "ball clay", a kaolinite rich material, in animal feed due to the high levels of dioxins (FDA 1997). Some bentonites contained significantly lower levels of these contaminants (Afriyie-Gyawu et al. 2008a); all proposed AF binders should be rigorously tested for the presence of heavy metals, dioxins/furans and other potentially hazardous substances. Uniform particle size NovaSil clay (UPSN) is a refined Ca-bentonite that has been selected to minimize the content of particles > 100 μm. This size selection is essential to diminish batch to batch differences. Expected low levels of contaminants and a more uniform particle size make UPSN a candidate for potential use as an AF sorbent in humans, upon verification of its safety and efficacy. Hence, the objectives of this study were to investigate the mineralogy and AF sorption characteristics of the refined UPSN and its parent NS, and to determine the safety of UPSN ingestion in a 3-month rodent study.

4.2 Materials and methods

4.2.1 Chemicals and samples

For all experiments, ultrapure deionized water (DI-H_2O 18MΩ cm^{-1}) was used. All reagents used in the sample preparation for mineralogical analyses were analytical grade. AFB_1 from *Aspergillus flavus* was purchased from Sigma Aldrich. UPSN clay was obtained from Texas Enterosorbents, Inc. (Bastrop, TX), and NS was obtained from Engelhard Chemical Corporation (Iselin, NJ). Levels of chlorinated dibenzo-p-dioxins/furans (CDDs/CDFs) in UPSN clay were measured by Columbia Analytical Services, Inc. (Houston, TX). Procedures followed the U.S. Environmental Protection Agency (USEPA) methods for sample preparation, clean-up and analysis with high resolution capillary column gas chromatography/high resolution mass spectrometry (USEPA Method 1613B). The analysis included the 17 USEPA priority compounds. Dioxin and furan levels in NS were previously reported by Afriyie-Gyawu et al. (2008a). UPSN and NS samples were sent to Columbia Analytical Services (Kelso, Washington) for analysis of As, Ba, Cd, Co, Cr, Mn, Mo, Ni, Pb, Sr and Zn. USEPA method 200.8 was used for As, Cd, Co, Cr, Mo, Ni, Pb and Zn, while method 6010B was used for Ba, Mn and Sr. Method 7471A was used for Hg analysis.

4.2.2 Mineralogical analyses

Mineralogical analyses included an initial sample evaluation to determine moisture content and to assess the presence of carbonate minerals, sulfides, manganese oxides and evaporites. Most of the mineralogical analyses were performed following standard procedures previously reported (Soukup et al. 2008), with minor modifications.

The presence of calcite ($CaCO_3$) was checked by using 1M hydrochloric acid (HCl). Presence of oxidizing/reducing components was evaluated using hydrogen peroxide (30% H_2O_2). Presence of magnetic minerals was assessed by using a magnetic stir bar. Occurrence of evaporite minerals was qualitatively estimated by measurement of electrical conductivity (EC) and acetone precipitation. For EC measurement, 10 g of each sample were weighed into 250 mL plastic bottles. The ratio of deionized water: solid used was 5:1. Samples were shaken for 30 min on a rotary shaker at room temperature and then centrifuged at 2000 rpm for 10 min. After centrifugation, the supernatants were used to measure the EC and pH sequentially. Two mL of the supernatants were mixed with the same volume of acetone to evaluate the presence of gypsum.

Other analyses included size fractionation, X-ray diffraction (XRD), Fourier transform infrared (FTIR) spectroscopy, scanning electron microscopy (SEM), transmission electron microscopy (TEM), cation exchange capacity (CEC) and extractable bases determination with ammonium acetate (NH_4OAc) and DI-water. The CEC determination was done following a slightly modified procedure reported by the U.S. Salinity Laboratory Staff (1969), while the extractable bases using NH_4OAc were determined with a method described by the U.S. Soil Survey Staff (1996); both analyses were done by the Soil Characterization Laboratory from the Soil and Crop Sciences Department at Texas A&M University using a mechanically controlled variable rate leaching device as reported by Holmgren et al. (1977). Additionally, extractable bases using DI-water were determined by the Soil, Water and Forage Testing Laboratory at

Texas A&M University, using a saturated paste extract method based on Rhoades and Clark (1978). An amount of 100 g of sample was used for this analysis. For NS, most of the mineralogical probes were performed, except for SEM and TEM.

Before size fractionation, carbonate-based cementing and flocculating materials were removed using sodium acetate buffer (pH 5). Organic matter (OM) was removed using 30% H_2O_2. Samples were then dispersed with 50 mL of pH 10 sodium carbonate (Na_2CO_3) solution. The sand fraction (> 53 μm) was separated using a 53 μm sieve. The clay fraction (< 2 μm) was separated from the silt fraction (2-53 μm) by centrifugation using pH 10 Na_2CO_3 as dispersant. Sand and silts weights were recorded after drying at 105 °C overnight. Clay suspensions were flocculated with sodium chloride (NaCl) and then dialyzed until EC measurements were close to the values of DI-water (< 2 μS cm^{-1}). Air dry fractionation of clays was done using an Octagon 200 sieve shaker (Endecotts). Percentage of particles > 100 μm, 100-45 μm and < 45 μm were calculated. Additionally, fractionation using water (without dispersant) was achieved using the hydrometer method according to Mildford (1997). This was important since the presence or concentrations of dispersants used for fractionation assays may not be relevant to explain the behavior of the clays *in vivo*.

XRD patterns were recorded on a Bruker D8 ADVANCE X-ray diffractometer. Sand and silt fractions were ground in a mortar, passed through a 140 mesh (105 μm) sieve and mounted as powder to collect the XRD patterns. Magnesium and potassium saturated clay suspensions were air dried on glass discs to obtain oriented films for XRD analysis. Mineral identification was determined using the EVA Bruker (Madison, WI)

computer software. Infrared patterns were recorded in a Spectrum 100 FTIR (Perkin Elmer, Inc.) using the diffuse reflectance infrared Fourier transform (DRIFT) method and the ATR method. For the DRIFT method, 0.005 g of sample was placed in a holder, using KBr as background material. For the ATR method, 1 mg of the clay fraction was mixed with 300 mg of KBr under a lamp to maintain dryness. Clay samples and KBr were mixed in a steel capsule for 30 seconds and then placed in a holder for reading.

Silt samples of UPSN were examined in a JEOL 6400 scanning electron microscope with energy dispersive X-ray (EDS) recording capability. For TEM analysis, clay samples from UPSN were mounted on a holey carbon grid, and images were recorded on a JEOL 2010 microscope with EDS capability.

4.2.3 AFB_1 sorption analyses

Isotherms were performed based on methods reported by Grant and Phillips (1998) and previously described in detail by Marroquin-Cardona et al. (2009). Isotherms were conducted in triplicate at pH 2 and 6.5. Computer-generated equilibrium isotherms were extrapolated from additive data and fit to the Langmuir model (based on r^2-values and randomness of the residuals). The parameters of Q_{max} (mol AFB_1 kg^{-1} additive) and K_d were estimated to delineate the maximum sorption to the surface and the affinity of the sorption interaction.

4.2.4 Rodent study experimental design

Authorization for the study was obtained by Texas A& M University, according to the animal use protocol (AUP) 2008-39. Sprague Dawley®™ (SD) rats were obtained from Harlan (Houston, TX). Animals in each group came from different litters to assure independence of samples. Four-week-old male (100-124 g) and female (75-99 g) rats were maintained on rodent feed 8604 Teklad Harlan (Madison, WI) meal form and water *ad libitum*. After a brief acclimation period (5 days), the rats (1 rat per cage) were allocated to either the control group (basal rodent diet) or one of the two treatment groups; 0.25% and 2% w/w inclusion of UPSN, with basal diet respectively. The corresponding groups consisted of 10 female and 10 male rats each. Dietary clay contents were based on the minimum effective dose (0.25% w/w) of NovaSil clay used in human studies (Afriyie-Gyawu et al. 2008b), and the highest level allowed (2% w/w) by the FDA as generally recognized as safe (GRAS) additive (Electronic Code of Federal Regulations, 2010). Animals were housed in a climate controlled environment (temperature 22–25°C), artificially illuminated (12 hr dark/12 hr light), and free from chemical contamination. Animals were inspected daily for general appearance, behavior, and signs of morbidity and mortality. Body weights were measured at baseline and every three days throughout the course of the study. Feed consumption was also recorded every three days. In total, body weight and feed consumption were recorded at 30 and 29 time points, respectively. After 13 weeks, final body weights were recorded, and blood was drawn via cardiac puncture under isoflurane anesthesia. Following euthanasia, organs and tissues of interest were removed and evaluated for gross abnormalities. Wet

weights of liver, kidneys, heart, lungs, brain, spleen, tibia, and uterus plus ovaries were recorded. Growth parameters measured included initial body weight (IBW), final body weight (FBW), total body weight gain (TBWG), total feed consumption (TFC) and feed conversion efficiency (FCE).

4.2.5 Hematological and serum biochemical analyses

Hematology and serum biochemical parameters were analyzed by the Clinical Pathology Lab, Texas Veterinary Medical Diagnostics Lab (TVMDL) (College Station, TX). Hematological analysis of whole blood samples was conducted using an Abbott CELL-DYN 3700 Hematology Analyzer (Abbott Laboratories, Abbott Park, IL) and included hemoglobin (Hb) concentration, mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV), percent corpuscular volume (PCV), platelets, red blood cell (RBC) and white blood cell (WBC) counts. Leukocyte differential analysis was performed manually by microscopic examination of blood smears and included neutrophils, lymphocytes, monocytes, and eosinophils. Serum biochemical parameters were assessed using an automated analyzer Modular P (Roche Diagnostics, Indianapolis, IN) and included albumin/globulin ratio (A/G), albumin (ALB), alkaline phosphatase (ALP), alanine aminotransferase (ALT), amylase (AMYL), aspartate aminotransferase (AST), blood urea nitrogen (BUN), calcium (Ca), cholesterol (CHOL), creatine kinase (CK), chloride (Cl), creatinine (CRT), gamma glutamyl-transferase (GGT), globulins (GLOB), glucose (GLUC), potassium (K), sodium (Na), Na/K ratio, phosphorous (P), total bilirubin (T-BIL), and total serum protein (TSP).

4.2.6 Iron, zinc, vitamins A and E

Serum micronutrients Fe and Zn were measured with a Hitachi 911 (Roche Laboratories, Indianapolis, IN) and a Perkin Elmer Analyst 100 (PerkinElmer, Shelton, CT), respectively. Vitamin A and E were measured by HPLC according to previously reported methods by Weinman et al. (1999) and Ruperez et al. (2004), respectively.

4.2.7 Statistical analysis

First, to investigate the weight gained over time among the absolute control and the two doses of UPSN, a linear mixed model was constructed with the fixed effect treatment, sex, and treatment*sex interaction. Time was included as a continuous variable and tested for interactions with treatment and sex. Differences in FBW were investigated with a similar model that included the fixed effect treatment and the covariates IBW and TFC as explanatory variables. Backward selection was done by removing one term at a time, starting with non-significant interactions ($p > 0.05$).

A second analysis was performed to investigate differences in biochemical parameters, relative organ weights and selected vitamins and minerals among the absolute control and the two doses of UPSN for males and females separately. A linear or a generalized linear mixed model was constructed with the fixed effect treatment as the only independent variable. The MIXED and GLIMMIX procedures were used for all statistical analyses using SAS 9.2 with Enterprise Guide 4.1. In all these analyses, the effect of subject (i.e. rat) was considered as random, since all animals came from different litters (non relatives in each group). All post-test comparisons were adjusted by the method of Tukey-Kramer. Finally, due to the nature of the data, monocyte counts

and T-BIL were analyzed with non-parametric tests using SPSS 14.0. Data for T-BIL were classified in categories before analysis. Kruskal-Wallis test followed by Mann-Whitney test for multiple comparisons were used to detect individual differences between groups.

4.3 Results

4.3.1 Dioxins/furans and mineral analyses

The only dioxins/furans detected in UPSN were octachlorodibenzo-p-dioxin (OCDD) and heptachorodibenzo-p-dioxin (HpCDD). The average toxic equivalents (TEQs) for these compounds were calculated to be 0.0015 pg g^{-1}. According to previous reports (Afriyie-Gyawu et al., 2008a), HpCDD and OCDD were the only dioxins in NS present above the limits of detection (LOD = 1.11 pg g^{-1} for HpCDD and 1.91 pg g^{-1} for OCDD). The combined toxic equivalent (TEQ) value calculated for both dioxins was 0.0466 pg g^{-1}. In general, the heavy metal analyses showed a similar profile for both clays (Table 3).

Table 3. Levels of heavy metals in Uniform Particle Size NovaSil and NovaSil

Metal	Conc. in UPSN (mg kg^{-1})	Conc. in NS (mg kg^{-1})	Amt. in 3 g UPSN (mg)	Amt. in 3 g NS (mg)	TDI/ PTDI (mg/kg^{-1} BW)	Based on 70 kg BW (mg)	Reference TDI
As	2.5	1.7	0.007	0.005	0.002	0.14	WHO, 1989
Ba	72.1	77.5	0.216	0.233	0.051	3.57	NSCFS, 2004
Cd	0.1	0.1	0.000	0.000	0.001	0.07	WHO, 1989
Co	1.8	1.4	0.005	0.004	0.001	0.07	RIVM, 2001
Cr	1.2	1.7	0.004	0.005	0.005	0.35	RIVM, 2001
Hg	0.003	0.009	0.000	0.000	0.0002	0.01	JECFA, 2007
Mn	223.0	172.0	0.669	0.516	0.030	2.10	OEHHA, 2004
Mo	0.2	0.1	0.001	0.000	0.010	0.70	RIVM, 2001
Ni	3.5	3.0	0.011	0.009	0.005	0.35	WHO, 1993
Pb	12.6	10.5	0.038	0.031	0.003	0.21	JEFCA, 2000
Sr	1150.0	1040.0	3.450	3.120	0.130	9.1	WHO, 2010
Zn	53.8	38.0	0.160	0.114	1.000	70.0	JECFA, 1982

Notes: TDI: Tolerable daily intake; PTDI: Proposed tolerable daily intake; RIVM: National Institute of Public Health and the Environment; NSCFS: Norwegian Scientific Committee for Food Safety; JECFA: Joint FAO/WHO Expert Committee on Food Additives; OEHHA: Office of Environmental Health Hazard Assessment; WHO: World Health Organization.

4.3.2 Mineralogical analyses

The results for sample evaluation and other mineralogical analyses are summarized in Table 4. Differences were observed in moisture content, electrical conductivity, CEC, gypsum precipitation and percentages of the different fractions in both, dry and wet states. The major minerals identified in both samples were montmorillonite and quartz (Figure 23). A lower relative intensity of quartz was observed in UPSN suggesting less content of this mineral. Calcite and potassium feldspars (e.g. sanidine) were also observed in both samples. Quartz was the major mineral identified in the sand fractions of both samples; however a broad smectite peak was still evident in both samples, suggesting the presence of smectite aggregates. Similarly, smectite, along with sanidine, albite, muscovite, and quartz minerals, were identified in the silt fraction of both samples.

Montmorillonite presence was confirmed in both samples after XRD analysis of the clay fraction exchanged with Mg and K. After treatment with Mg and glycerol, the d-space of the most intense montmorillonite peak increased from ~15 to ~18 Å. Air dried samples exchanged with K showed a peak of ~12 Å. After heating at 330°C and 550°C, the K exchanged samples depicted a peak of ~10 Å and ~9.8 Å, respectively. This pattern of collapse of the major 15 Å peak was expected for montmorillonites. Montmorillonite was also confirmed with KBr-DRIFT and ATR. Both samples showed a characteristic band of montmorillonite of 3631 cm^{-1} (UPSN) and 3619 cm^{-1} (NS) in the ATR pattern. Similarly, KBr-DRIFT patterns displayed a confirmatory montmorillonite band at 3632 cm^{-1} (UPSN) and 3623 cm^{-1} (NS).

Table 4. Characterization of Uniform Particle Size NovaSil and NovaSil

Parameter	UPSN	NS
Moisture %	6.7	12.1
pH	7.5	7.1
EC (µS)	304.1	535.0
HCl reaction	Little	Little
H_2O_2 reaction	ND	Little
Magnetic minerals	ND	Little
Gypsum precipitation	ND	Evident
Dry state fractions (%)		
> 100µm	2	20
100-45µm	67	30
< 45µm	31	50
Wet sate fractions (%) (water + Na_2CO_3)		
>53µm	1.0	15.0
2-53µm	23.0	20.0
<2µm	76.0	65.0
Wet state fractions (%) (water)		
>50 µm	0	25
2-50 µm	25	67
<2 µm	75	8
CEC (cmol kg^{-1})	89.2	82.9
Extractable bases (cmol kg^{-1})(NH_4OAc)		
Ca	98.4	85.8
Mg	8.7	9.1
Na	0.5	0.5
K	1.7	1.5
Soluble bases (mEq L^{-1})(DI-water)*		
Ca	8.1	20.4
Mg	0.7	1.6
Na	0.9	1.4
K	0.6	0.8

Notes: * Soluble bases were measured in DI-water vacuum extracts from a paste according to Rhoades and Clark 1978; ND: non-detectable.

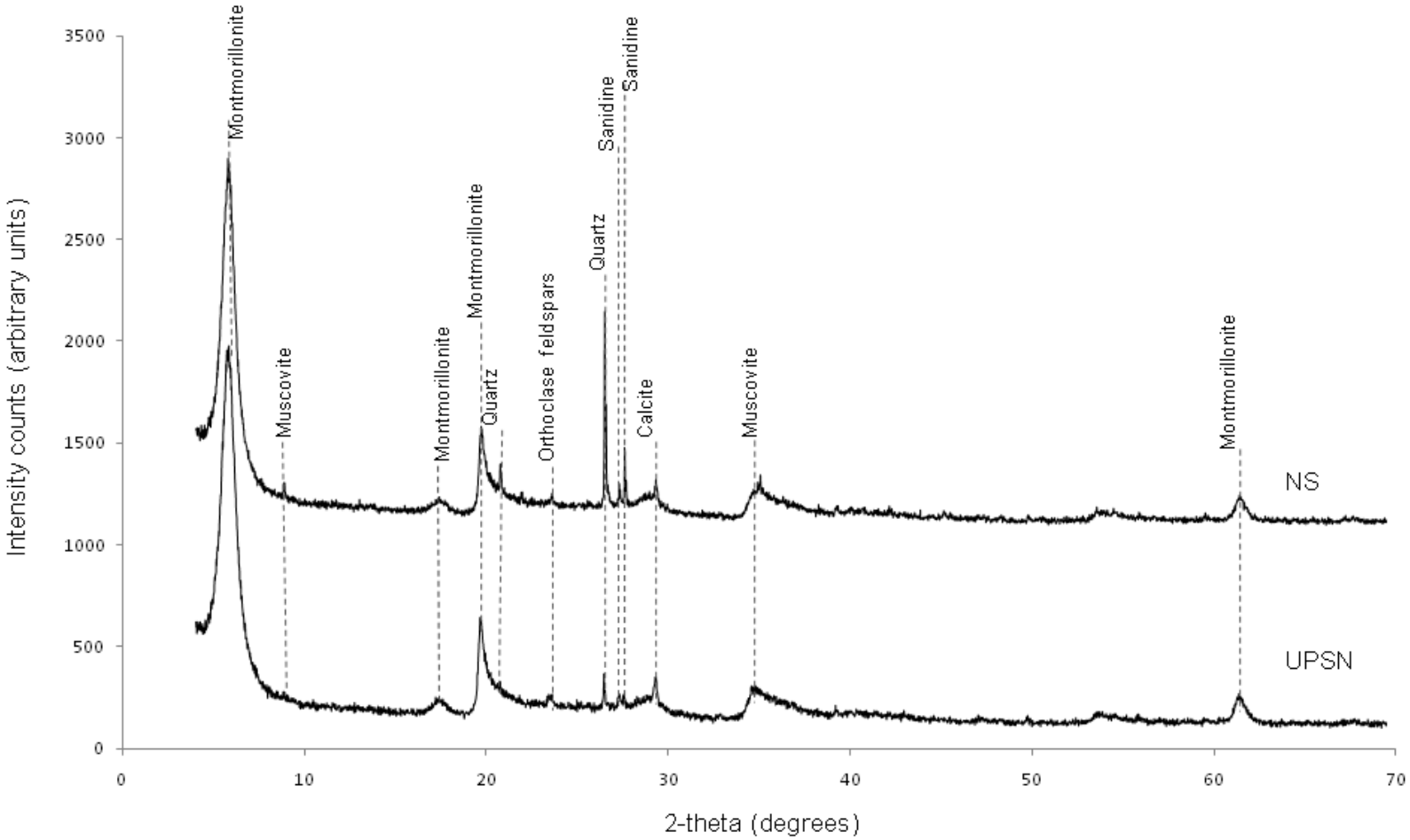

Figure 24. Powder mounts XRD patterns of UPSN and parent NS. Minerals identified in both samples included montmorillonite (15.23, 5.1, 4.48, 1.49 Å), mica (9.9, 2.5 Å), quartz (3.3 Å), calcite (3.0 Å), orthoclase feldspars (3.7 Å) and sanidine (3.24, 3.20 Å). Quartz relative intensities in UPSN were lower than in parent NS.

Additionally, bands of AlFeOH deformation (884 and 882 cm^{-1}) and AlMgOH (842 and 845 cm^{-1}) were observed for UPSN and NS, respectively. SEM findings for the silt fraction of UPSN confirmed the presence of feldspars and mica. Additionally, smectite particles were noted in the silts (Figure 25A). The main feature observed in the TEM images was the folding tendency of montmorillonite particles (Figure 25B). The particles are shown as thin films of considerable length (3-5 µm) that fold many times and can be described like a "veil fabric".

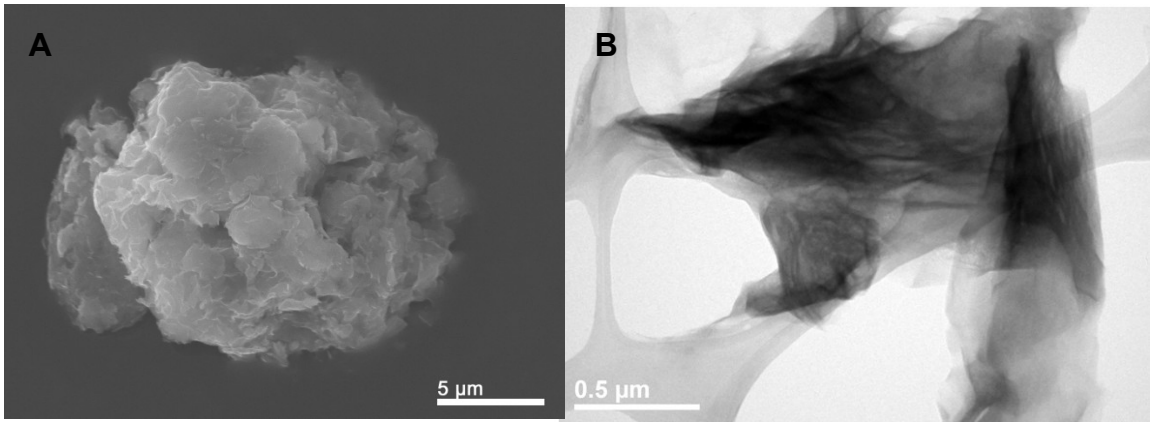

Figure 25. SEM and TEM images of smectite particles in UPSN. A) SEM image of smectite particle in UPSN. The particle is depicting a globular morphology with dimensions of ~25 µm x 15 µm, according to the scale bar. B) TEM image of smectite particle displaying complex morphology of sheets and rolls. This morphology is commonly observed for good aflatoxin adsorbents, according to Mulder et al. (2008).

4.3.3 AFB_1 sorption analyses

The parameters of Q_{max} and K_d were similar for both samples at both pH levels. Samples fitted the Langmuir model ($r^2 \geq 0.92$) and depicted the favorable AF sorption pattern characterized as L-shape (Figure 26). Specifically at pH 6.5, and L2-pattern was documented for both samples (plateau has been reached); while at pH2 the sorption pattern more closely resembled an L1-pattern (in process of reaching the plateau). The Q_{max} values calculated for UPSN and NS at pH 2 were 0.39 ± 0.01 and 0.40 ± 0.03 mol AFB_1 kg^{-1}, while at pH 6.5 Q_{max} values were 0.44 ± 0.05 and 0.41 ± 0.02 mol AFB_1 kg^{-1}, respectively. A slight higher binding capacity was noted for UPSN at pH 6.5 when compared to NS.

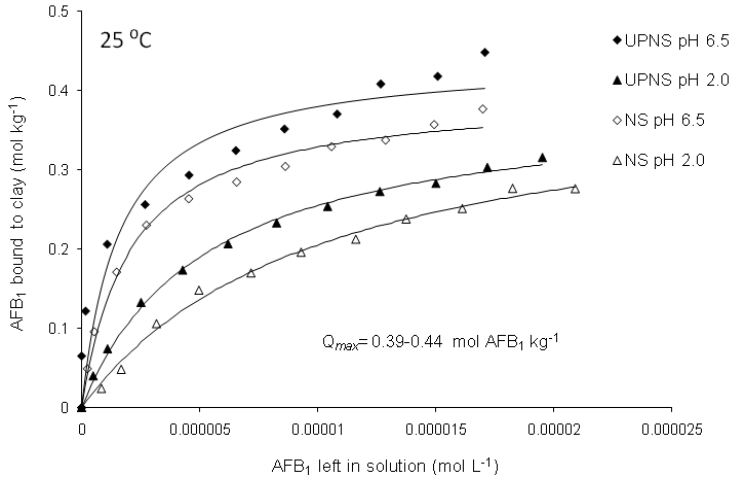

Figure 26. AFB_1 adsorption onto UPSN and NS at pH 6.5 and 2. Both samples depicted an L-shape pattern characteristic of planar arrangement of molecules adsorbed on the surface. The bend on the pattern indicates that the sorbent is reaching saturation as the concentration of the solute is increased. The Q_{max} values calculated for both bentonites were similar at both pH levels, ranging from 0.39 to 0.44 mol AFB_1 kg^{-1}.

4.3.4 Rodent study outcomes

No apparent adverse effects due to consumption of either dose of UPSN were noticed throughout the study. All rats remained healthy and active without noticeable changes in behavior. Males gained more weight than the females ($p < 0.0001$), as expected. Gained weight across time was different in at least on of the treatment groups ($p = 0.0099$) and the effect of treatment was different between males and females ($p = 0.0007$). There was no difference in weight gained in the males across all time points (a total of 30 time points recorded) and groups. In contrast, females in the control group gained more weight than the females in the 2% UPSN group by time 15 (day 43) ($p = 0.0351$) and consecutively up to time 30 (day 88) ($p = 0.0026$), but not on time 10 (day 28) or before. Similarly, females in the 0.25% UPSN group gained more weight than the females in the 2% UPSN group by time 30 (day 88) ($p = 0.0362$) but not on time 25 (day 73) or before. Significant differences were observed between the control and the treatment groups for some of the growth parameters in the females (Table 5). TFC and IBW were not significant factors for explaining the differences in FBW of the females.

4.3.5 Hematological and serum biochemistry analyses

No statistically significant differences were observed between the control and the UPSN groups for most of the biochemical parameters measured (Tables 6 and 7). Nevertheless, some significant differences were registered in at least one of the treatment groups, for either females or males. Importantly, most of the parameters measured in this study fell between the normal ranges reported for control animals (< 6 months old) in several studies and did not show patterns of dose dependency.

Table 5. Growth parameters of SD rats receiving dietary UPSN at 0.25 and 2% for 13 weeks

Group	Control	UPSN 0.25%	UPSN 2%
Females			
FBW (g)	243.85 ± 13.49	242.41 ± 10.29[a]	231.42 ± 6.67[*b]
FCE	13.24 ± 2.74	13.60 ± 2.33[a]	16.45 ± 2.72[*b]
IBW (g)	99.52 ± 4.75	95.88 ± 4.93	94.72 ± 3.25[*]
TBWG (g)	144.33 ± 10.12	146.53± 9.43[a]	136.70 ± 7.23[b]
TFC (g)	1899.17 ± 341.57	1982.55 ± 292.75	2244.73 ± 368.36
Males			
FBW (g)	406.45 ± 19.99	423.65 ± 13.8	424.88 ± 13.89
FCE	7.42 ± 0.70	7.42 ± 0.32	7.19 ± 0.25
IBW (g)	120.87 ± 3.65	122.37 ± 4.20	124.42 ± 4.64
TBWG (g)	285.58 ± 20.18	301.28 ± 12.95	300.46 ± 12.14
TFC (g)	2110.08 ± 104.45	2233.62 ± 102.72	2160.43 ± 42.07

Notes: Means with asterisk are significantly different from the control at the 0.05 level. Means with different letters are significantly different between USPN treatment groups at the 0.05 level. Data are reported as mean values ± standard deviation. FBW: Final body weight; FCE: Feed conversion efficiency; IBW: Initial body weight; TBWG: Total body weight gain; TFC: Total feed consumption.

Table 6. Hematological parameters of SD rats receiving dietary UPSN at 0.25 and 2% for 13 weeks

	Group				
Parameter	Control	UPSN 0.25%	UPSN 2%	Normal values	Reference
Females					
Eosinophils μL^{-1}	25.30 ± 29.91	25.30 ± 37.83	25.10 ± 35.23	~ 196.50 ± 216.10	B
Hb (g dL^{-1})	14.19 ± 0.33	13.96 ± 0.59	13.77 ± 0.34*	14.30 ± 0.83	C
Lymphocytes μL^{-1}	3755.10 ± 907.92	5654.00 ± 1992.89*	4497.20 ± 855.67	6880.00 ± 2630.00	B
MCH (pg)	18.44 ± 1.70	18.33 ± 2.30	19.14 ± 1.91	19.8 ± 0.94	C
MCHC (g dL^{-1})	34.43 ± 0.72	33.30 ± 1.00*	33.36 ± 0.55*	34.40 ± 0.50	B
MCV (fL)	55.85 ± 1.76	57.72 ± 2.40	58.36 ±1.55*	57.90 ± 2.10	B
Monocytes μL^{-1}	0 ± 0.00	81.60 ± 70.85*[a]	0 ± 0.00[b]	~19.60 ± 58.90	B
Neutrophils μL^{-1}	163.70 ± 111.49	288.30 ± 373.30[a]	44.70 ± 62.04[b]	870.00 ± 660.00	B
PCV (%)	41.24 ± 1.53	41.96 ± 2.07	41.30 ± 1.42	38.10 ± 2.43	C
Platelets ($x10^3$) μL^{-1}	468.20 ± 77.83	619.40 ± 224.76[a]	344.80 ± 85.57[b]	471.68 ± 38.77	A
RBCs ($x10^6$) μL^{-1}	7.39 ± 0.29	7.27 ± 0.29	7.08 ± 0.31	7.02 ± 0.40	B
WBC μL^{-1}	3944.00 ± 962.96	6049.00 ± 2267.71*	4567.00 ± 872.60	7840.00 ± 2950.00	B
Males					
Eosinophils μL^{-1}	134.00 ± 109.88	49.30 ± 86.83	39.60 ± 60.57	~89.30 ± 114.80	B
Hb (g dL^{-1})	14.51 ± 0.46	14.40 ± 0.36	15.05 ± 0.78	14.70 ± 1.22	C
Lymphocytes μL^{-1}	6265.60 ± 1994.52	6951.40 ± 1839.27	6047.30 ± 1083.73	6880.00 ± 2630.00	B
MCH (pg)	18.78 ± 0.45	18.05 ± 0.41*	18.46 ± 0.50	19.00 ±1.09	C
MCHC (g dL^{-1})	33.77 ± 0.71	32.59 ± 0.64*[a]	33.82 ± 0.58[b]	34.10 ± 0.40	B
MCV (fl)	55.78 ± 2.25	55.49 ± 1.63	54.61 ± 1.70	59.50 ± 2.00	B
Monocytes μL^{-1}	18.80 ± 54.70	95.30 ± 96.04	19.10 ± 31.57	~38.20 ± 63.80	B
Neutrophils μL^{-1}	819.90 ± 669.78	517.00 ± 623.21[a]	79.10 ± 50.89*[b]	870.00 ± 660.00	B
PCV (%)	43.10 ± 1.73	44.22 ± 1.48	44.50 ± 1.96	39.90 ± 3.49	C
Platelets ($x10^3$) μL^{-1}	418.60 ± 133.39	692.50 ± 145.47*[a]	374.00 ± 81.09[b]	471.68 ± 38.77	A
RBCs ($x10^6$) μL^{-1}	7.73 ± 0.32	7.97 ± 0.31	8.15 ±0.28*	7.82 ± 0.83	C
WBC μL^{-1}	7238.00 ± 2462.55	7613.00 ± 2293.36	6185.00 ± 1106.74	7840.00 ± 2950.00	B

Notes: Means with asterisk are significantly different from the control at the 0.05 level. Means with different letters are significantly different between UPSN treatment groups at the 0.05 level. Data are reported as mean values ± standard deviation. Hb: hemoglobin; MCH: mean cell hemoglobin; MCHC: mean corpuscular hemoglobin concentration; MCV: mean corpuscular volume; PCV: packed cell volume; RBC: red blood cell; WBC: white blood cell. References: A) Almodovar et al. 1985; B) Lillie et al. 1996; C) Woldford et al. 1986.

Table 7. Serum biochemical parameters of SD rats receiving dietary UPSN at 0.25 and 2% for 13 weeks

	Group				
Parameter	Control	UPSN 0.25%	UPSN 2%	Normal values	Reference
Females					
A/G Ratio	2.41 ± 0.11	2.18 ± 0.21*	2.41 ±0.22	2.13 ± 0.18	A
ALB (g dL^{-1})	3.74 ± 0.15	3.79 ± 0.10	3.77 ± 0.13	3.41 ± 0.23	C
ALP (U L^{-1})	58.40 ± 14.23	67.60 ± 17.10	69.20 ± 7.87	117 ± 41.7	B
ALT (U L^{-1})	54.70 ± 9.47	51.00 ± 8.75	51.60 ± 9.77	44.00 ± 23.9	D
AMYL (U L^{-1})	1258.40 ± 112.41	1413.50 ± 268.12	1341.70 ± 138.95	1703 ± 164.32	A
AST (U L^{-1})	138.20 ± 42.38	98.40 ± 24.23*	103.40 ± 24.96	93.00 ± 30.3	D
BUN (mg dL^{-1})	18.44 ± 1.70	18.33 ± 2.30	19.14 ± 1.91	21.00 ± 3.90	D
Ca (mg/dL)	9.34 ± 0.32	9.80 ± 0.25*[a]	9.53 ± 0.22[b]	10.36 ± 0.32	C
CHOL (mg dL^{-1})	86.73 ± 9.33	92.43 ± 11.80	90.35 ± 10.52	89.00 ± 23.00	D
CK (U L^{-1})	815.11 ± 360.20	262.70 ± 102.96*	558.20 ± 421.90	210.00 ± 109	C
Cl (mEq L^{-1})	94.50 ± 19.54	101.90 ± 1.37	101.50 ± 0.84	104.00 ± 2.40	B
CRT (mg dL^{-1})	0.29 ± 0.03	0.30 ± 0.03	0.32 ± 0.02	0.55 ± 0.10	C
GGT (U L^{-1})	<3.0	<3.0	<3.0	<3.0	A
GLOB (g dL^{-1})	1.55 ± 0.08	1.75 ± 0.16*	1.57 ± 0.14	1.91 ± 0.09	A
GLUC (mg dL^{-1})	180.10 ± 15.62	192.00 ± 20.48	190.00 ± 20.79	199.80 ± 21.60	C
K (mEq L^{-1})	5.71 ± 1.33	4.89 ± 0.32	4.68 ± 0.19*	4.76 ± 0.44	C
Na (mEq L^{-1})	129.80 ± 25.68	140.30 ± 1.06*	141.60 ± 0.70*	142.00 ± 2.00	C
Na/K Ratio	23.04 ± 2.99	28.80 ± 1.78*	30.32 ± 1.28*	~29	C
P (mg dL^{-1})	5.56 ± 0.87	4.94 ± 0.61	5.30 ± 0.37	5.80 ± 1.10	B
T-BIL (mg dL^{-1})	0.09 ± 0.05	0.07 ± 0.06	0.13 ± 0.02	0.30 ± 0.24	D
TP (g dL^{-1})	5.29 ± 0.21	5.54 ± 0.17*	5.34 ± 0.19	6.17 ± 0.33	C

Notes: Means with asterisk are significantly different from the control at the 0.05 level. Means with different letters are significantly different between UPSN treatment groups at the 0.05 level. Data are reported as mean values ± standard deviation. ALB: albumin; ALP: alkaline phosphatase; ALT: alanine aminotransferase; AMYL: amylase; AST: aspartate aminotransferase; BUN: blood urea nitrogen; CHOL: cholesterol; CK: creatine kinase; CTR: creatinine; GGT: gamma-glutamyl transferase; GLOB: globulin; GLUC: glucose; T-BIL: total bilirubin; TP: total protein. References: A) Afriyie-Gyawu et al. 2005; B) Kohn and Clifford 2002; C) Lillie et al. 1996; D) Woldford et al. 1986.

Table 7. continued

Parameter	Group			Normal values	Reference
	Control	UPSN 0.25%	UPSN 2%		
Males					
A/G Ratio	1.93 ± 0.15	1.75 ± 0.17	1.82 ± 0.10	1.37 ± 0.18	A
ALB (g dL^{-1})	3.42 ± 0.15	3.67 ± 0.20*	3.70 ± 0.16*	3.40 ± 0.20	B
ALP (U L^{-1})	79.20 ± 9.47	60.40 ± 14.35*	58.40 ± 8.45*	130 ± 43.7	B
ALT (U L^{-1})	61.30 ± 19.38	49.20 ± 8.79[a]	40.20 ± 3.82*[b]	49.00 ± 24.1	B
AMYL (U L^{-1})	2316.00 ± 211.13	2329.80 ± 274.48	2305.20 ± 192.53	2671.00 ± 89.00	A
AST (U L^{-1})	116.80 ± 49.97	104.40 ± 31.13[a]	70.30 ± 13.42*[b]	97.00 ± 28.40	D
BUN (mg dL^{-1})	19.41 ± 2.63	20.98 ± 2.50	20.88 ± 3.08	20.00 ± 3.20	D
Ca (mg dL^{-1})	9.12 ± 0.39	9.90 ± 0.18*	9.82 ± 0.30*	10.48 ± 0.28	C
CHOL (mg dL^{-1})	85.37 ± 10.16	95.70 ± 9.79	97.69 ± 12.03*	75.00 ± 19.80	D
CK (U L^{-1})	432.22 ± 227.32	338.80 ± 151.83	271.60 ± 92.34	222.00 ± 109.00	C
Cl (mEq L^{-1})	98.30 ± 2.26	100.20 ± 0.92	101.00 ± 2.30*	103.00 ± 1.90	B
CRT (mg dL^{-1})	0.26 ± 0.03	0.29 ± 0.03	0.28 ± 0.02	0.54 ± 0.08	C
GGT (U L^{-1})	<3.0	<3.0	<3.0	<3.0	A
GLOB (g dL^{-1})	1.78 ± 0.15	2.11 ± 0.14*	2.04 ± 0.11*	2.51 ± 0.25	A
GLUC (mg dL^{-1})	192.70 ± 37.05	214.20 ± 39.70	192.10 ± 19.83	212.40 ± 28.8	C
K (mEq L^{-1})	7.23 ± 0.66	5.11 ± 0.38*	5.38 ± 0.27*	5.30 ± 0.39	C
Na (mEq L^{-1})	135.20 ± 2.74	140.80 ± 0.63*	142.30 ± 3.30*	142.00 ± 2.00	C
Na/K Ratio	18.86 ± 1.74	27.69 ± 2.11*	26.51 ± 1.36*	~26	C
P (mg dL^{-1})	5.92 ± 0.39	6.08 ± 0.59	6.15 ± 0.22	7.30 ± 1.50	B
T-BIL (mg dL^{-1})	0.01 ± 0.03	0.07 ± 0.06	0.09 ± 0.05*	0.30 ± 0.16	D
TP (g dL^{-1})	5.20 ± 0.25	5.78 ± 0.17*	5.74 ± 0.22*	5.85 ± 0.23	C

Notes: Means with the same letter are not significantly different at the 0.05 level. Means with different letters are significantly different between UPSN treatment groups at the 0.05 level. Data are reported as mean values ± standard deviation. ALB: albumin; ALP: alkaline phosphatase; ALT: alanine aminotransferase; AMYL: amylase; AST: aspartate aminotransferase; BUN: blood urea nitrogen; CHOL: cholesterol; CK: creatine kinase; CTR: creatinine; GGT: gamma-glutamyl transferase; GLOB: globulin; GLUC: glucose; T-BIL: total bilirubin; TP: total protein. References: A) Afriyie-Gyawu et al. 2005; B) Kohn and Clifford 2002; C) Lillie et al. 1996; D) Woldford et al. 1986.

4.3.6 Iron, zinc, vitamin A and E analyses

No statistically significant differences were observed between the control and the treatment groups for Fe and vitamin A, in either males or females. Differences were observed for Zn, in both sexes of the 2.0% UPSN group when compared to the control (Table 8). However, Zn values were still within the ranges reported by other authors for 28 week old control animals (Afriyie-Gyawu et al. 2005). Serum vitamin E was significantly increased for both sexes in the UPSN groups (Table 8).

4.3.7 Relative organ weights

Isolated differences were observed in relative organ weights for both males and females (Table 9). However, none of the differences registered for relative organ weights suggested dose dependency.

4.4 Discussion

4.4.1 Mineral characteristics and AFB_1 sorption analyses

The levels of dioxin/furans in UPSN were lower than in parent NS. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) has placed a provisional tolerable daily intake (PTDI) value for dioxins of ~2.3 pg WHO-TEQ kg-bw^{-1} day^{-1} (JECFA 2001) for humans.

Table 8. Serum Fe, Zn, vitamin A and E of SD rats receiving dietary UPSN at 0.25 and 2% for 13 weeks

	Group				
Parameter	Control	UPSN 0.25%	UPSN 2%	Normal values	Reference
Females					
Iron (µg dL^{-1})	254.67 ± 38.20	245.74 ± 33.98	246.47 ± 60.02	220 ± 130	B
Zinc (µg mL^{-1})	1.24 ± 0.18	1.34 ± 0.09[a]	1.12 ± 0.10[b]	1.05 ± 0.09	A
Vitamin A (µg L^{-1})	267.31 ± 27.78	275.50 ± 33.92	271.01 ± 19.11	254.10 ± 6.00	A
Vitamin E (µg L^{-1})	3560.00 ± 40.00	9150.00 ± 1350[*]	8500.00 ± 1040.00[*]	NA	
Males					
Iron (µg dL^{-1})	115.89 ± 10.92	126.87 ± 19.99	123.45 ± 16.62	152 ± 70	B
Zinc (µg mL^{-1})	1.50 ± 0.09[a]	1.51 ± 0.13[a]	1.24 ± 0.09[*b]	1.29 ± 0.04	A
Vitamin A (µg L^{-1})	530.80 ± 39.44	489.50 ± 80.10	509.40 ± 36.22	567.40 ± 24.80	A
Vitamin E (µg L^{-1})	3440.00 ± 50.00	7860.00 ± 2690.00[*]	7430.00 ± 870.00[*]	2106.17 ± 1261.98	E

Notes: Means with asterisk are significantly different from the control at the 0.05 level. Means with different letters are significantly different between UPSN treatment groups at the 0.05 level. Data are reported as mean values ± standard deviation. NA: not-available. References: A) Afriyie-Gyawu et al. 2005; B) Kohn and Clifford 2002; E) Seifi et al. 2009.

Table 9. Relative organ weights of SD rats receiving dietary UPSN at 0.25 and 2% for 13 weeks

Group	Control	UPSN 0.25%	UPSN 2%
Females			
Brain	1.63 ± 0.11	1.60 ± 0.07[a]	1.66 ± 0.04*[b]
Heart	0.82 ± 0.06	0.81 ± 0.06	0.81 ± 0.05
L. kidney	0.80 ± 0.07	0.79 ± 0.08	0.71 ± 0.04*
Liver	6.82 ± 0.40	6.92 ± 0.71	6.35 ± 0.27
Lung	1.20 ± 0.10	1.17 ± 0.06	1.11 ± 0.07
R. kidney	0.79 ± 0.06	0.77 ± 0.07	0.72 ± 0.05
Spleen	0.52 ± 0.06	0.53 ± 0.06	0.51 ± 0.06
Tibia	0.75 ± 0.13	0.70 ± 0.04	0.62 ± 0.07
Uterus	0.63 ± 0.13	0.59 ± 0.12	0.55 ± 0.12
Males			
Brain	1.65 ± 0.09	1.75 ± 0.15	1.69 ± 0.24
Heart	1.36 ± 0.09	1.32 ± 0.10[a]	1.42 ± 0.11[b]
L. kidney	1.31 ± 0.11	1.33 ± 0.12	1.32 ± 0.08
Liver	12.34 ± 0.82	12.10 ± 0.89	11.35 ± 0.63*
Lung	1.64 ± 0.21	1.65 ± 0.22	1.61 ± 0.14
R. kidney	1.35 ± 0.14	1.39 ± 0.12	1.33 ± 0.09
Spleen	0.77 ± 0.07	0.78 ± 0.08	0.79 ± 0.09
Tibia	1.04 ± 0.04	1.06 ± 0.03	1.14 ± 0.10

Notes: Means with asterisk are significantly different from the control at the 0.05 level. Means with different letters are significantly different between UPSN treatment groups at the 0.05 level. The relative organ weights (ROWs) were calculated by dividing the wet weight of each organ by the final body weight of the rat just before termination. ROWs have been multiplied by a factor of 10^2. Data are reported as mean values ± standard deviation.

The levels found in both UPSN and NS are below these PTDIs, hence negligible risk of dioxin/furan exposure is expected from these clays. The metal analysis for both clays also showed similarities. The amount of NS previously used in human trials was equal to 3 g/day/participant. In either clay, the concentrations of trace metals were below the TDIs or PTDIs that have been reported from different sources (Table 1). This is particularly important for future applications of UPSN in studies involving human subjects. The mineralogical analyses for UPSN and parent NS revealed similar structural, morphological and chemical characteristics, as expected. Similar pH values were obtained for both clays. Previous studies have found pH values ranging from 6.7 to 9.6 (Marroquin-Cardona et al. 2009) and 7.1 to 9.6 (Kannewischer et al. 2006) for similar bentonite samples. Small differences were observed in moisture content, electrical conductivity, CEC, gypsum precipitation and the particle size distribution in dry and wet sates. Differences in moisture content may be related to batch to batch variations of the clay, while the greater electrical conductivity values obtained for NS are likely related to the presence of gypsum in this sample. The slightly higher CEC obtained for UPSN (89.2 cmol kg^{-1}) could be related to the increased proportion of < 2 μm particles in this clay, as revealed by the wet state fractionations. Common CEC values for smectites fall between 80-150 cmol kg^{-1} (Sparks 1995; Deer et al. 1978). Different authors have reported similar CEC ranges for other natural smectites (Kannewischer et al. 2006). High CEC values reflect the high capacity of the clays to potentially exchange/interact with diverse cationic components. These components have to be able to reach the interlayer galleries of the clays, implying that there are size,

charge and polarity restrictions. Regarding particle size in dry state, NS had a higher percentage of particles < 100 μm (20%) compared to UPSN (2%). UPSN had more particles in the size range of 100-45 μm (67%) and less particles with size < 45 μm (31%). This is attributed to the selection procedure used for UPSN. Importantly, in the wet state using water, UPSN had more ≤ 2 μm particles (75%) than in the dry state. This suggests that previous dry selection of particle size does not alter wet state particle size, which is the expected "active" state of these clays upon ingestion by animals and humans. Montmorillonite was the major mineral identified by XRD and FTIR in the clay fractions of both samples. Sand and silt fractions of both clays contained montmorillonite, quartz, feldspars and muscovite particles, as revealed by XRD and confirmed by SEM in UPSN. These findings were expected, since NS is the parent material from which UPSN has been selected. Although not considering safety, Dixon et al. (2008) proposed a criteria for selection of effective AF adsorbents based on several mineral characteristics, including Langmuir adsorption effectiveness of 0.30 (30 points), smectite predominance by XRD (30 points), CEC of 70 (5), pH ranging from 6.5-8.5 (5 points), and FTIR evidence of structural Mg and Fe (10). According to this criterion, both UPSN and NS are very effective AF adsorbents.

The pattern of AF sorption for both samples was the same, and the Q_{max} values at two pH levels were similar. Similar findings have been previously reported for other NS samples (Marroquin-Cardona et al. 2009; Phillips et al. 2009). The slight increase in Q_{max} observed for UPSN may be related to the higher amount of smectite particles in this sample, as revealed in the wet state fractionation analyses.

4.4.2 Animal study

None of the differences observed for the growth parameters occurred in both sexes at both levels of UPSN. The statistical analysis of hematological and serum biochemistry parameters revealed some statistically significant differences in both sexes. Nevertheless, all of these differences fall between the ranges reported for control animals in different studies (Almodovar-Cuevas et al. 1985, Wolford et al. 1986). Na and Na/K ratio in males and females of UPSN groups were significantly greater than the control. According to the extractable bases determinations, these clays are able to leach some cations by means of exchange processes. Although the concentrations of NH_4OAc used for the CEC and extractable bases determinations are higher than what can be expected in simulated physiological conditions, acetate and NH_4OAc have been used as ingredients of Simulated Intestinal Fluid (Galia et al. 1998; Persson et al. 2009) for dissolution of pharmaceutical drugs. The K levels in the males treated with UPSN were significantly lower than the controls, but no dose dependency was observed for this parameter. A possible explanation for the lower serum K levels in the males could be related to the cation exchange capacity of UPSN. It is possible that UPSN was able to exchange Na ions (in the clay) for K ions (from the diet). This phenomenon has been demonstrated with Na-cation-exchange resins that are orally administered to treat hyperkalemia (Elder 2005).

Serum Ca levels were significantly greater in females treated with 0.25% UPSN and in males from both UPSN groups. Despite this, the Ca levels were within the normal range for < 6 month old rats and no evidence of dose dependency was found. In a similar

study using SD rats, Afriyie-Gywau et al. (2005) found a significant increase in serum Ca levels in females ingesting 0.5 and 2.0% of NS in the diet. Although not significant, the males of that study also showed increased levels of serum Ca. Using simulated gastric conditions for edible soils, Hodda et al. (2004) showed that bioavailability of Ca was correlated with amounts of Ca originally present in the soils. The potential supplementation of Ca by clays has also been suggested by Wiley and Katz (1998) and Wilson (2003). In our study, based on the XRD patterns, we suggest that calcite dissolution and exchangeable Ca from montmorillonite may act as potential sources of Ca supplementation.

In the mineral and vitamin analysis, no significant differences were observed for Fe and vitamin A in the UPSN groups for either sex. However, significantly lower levels of serum Zn were detected in females and males from the 2% UPSN treatment group when compared to the control. Normal ranges of serum Zn for 3 month old rats are not reported in the literature, but the Zn levels in our study fell in the ranges previously reported for control SD rats that were 28 weeks old, according to Afriyie-Gyawu et al. (2005). Nonetheless, these findings are important since previous studies using a similar bentonite (hydrated sodium calcium aluminosilicate; HSCAS) reported potential interactions with Zn. Chung and Baker (1990) observed that chickens fed 1% HSCAS in the diet had significantly ($p < 0.05$) lower Zn levels measured in the tibia. Zn tibia concentration is a more reliable indicator of Zn levels in the body (Wedekind and Baker 1990) since plasma Zn levels represent short-term changes in dietary zinc and can be influenced by factors independent of diet intake (Wallwork 1987). Based on results from

this study and Chung and Baker (1990), it can be suggested that Zn interactions may become important at higher inclusion levels of bentonites but not at lower levels. Regarding vitamin E, a significant increase in serum levels was noted for both doses of UPSN in both sexes. Afriyie-Gyawu et al. (2008b) observed a similar phenomenon in males during a human 3 month trial with NS clay. In our study, a treatment effect was clear and this could be related to a potential protective function the clay may exert on the vitamin. Specifically, it is possible that UPSN may prevent complete oxidation of the vitamin in the stomach, hence making it more bioavailable in the intestines. More work is warranted to determine the mechanism of this effect.

In summary, UPSN showed similar AF sorption and mineral characteristics as parent NS; they are mainly composed of montmorillonite but also contained mica, feldspars and quartz minerals. We did not find evidence of overt toxicity due to the addition of UPSN at the levels used in the present study. Nevertheless, our findings with Na, Zn, K and vitamin E are important and further research on this clay is warranted.

V. TOXICOLOGICAL EVALUATION OF DIETARY SODIUM BENTONITE AS A POTENTIAL AFLATOXIN ENTEROSORBENT

5.1 Introduction

AFB_1 is a potent hepatocarcinogen (Busby and Wogan 1984; Lopez et al. 2002) with genotoxic (Smela et al. 2001), immunotoxic (Hinton et al. 2003; Turner et al. 2003) and antinutritional (Pimpukdee et al. 2004) effects. AFB_1 is a group 1 carcinogen (IARC 2002) mainly produced by *Aspergillus flavus* and *A. parasiticus* fungi that commonly contaminate maize and peanuts (CAST 2003). The FDA (2011) has established action levels ranging from 20 μg kg^{-1} to 300 μg kg^{-1} for different feeds, according to their intended use for farm animals. The addition of bentonite in feeds (Harvey et al. 1991; Smith et al. 1994) is a feasible and promising method to reduce AFB_1 exposure. Bentonites are montmorillonite-rich minerals that are commonly used as anti-caking agents or flow promoters, and are generally recognized as safe additives (GRAS) when used at levels not exceeding 2%. Montmorillonite is the most abundant mineral in bentonites and it has been reported as the active ingredient for AF binding. By adsorbing AFs from the diet, montmorillonite reduces toxin bioavailability to the blood and organs (Phillips 1999). Currently, a wide variety of purported AF binders are available in the marketplace, many of them having multi-mycotoxin binding claims. Of the clay-based-additives, calcium bentonites have been extensively investigated *in vitro* and *in vivo* and have proven to effectively protect several species from the negative effects of AFB_1 (Smith et al. 1994; Phillips et al. 2006; Ledoux et al. 1999). On the other hand, sodium

bentonites have also been observed to prevent AF's effects on dairy cattle (Diaz et al. 2004), but their potential interactions with other additives (Shryock et al. 1994), anticoccidials (Gray et al. 1998) and probable interactions with vitamin A (Briggs and Fox 1956; Laughland and Phillips 1956), as well as reports citing incomplete protection against AF effects (Abdel et al.1999; Voss et al. 1993), generates concern requiring more safety and efficacy studies on this material.

Due to their natural origin, bentonites may contain undesirable contaminants like heavy metals, dioxins and furans. For these reasons, *in vivo* safety evaluation is essential before these materials are widely included in foods or feeds. Hence, our objectives were to elucidate the mineral characteristics of a sodium bentonite with potential AF sorption abilities and to determine its safety in a 13-week rodent model using Sprague Dawley rats.

5.2 Materials and methods

5.2.1 Sample information and chemicals

Na-bentonite (Na-BENT) was obtained from AMCOL Specialty Minerals (Hoffman Estates, IL). For all experiments, ultrapure water (UP-H_2O) was prepared by processing distilled water through a PURELAB Ultra (ELGA Woodridge, IL). AFB_1 from *Aspergillus flavus* was purchased from Sigma Aldrich (CAS 1162-65-8). All other reagents used in the sample preparation steps for the mineralogical analyses were analytical grade.

5.2.2 Dioxins and selected heavy metals

Data for the 17 priority chlorinated dibenzo-p-dioxins/furans (CDDs/CDFs) in Na-BENT were performed by Analytical Perspectives (Wilmington, NC). Procedures followed the US Environmental Protection Agency (USEPA) method 8290 for sample preparation, clean-up and analysis with high resolution gas chromatography/high resolution mass spectrometry (HRGC/HRMS). Measurements of arsenic (As), cadmium (Cd), lead (Pb) and mercury (Hg) were also performed by the same laboratory. Samples were digested and prepared according to method SW846-3051 using a PerkinElmer Multiwave 3000 Anton Paar Microwave Reaction System. For mercury analyses, a PerkinElmer FIMS-100 CVAA analyzer was used following EPA method 7470A. Other metals were analyzed on a PerkinElmer ELAN 6100 ICP-MS using EPA method 6020.

5.2.3 Mineralogical analyses

Mineralogical analyses included an initial sample evaluation to determine moisture content and to assess the presence of carbonate minerals, sulfides, manganese oxides and evaporites. Other analyses included X-ray diffraction (XRD), Fourier transform infrared (FTIR) spectroscopy, scanning electron microscopy (SEM), transmission electron microscopy (TEM), cation exchange capacity (CEC), and extractable bases determinations in water and ammonium acetate (NH_4OAs). Most mineralogical analyses were performed following standard procedures previously reported (Soukup et al. 2008) with minor modifications. The moisture content was determined by subtracting the oven dry weight (110 °C) from the air dry weight of 5 g of Na-BENT. The resulting value was expressed as % of moisture. Calcite ($CaCO_3$)

presence was determined by using 1M hydrochloric acid (HCl). The presence of oxidizing/reducing components was evaluated by addition of hydrogen peroxide (H_2O_2 30%). Presence of magnetic minerals was assessed by using a magnetic stir bar. Occurrence of evaporite minerals was qualitatively estimated by measurement of electrical conductivity (EC) and acetone precipitation. For EC measurement, 10 g of sample were weighed into 250 mL plastic bottles, the ratio of deionized water:solid used was 25:1. The sample was shaken for 30 min on a rotary shaker at room temperature and then centrifuged at 2000 rpm for 10 min. After centrifugation, the supernatant was used to measure the EC and pH sequentially. In order to evaluate the presence of gypsum, 2 mL of the supernatant were collected in a glass tube followed by the addition of an equal volume of acetone.

CEC and extractable bases determination in NH_4OAc were done by the Soil Characterization Laboratory from the Soil and Crop Sciences Department at Texas A&M University, using a mechanically controlled variable rate leaching device as reported by Holmgren et al. (1977). Soluble bases using DI-water were determined by the Soil, Water and Forage Testing Laboratory at Texas A&M University, using a saturated paste extract method based on Rhoades and Clark (1978). An amount of 100 g of sample was used for this analysis.

Prior to fractionation, removal of cementing and flocculating materials was done by treatment with pH 5 sodium acetate buffer at 90°C for 30 min, in duplicates. Organic matter (OM) was removed by using 30% H_2O_2. Na-BENT (same previously used for EC and pH measurements) was mixed with 50 mL of pH 10 sodium carbonate (Na_2CO_3)

dispersion solution, shaken and centrifuged at 2000 rpm for 10 min. The sand fraction was separated by passing the precipitated slurry through a 53 μm sieve. The silt fraction (2-53 μm) was separated from the clay fraction (<2 μm) by a centrifugation procedure based on Stokes' law. A pH 10 solution of Na_2CO_3 was used as dispersant. All supernatants (containing the clay fraction) were collected into beakers. Collected sand and silts were dried overnight at 105°C before weighing. The clay fraction was further flocculated with sodium chloride (NaCl) and then dialyzed until EC measurements were close to the water EC values (< 2 μS cm^{-1}).

XRD patterns were recorded in a Bruker D8 ADVANCE X-ray diffractometer. Sand and silt fractions were ground in a mortar and passed through a 140 mesh (105 μm) sieve and mounted as powder to collect the XRD patterns. Magnesium (Mg) and potassium (K) saturated clay were air dried on glass discs to obtain oriented films for XRD analysis. Mineral identification was determined using EVA (Bruker, Madison, WI) computer software. Infrared patterns were recorded in a Spectrum 100 FTIR (PerkinElmer, Inc.) using the diffuse reflectance infrared Fourier transform (DRIFT) method and the ATR method. For the DRIFT method, 0.005 g of sample was placed in a holder, using KBr as background material. For the ATR method, 1 mg of the clay fraction was mixed with 300 mg of KBr under a lamp to maintain dryness. Clay sample and KBr were mixed in a steel capsule for 30 seconds and then placed in a holder for reading.

Silt samples were examined in a JEOL 6400 scanning electron microscope with energy dispersive X-ray (EDS) recording capability. For TEM analysis, a clay sample

was mounted on a holey carbon grid, and images were recorded in a JEOL 2010 microscope with EDS capability.

5.2.4 AFB_1 sorption analyses

Isotherms were performed based on methods reported by Grant and Phillips (1998) and previously described in detail by Marroquin-Cardona et al. (2009). Isotherms were conducted in triplicate at pH 2 and 6.5. Computer-generated equilibrium isotherms were extrapolated from additive data and fit to the Langmuir model (based on r^2-values and randomness of the residuals). The parameters of Q_{max} (mol AFB_1 kg^{-1} additive) and K_d were estimated to delineate the maximum sorption to the surface and the affinity of the sorption interaction.

5.2.5 Rodent study experimental design

Authorization for the study was obtained by Texas A& M University, according to the animal use protocol (AUP) 2008-39. Sprague Dawley®™ (SD) rats were obtained from Harlan (Houston, TX). Animal subjects in each group came from different litters to assure independence of samples. Four-week-old female (75-99 g) and male (100-124 g) rats were maintained with meal rodent feed 8604 Teklad Harlan (Madison, WI) and water *ad libitum*. After a brief acclimation period (5 days), the rats (1 rat per cage) were allocated to each particular group, including one control and two treatment groups. Each group consisted of 10 male and 10 female rats. The control group received basal rodent diet thorough the study while the treatment groups received formulations containing basal rodent diet with low (0.25%) and high (2%) concentrations of Na-BENT. Dietary clay concentrations were based on the minimum effective dose (0.25% w/w) of NovaSil

clay used in human studies for AF adsorption and the highest level allowed (2.0% w/w) by the FDA for use as a GRAS additive (Electronic Code of Federal Regulations 2010). Animals were housed in a climate controlled environment (temperature 22–25°C), artificially illuminated (12 hr dark/12 hr light) and free from chemical contamination. General appearance, behavior and signs of morbidity and mortality were inspected daily. Body weights were measured initially and every three days throughout the course of the study. Feed consumption was also recorded every three days. In total, body weight and feed consumption were recorded at 30 and 29 time points, respectively. Before termination, blood was drawn via cardiac puncture under isoflurane anesthesia. Following euthanasia, organs and tissues of interest were removed and evaluated for gross abnormalities. Wet weights of liver, kidneys, heart, lungs, brain, spleen, tibia, and uterus plus ovaries were recorded. The present study was simultaneously done with another trial investigating the safety of a refined Ca-bentonite (UPSN) (Marroquin-Cardona et al. 2011). Both studies used the same control group as a reference.

5.2.6 Hematological and serum biochemical analyses

Hematology and serum biochemical parameters were analyzed by the Clinical Pathology Lab, Texas Veterinary Medical Diagnostics Lab (TVMDL) (College Station, TX). Hematological analysis of whole blood samples was conducted using an Abbott CELL-DYN 3700 Hematology Analyzer (Abbott Laboratories, Abbott Park, IL) and included hemoglobin (Hb) concentration, mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV), percent corpuscular volume (PCV), platelets, red blood cell (RBC) and white blood cell

(WBC) counts. Leukocyte differential analysis was performed manually by microscopic examination of blood smears and included neutrophils, lymphocytes, monocytes, and eosinophils. Serum biochemical parameters were assessed using an automated analyzer Modular P (Roche Diagnostics, Indianapolis, IN) and included albumin/globulin ratio (A/G), albumin (ALB), alkaline phosphatase (ALP), alanine aminotransferase (ALT), amylase (AMYL), aspartate aminotransferase (AST), blood urea nitrogen (BUN), calcium (Ca), cholesterol (CHOL), creatine kinase (CK), chloride (Cl), creatinine (CRT), gamma glutamyl-transferase (GGT), globulins (GLOB), glucose (GLUC), potassium (K), sodium (Na), Na/K ratio, phosphorous (P), total bilirubin (T-BIL) and total serum protein (TSP).

Serum micronutrients iron (Fe) and zinc (Zn) were measured with a Hitachi 911 (Roche Laboratories, Indianapolis, IN) and a Perkin Elmer Analyst 100 (PerkinElmer, Shelton, CT), respectively. Vitamin A and E were measured by HPLC according to previously reported methods by Weinman et al. (1999) and Rupérez et al. (2004).

5.2.7 Statistical analysis

First, to investigate the weight gained over time among the absolute control and the two doses of UPSN, a linear mixed model was constructed with the fixed effect treatment, sex, and treatment*sex interaction. Time was included as a continuous variable and tested for interactions with treatment and sex. Differences in final body weight (FBW) were investigated with a similar model that included the fixed effect treatment and the covariates initial body weight (IBW) and total feed consumption

(TFC) as explanatory variables. Backward selection was done by removing one term at a time, starting with non-significant interactions ($p > 0.05$).

A second analysis was performed to investigate differences in biochemical parameters, relative organ weights and selected vitamins and minerals among the absolute control and the two doses of UPSN for males and females separately. A linear or a generalized linear mixed model was constructed with the fixed effect treatment as the only independent variable. The MIXED and GLIMMIX procedures were used for all statistical analyses using SAS 9.2 with Enterprise Guide 4.1. In all these analyses, the effect of subject (i.e. rat) was considered as random, since all animals came from different litters (non relatives in each group). All post-test comparisons were adjusted by the method of Tukey-Kramer. Finally, monocyte counts and T-BIL were analyzed with non-parametric tests using SPSS 14.0. Data for T-BIL were classified in categories before analysis. Kruskal-Wallis test followed by Mann-Whitney test for multiple comparisons were used to detect individual differences between groups.

5.3 Results

Dioxins/furans detected in Na-BENT were tetrachlorodibenzo-p-dioxin, hexachlorodibenzo-p-dioxin, heptachlorodibenzo-p-dioxin, tetrachlorodibenzo-p-furan, and pentachlorodibenzo-p-furan. The average calculated dioxin/furan toxic equivalents (TEQs) for Na-BENT were 0.0811 pg g^{-1}. The heavy metal levels found in Na-BENT were 0.01 mg g^{-1} for As, 0.003 mg g^{-1} for Cd, <0.00003 mg g^{-1} for Hg, and 0.03 mg g^{-1} for Pb. Using the tolerable daily intake (TDI) levels reported by the JECFA (2000; 2007;

WHO 1989), the TDIs calculated for a 70 kg BW person, are 0.14 mg for As, 0.07 mg for Cd, 0.01 mg for Hg, and 0.21 mg for Pb.

5.3.1 Mineralogical analyses

Little reaction following HCl application suggested that calcite and other carbonate minerals were not present in significant amounts in Na-BENT (Table 10). Similarly, little or no reaction following H_2O_2 treatment suggested that Na-BENT does not contain significant amounts of oxidizing/reducing components (e.g. manganese oxides) or organic matter. The pH of the sample was 8.3 and it is in the ranges reported for clay samples containing smectites (Marroquin-Cardona et al. 2009; Kannewischer et al. 2006). The EC values observed suggested that gypsum was present in this sample. This finding was confirmed by XRD powder mounts, in which a gypsum peak (7.6 Å) was evident (Figure 27). As a moderate soluble sulfate mineral, gypsum is able to increase EC readings when dissolved. The percentages calculated for each fraction showed that this sample is mainly composed of clay particles (90%). CEC values were calculated to be 90.5 cmol kg^{-1}. The major minerals identified in Na-BENT powder mount XRD were montmorillonite (12.5 Å), gypsum (7.6 Å) and quartz (3.3 Å) (Figure 27).

Table 10. Mineral characteristics of Na-BENT

Parameter	Na-BENT
Moisture %	6.4
pH	8.3
EC (µS cm^{-1})	751.0
HCl reaction	Little
H_2O_2 reaction	Little
Magnetic minerals	ND
Free iron oxides (%)	0.8
Sand (>53µm) (%)	3.0
Silt (2-53µm) (%)	7.0
Clay (<2µm) (%)	90.0
Mica (%)	2.1
CEC (cmol kg^{-1})	90.5
Extractable cations (cmol kg^{-1})	
Ca	32.5
K	1.4
Mg	19.9
Na	58.8
Soluble cations DI-water (mEq L^{-1})*	
Ca	2.7
K	0.5
Mg	1.6
Na	31.9

Notes: *Soluble bases were measured in DI-water vacuum extracts from a paste according to Rhoades and Clark, 1978; ND: non-detected.

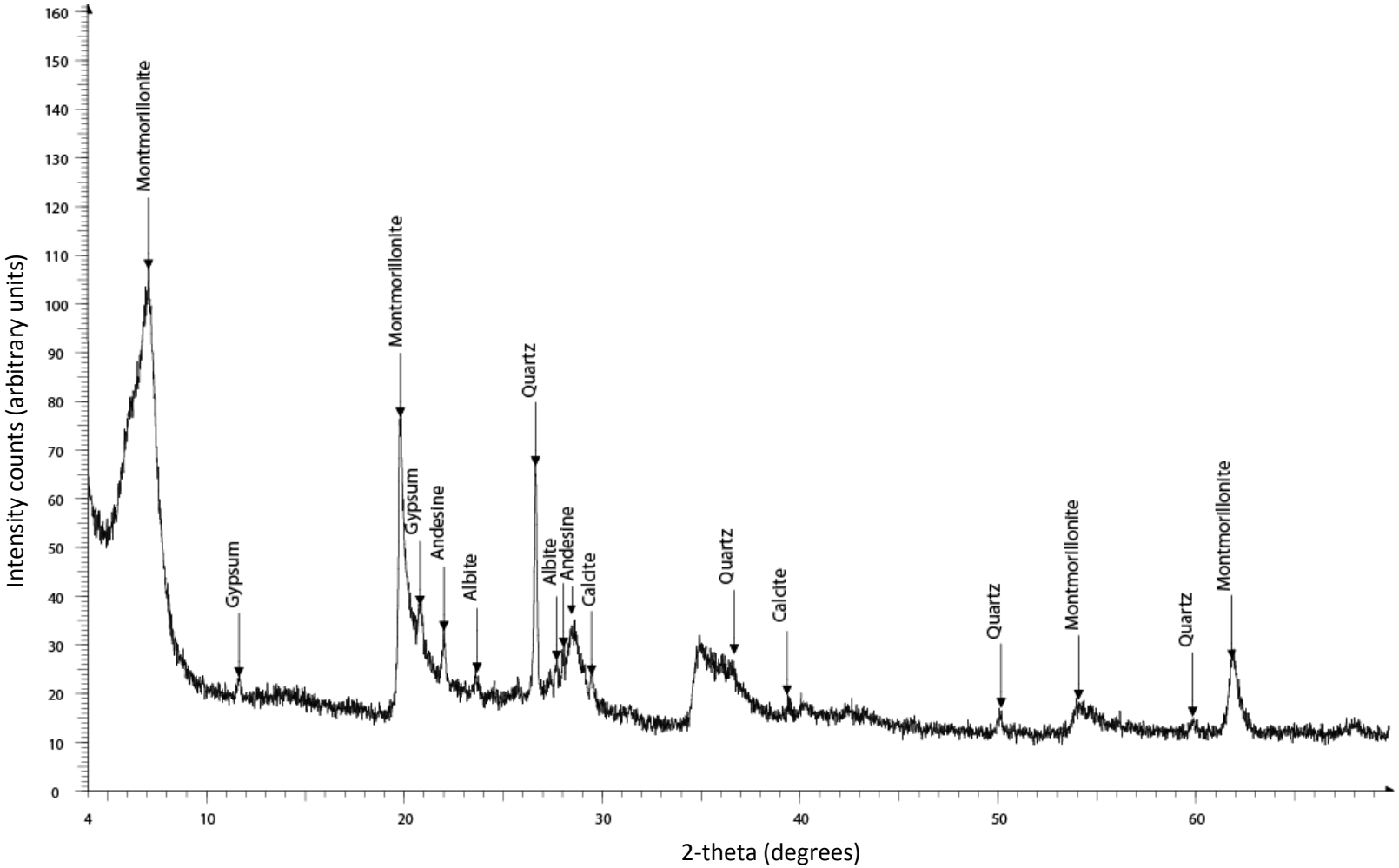

Figure 27. Powder mount XRD pattern of Na-bentonite. Minerals identified included montmorillonite (12.5, 4.48, 1.49 Å), gypsum (7.62 and 4.28 Å) quartz (3.34 Å), calcite (3.03 Å), orthoclase feldspars (3.75 Å) and andesine (4.04, 3.22 and 3.18 Å).

Peaks for quartz and mica (10.0 Å) as well as peaks of feldspars from the plagioclase group (andesite) and the orthoclase group (sanidine and albite) were observed in the sand and silt fractions. Montmorillonite presence was confirmed after XRD analysis of the clay fraction exchanged with Mg and K. After treatment with Mg and glycerol, the d-space of the most intense montmorillonite peak increased from ~12 to ~18 Å. Samples exchanged with K and air dried showed a peak of ~12 Å, which is in the range expected for montmorillonites (10-14 Å). After heating the samples exchanged with K at 330°C and 550°C, the ~12 Å peak changed to ~10 Å respectively. A quartz peak was observed in the clay fraction saturated with Mg (air dried), glycerol and K saturated, which suggests silica could be present. Opal was verified in the KBr pattern where a 622 cm^{-1} band was observed. The characteristic bands of montmorillonite were observed in the DRIFT (3636 cm^{-1}) and ATR (3632 cm^{-1}) patterns, confirming XRD findings. SEM findings for Na-BENT revealed a variety of particle shapes and compositions. Important findings included a small particle compatible with monazite which contained phosphorous (P) and some lanthanides and actinides like cerium (Ce), neodymium (Nd), samarium (Sm) and protactinium (Pa) (Figure 28A), and a particle composed of calcium phosphate compatible with hydroxyapatite ($Ca_5(PO_4)_3(OH)$) (Figure 28B). The main phenomenon observed in the TEM images was the folding tendency of the montmorillonite particles as illustrated in Figure 28C. The particles are shown as thin films of considerable length (3-5 μm). Moiré patterns were also observed in some particles (Figure 28D).

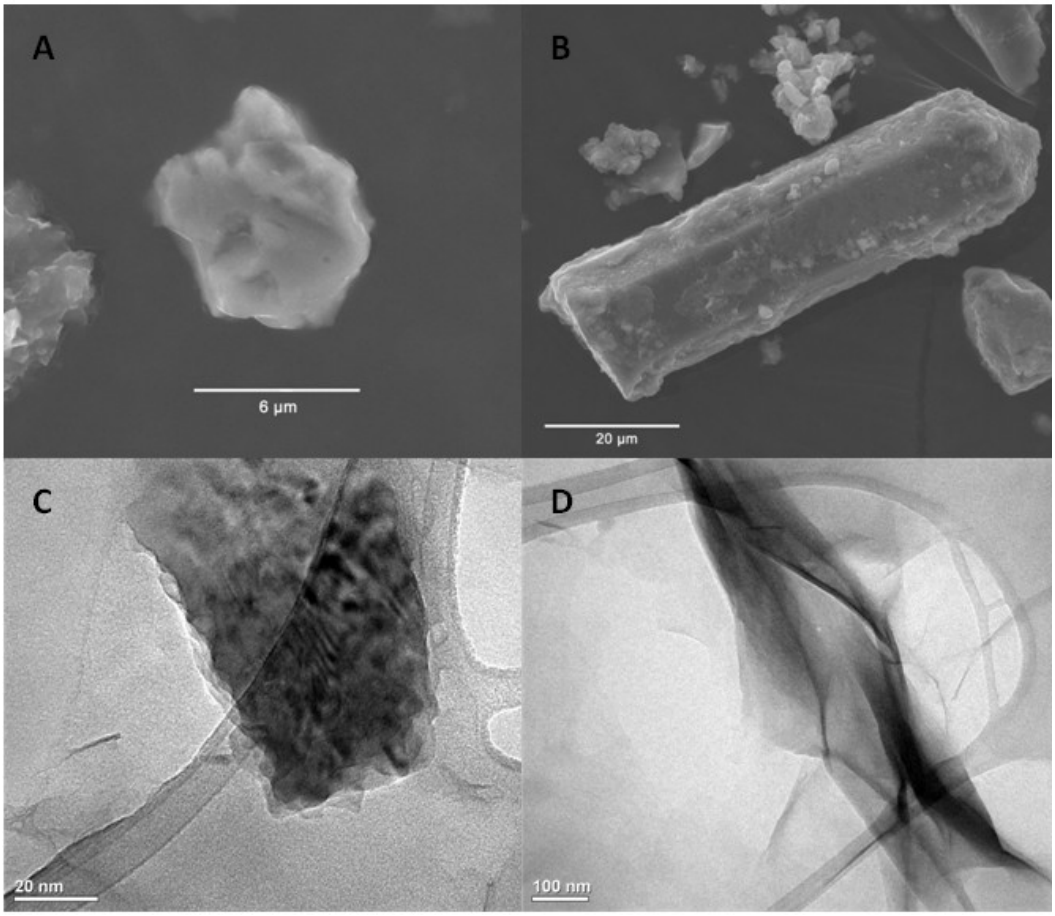

Figure 28. SEM and TEM images of Na-bentonite. A) SEM image of monazite particle in Na-BENT sample. B) Hydoxyapatite particle in Na-BENT. C) Moiré patterns observed in platelets of Na-BENT. D) Smectite particle depicting sheets and rolls morphology with an approximate length of 1 µm, according to the scale bar. This morphology is commonly observed for effective aflatoxin adsorbents, according to Mulder et al. (2008).

5.3.2 AFB_1 sorption analyses

The parameters of Q_{max} and K_d calculated for Na-BENT demonstrated the effectiveness of the clay *in vitro*. Na-BENT depicted a favorable AF sorption pattern characterized as L shape, at both pH levels (Figure 29). Specifically, an L2 shape was documented for Na-BENT at pH 6.5 with a calculated Q_{max} value of 0.34 mol AFB_1 kg^{-1}. A slightly higher Q_{max} of 0.39 mol AFB_1 kg^{-1} was calculated at pH 2.0, for which an L1 pattern was observed.

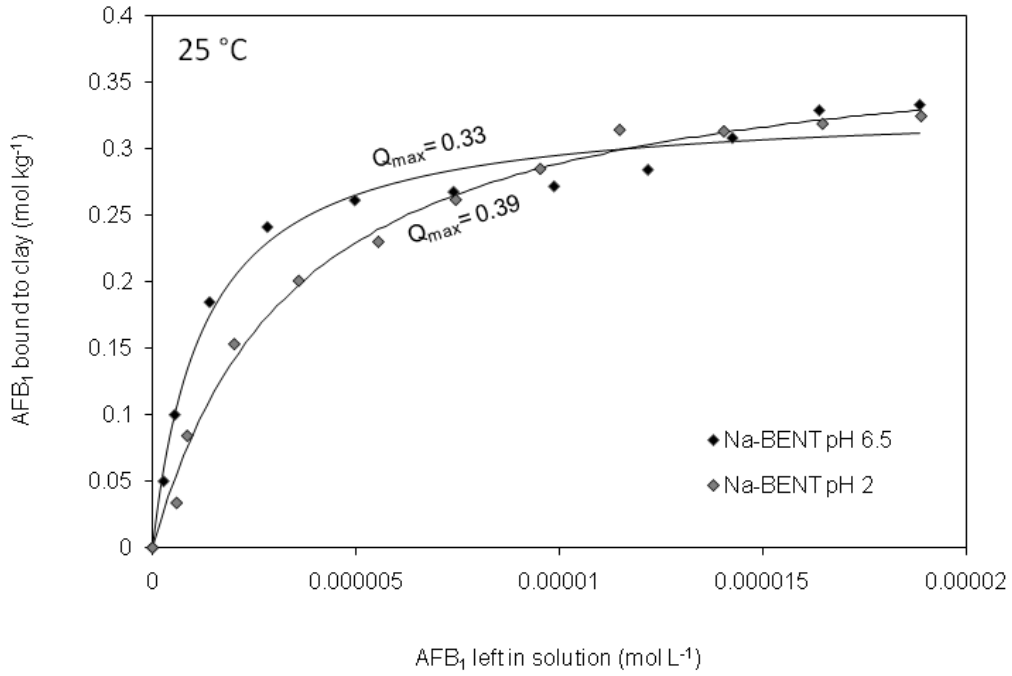

Figure 29. AFB_1 adsorption isotherms onto Na-BENT at pH 6.5 and 2. At both pH levels, Na-BENT depicted an L-shape pattern characteristic of planar arrangement of molecules adsorbed on the surface. The bend on the pattern indicates that the sorbent is reaching saturation as the concentration of the solute is increased (pH 6.5). The Q_{max} values calculated were similar at both pH levels, with 0.33 and 0.39 mol AFB_1 kg^{-1} at pH 6.5 and 2, respectively.

5.3.3 Rodent study outcomes

No apparent toxicity effects due to consumption of bentonite at either dose were noticed throughout the study. All rats, with the exception of two males, remained healthy and active without noticeable changes in behavior. Two males of the 0.25% Na-BENT group had black discharge from one eye and they were treated with topic antibiotic ointment. The discharge disappeared within 4 days.

Males gained more weight than the females ($p \leq 0.0001$), as expected. The interaction between treatment * gender was not significant ($p = 0.6780$). Weight gain across time was different in at least one of the treatment groups ($p \leq 0.0001$). Rats in the control group gained more weight than rats in the 2% group by time 5 ($p = 0.0019$) and consecutively up to time 30 ($p \leq 0.0001$). Similarly, animals in the control group gained more weight than 0.25% by time 20 ($p = 0.0131$) and consecutively at time 30 ($p = 0.0002$). Significant differences were observed between the control and the treatment groups for some of the growth parameters in the females (Table 11). TFC and IBW were not significant factors for explaining the differences in FBW of the females. Similarly, in males, IBW was not a significant factor for explaining the differences in FBW. However, in males there was a linear relationship between TFC ($p = 0.0278$ for TFC * treatment interaction) and FBW in both, the 0.25% and 2% Na-BENT groups, meaning that TFC influenced FBW.

Table 11. Growth parameters of SD rats receiving dietary Na-BENT at 0.25 and 2% for 13 weeks

Group	Control	Na-BENT 0.25%	Na-BENT 2%
Females			
FBW (g)	243.85 ± 13.49	231.03 ± 7.46*	226.60 ± 15.63*
FCE (g)	13.24 ± 2.74	14.72 ± 1.74	15.82 ± 1.69
IBW (g)	99.52 ± 4.75	95.30 ± 3.14	86.33 ± 4.62*
TBWG (g)	144.33 ± 10.12	135.73 ± 7.35	140.27 ± 13.70
TFC (g)	1899.17 ± 341.57	1995.90 ± 231.58	2205.14 ± 169.50
Males			
FBW (g)	406.45 ± 19.99	397.39 ± 24.44	392.73 ± 19.19
FCE (g)	7.42 ± 0.70	7.78 ± 0.53	7.68 ± 0.45
IBW (g)	120.87 ± 3.65	121.83 ± 6.40[a]	115.66 ± 4.96*[b]
TBWG (g)	285.58 ± 20.18	275.56 ± 21.93	277.07± 21.89
TFC (g)	2110.08 ± 104.45	2135.83 ± 101.44	2120.53 ± 66.08

Notes: Means with asterisk are significantly different from the control at the 0.05 level. Means with different letters are significantly different between Na-BENT groups at the 0.05 level. Data are reported as mean values ± standard deviation. FBW: Final body weight; FCE: Feed conversion efficiency; IBW; Initial body weight; TBWG: Total body weight gain; TFC: Total feed consumption

5.3.4 Hematological and serum biochemistry analyses

Some differences between the control and at least one of the Na-BENT treatment groups were observed for the hematological and vitamin and metal serum parameters (Table 12). Most relevant findings included the increased vitamin E levels observed in both females and males of Na-BENT groups. Another important finding in the females was the reduction and increment of the RBC counts and MCV values respectively, in the Na-BENT groups. In the males of Na-BENT groups, the most important finding besides vitamin E, was the reduction in serum levels of Zn. Males of Na-BENT groups had significantly lower Zn levels than the controls, yet the levels were within normal ranges previously reported for control 28 weeks old SD rats, according to Afriyie-Gyawu et al. (2005). Normal ranges of serum Zn for 3 month old rats are not available in the literature. Concerning serum vitamin levels, the males on 2% Na-BENT had significantly lower vitamin A levels than the control group.

Table 12. Hematological parameters and serum Fe, Zn, Vitamins A and E of SD rats receiving dietary Na-BENT

	Control	Na-BENT 0.25%	Na-BENT 2%	Normal values	Reference
Females					
Hb (g dL^{-1})	14.19 ± 0.33	13.90 ± 0.72	13.68 ± 0.31*	14.30 ± 0.83	F
MCH (pg)	18.44 ± 1.70	18.66 ± 1.98	19.62 ± 3.10	19.8 ± 0.94	F
MCHC (g dL^{-1})	34.43 ± 0.72	34.20 ± 0.71	33.95 ± 0.79	34.40 ± 0.50	D
MCV (fl)	55.85 ± 1.76	57.96 ± 1.00*	57.77 ± 1.20*	57.90 ± 2.10	D
PCV (%)	41.24 ± 1.53	40.65 ± 1.99	40.32 ± 1.03	38.10 ± 2.43	F
Platelets ($x10^3$) μL^{-1}	468.20 ± 77.83	483.00 ± 138.59	484.66 ± 280.62	471.68 ± 38.77	B
RBCs ($x10^6$) μL^{-1}	7.39 ± 0.29	7.02 ± 0.36*	6.98 ± 0.21*	7.02 ± 0.40	D
WBC μL^{-1}	3944.00 ± 962.96	5029.00 ± 1314.55[a]	12595.00 ± 4759.51*	7840.00 ± 2950.00	C
Fe (µg dL^{-1})	254.67 ± 38.20	247.73 ± 52.63	233.02 ± 40.54	220 ± 130	C
Zn (µg mL^{-1})	1.24 ± 0.18	1.16 ± 0.11	1.14 ± 0.14	1.05 ± 0.09	A
Vitamin A (µg L^{-1})	267.31 ± 27.78	270.12 ± 17.75	250.16 ± 31.42	254.10 ± 6.00	A
Vitamin E (µg L^{-1})	3560.00 ± 40.00	8030.00 ± 1220*	9180.00 ± 1210*	NA	
Males					
Hb (g dL^{-1})	14.51 ± 0.46	14.98 ± 0.56[a]	14.22 ± 0.35[b]	14.70 ± 1.22	F
MCH (pg)	18.78 ± 0.45	18.76 ± 0.27	18.75 ± 0.59	19.00 ±1.09	F
MCHC (g dL^{-1})	33.77 ± 0.71	34.05 ± 0.53	33.58 ± 0.37	34.10 ± 0.40	D
MCV (fl)	55.78 ± 2.25	55.07 ± 1.06	55.84 ± 1.40	59.50 ± 2.00	D
PCV (%)	43.10 ± 1.73	44.00 ± 1.94	42.34 ± 0.80	39.90 ± 3.49	F
Platelets ($x10^3$) μL^{-1}	418.60 ± 133.39	543.10 ± 145.15	668.10 ± 122.03*	471.68 ± 38.77	B
RBCs ($x10^6$) μL^{-1}	7.73 ± 0.32	7.99 ± 0.31[a]	7.58 ± 0.19[b]	7.82 ± 0.83	F
WBC μL^{-1}	7238.00 ± 2462.55	8235.00 ± 1509.11	10850.00 ± 2912.90*	7840.00 ± 2950.00	C
Fe (µg dL^{-1})	115.89 ± 10.92	109.96 ± 16.69	124.32 ± 23.81	152 ± 70	C
Zn (µg mL^{-1})	1.50 ± 0.09	1.33 ± 0.09*	1.23 ± 0.08*	1.29 ± 0.04	A
Vitamin A (µg L^{-1})	530.80 ± 39.44	519.74 ± 77.78	441.55 ± 67.45*	567.40 ± 24.80	A
Vitamin E (µg L^{-1})	3440.00 ± 50.00	7210.00 ± 870*	8570.00 ± 1300*	2106.17 ± 1261.98	E

Notes: Means with asterisk are significantly different from the control at the 0.05 level. Means with different letters are significantly different between Na-BENT groups at the 0.05 level. Data are reported as mean values ± standard deviation. Hb: hemoglobin; MCH: mean cell hemoglobin; MCHC: mean corpuscular hemoglobin concentration; MCV: mean corpuscular volume; PCV: packed cell volume; RBC: red blood cell; WBC: white blood cell. References: A) Afriyie-Gyawu et al. 2005; B) Almodovar et al. 1985; C) Kohn and Clifford 2002; D) Lillie et al. 1996; E) Seifi et al. 2009; F) Woldford et al. 1986.

Table 13. Serum biochemical parameters of SD rats receiving dietary Na-BENT at 0.25 and 2% for 13 weeks

Parameter	Treatment				
	Control	Na-BENT 0.25%	Na-BENT 2%	Normal values	Reference
Females					
A/G Ratio	2.41 ± 0.11	2.22 ± 0.18*	2.38 ± 0.15	2.13 ± 0.18	D
ALB (g dL^{-1})	3.74 ± 0.15	3.74 ± 0.18	3.84 ± 0.13	3.41 ± 0.23	A
ALP (U L^{-1})	58.40 ± 14.23	57.70 ± 19.13	71.10 ± 19.67	117 ± 41.7	B
ALT (U L^{-1})	54.70 ± 9.47	56.78 ± 9.52	50.10 ± 6.95	44.00 ± 23.9	C
AMYL (U L^{-1})	1258.40 ± 112.41	1247.30 ± 155.68	1531.10 ± 447.64	1703 ± 164.32	D
AST (U L^{-1})	138.20 ± 42.38	155.77 ± 52.67	92.80 ± 16.98*	93.00 ± 30.3	C
BUN (mg dL^{-1})	18.44 ± 1.70	18.66 ± 1.98	19.62 ± 3.10	21.00 ± 3.90	C
Ca (mg dL^{-1})	9.34 ± 0.32	9.62 ± 0.24[a]	10.02 ± 0.41*[b]	10.36 ± 0.32	A
CHOL (mg dL^{-1})	86.73 ± 9.33	85.01 ± 11.97	85.53 ± 4.59	89.00 ± 23.00	C
CK (U L^{-1})	815.11 ± 360.20	1124.11 ± 802.88[a]	363.80 ± 116.44*[b]	210.00 ± 109	A
Cl (mEq L^{-1})	94.50 ± 19.54	101.50 ± 1.43	101.20 ± 1.13	104.00 ± 2.40	B
CRT (mg dL^{-1})	0.29 ± 0.03	0.33 ± 0.03	0.31 ±0.02	0.55 ± 0.10	A
GGT (U L^{-1})	<3.0	<3.0	<3.0	<3.0	D
GLOB (g dL^{-1})	1.55 ± 0.08	1.69 ± 0.16	1.62 ± 0.11	1.91 ± 0.09	D
GLUC (mg dL^{-1})	180.10 ± 15.62	181.70 ± 23.35	186.50 ± 29.45	199.80 ± 21.60	A
K (mEq L^{-1})	5.71 ± 1.33	4.91 ± 0.34†	4.57 ± 0.53†	4.76 ± 0.44	A
Na (mEq L^{-1})	129.80 ± 25.68	140.60 ± 0.97†	140.40 ± 1.35†	142.00 ± 2.00	A
Na/K Ratio	23.04 ± 2.99	28.77 ± 1.92*	31.08 ± 3.42*	~29	A
P (mg dL^{-1})	5.56 ± 0.87	5.51 ± 0.40	5.29 ± 0.45	5.80 ± 1.10	B
T-BIL (mg dL^{-1})	0.09 ± 0.05	0.14 ± 0.02	0.10 ± 0.04	0.30 ± 0.24	C
TP (g dL^{-1})	5.29 ± 0.21	5.43 ± 0.28	5.46 ± 0.20	6.17 ± 0.33	A

Notes: Means with asterisk are significantly different from the control at the 0.05 level. † These parameters resulted significantly different from the control when an outlier was removed. Means with different letters are significantly different between Na-BENT groups at the 0.05 level. Data are reported as mean values ± standard deviation. ALB: albumin; ALP: alkaline phosphatase; ALT: alanine aminotransferase; AMYL: amylase; AST: aspartate aminotransferase; BUN: blood urea nitrogen; CHOL: cholesterol; CK: creatine kinase; CTR: creatinine; GGT: gamma-glutamyl transferase; GLOB: globulin; GLUC: glucose; T-BIL: total bilirubin; TP: total protein. References: A) Afriyie-Gyawu et al. 2005; B) Kohn and Clifford 2002; C) Lillie et al. 1996; D) Woldford et al. 1986.

Table 13. continued

Parameter	Group				
	Control	Na-BENT 0.25 %	Na-BENT 2.0 %	Normal values	Reference
Males					
A/G Ratio	1.93 ± 0.15	1.80 ± 0.14	1.79 ± 0.13	1.37 ± 0.18	D
ALB (g dL^{-1})	3.42 ± 0.15	3.68 ± 0.15*	3.64 ± 0.11*	3.40 ± 0.20	B
ALP (U L^{-1})	79.20 ± 9.47	69.20 ± 15.87[a]	91.10 ± 19.15[b]	130 ± 43.7	B
ALT (U L^{-1})	61.30 ± 19.38	56.40 ± 14.45	51.70 ± 9.93	49.00 ± 24.1	B
AMYL (U L^{-1})	2316.00 ± 211.13	2386.40 ± 211.69	2461.68 ± 214.64	2671.00 ± 89.00	D
AST (U L^{-1})	116.80 ± 49.97	105.40 ± 26.47	89.40 ± 23.17	97.00 ± 28.40	C
BUN (mg dL^{-1})	19.41 ± 2.63	22.03 ± 2.46	22.05 ± 1.92	20.00 ± 3.20	C
Ca (mg dL^{-1})	9.12 ± 0.39	9.67 ± 0.19*	9.91 ± 0.30*	10.48 ± 0.28	A
CHOL (mg dL^{-1})	85.37 ± 10.16	92.38 ± 13.49	89.22 ± 9.12	75.00 ± 19.80	C
CK (U L^{-1})	432.22 ± 227.32	368.30 ± 86.66	399.36 ± 248.12	222.00 ± 109.00	A
Cl (mEq L^{-1})	98.30 ± 2.26	100.60 ± 0.97	100.20 ± 1.35	103.00 ± 1.90	B
CRT (mg dL^{-1})	0.26 ± 0.03	0.29 ± 0.02	0.28 ± 0.01	0.54 ± 0.08	A
GGT (U L^{-1})	<3.0	<3.0	<3.0	<3.0	D
GLOB (g dL^{-1})	1.78 ± 0.15	2.05 ± 0.18*	2.04 ± 0.12*	2.51 ± 0.25	D
GLUC (mg dL^{-1})	192.70 ± 37.05	186.60 ± 23.14	204.20 ± 16.90	212.40 ± 28.8	A
K (mEq L^{-1})	7.23 ± 0.66	5.30 ± 0.30*	5.16 ± 0.13*	5.30 ± 0.39	A
Na (mEq L^{-1})	135.20 ± 2.74	140.60 ± 0.97*	140.50 ± 2.01*	142.00 ± 2.00	A
Na/K Ratio	18.86 ± 1.74	26.62 ± 1.51*	27.23 ± 0.81*	~26	A
P (mg dL^{-1})	5.92 ± 0.39	6.18 ± 0.24	6.24 ± 0.47	7.30 ± 1.50	B
T-BIL (mg dL^{-1})	0.01 ± 0.03	0.11 ± 0.02*	0.02 ± 0.04	0.30 ± 0.16	C
TP (g dL^{-1})	5.20 ± 0.25	5.73 ± 0.27*	5.68 ± 0.13*	5.85 ± 0.23	A

Notes: Means with asterisk are significantly different from the control at the 0.05 level. † These parameters resulted significantly different from the control when an outlier was removed. Means with different letters are significantly different at the 0.05 level. Data are reported as mean values ± standard deviation. ALB: albumin; ALP: alkaline phosphatase; ALT: alanine aminotransferase; AMYL: amylase; AST: aspartate aminotransferase; BUN: blood urea nitrogen; CHOL: cholesterol; CK: creatine kinase; CTR: creatinine; GGT: gamma-glutamyl transferase; GLOB: globulin; GLUC: glucose; T-BIL: total bilirubin; TP: total protein. References: A) Afriyie-Gyawu et al. 2005; B) Kohn and Clifford 2002; C) Lillie et al. 1996; D) Woldford et al. 1986.

Other important outcomes in the females of both Na-BENT groups included increased Na/K ratio, and potentially increased and decreased serum Na and K, respectively (Table 13). Both of these parameters were significantly different only when their respective values in an outlier (rat 3) from the control group were removed. No specific reason other than better explanation of the data justified the removal of the outlier. Similar results for these parameters were observed for the males of Na-BENT group, for which serum Na and Na/K ratio were increased and serum K was decreased, when compared to the control (Table 13).

Calcium was also significantly increased in the males of Na-BENT groups. In the present study, the females of 2% Na-BENT had significantly increased levels of Ca but not the ones from the 0.25% group.

The rest of the parameters measured in blood and serum were either not significantly different from the control group or if different from the control, they fell in the ranges of normally expected values. Similarly, even when several parameters were significantly different from the control, the majority was found in the normal ranges reported for control animals in several studies (Tables 12 and 13) and no evidence of dose dependency was evident.

5.3.5 Relative organ weights

Isolated differences were observed for some of the relative organ weights for males (Table 14). Specifically, liver and brain weights of males of both Na-BENT groups were different when compared to the control. However, this was not observed in the females and there was not a dose dependency trend.

Table 14. Relative organ weights of SD rats receiving dietary Na-BENT at 0.25 and 2.0% for 13 weeks

Group	Control	Na-BENT 0.25 %	Na-BENT 2 %
Females			
Brain	1.63 ± 0.11	1.58 ± 0.07	1.59 ± 0.08
Heart	0.82 ± 0.06	0.80 ± 0.05	0.82 ± 0.07
L.kidney	0.80 ± 0.07	0.75 ± 0.04	0.73 ± 0.05
Liver	6.82 ± 0.40	6.30 ± 0.74	6.51 ± 0.88
Lung	1.20 ± 0.10	1.17 ± 0.08	1.15 ± 0.13
R. kidney	0.79 ± 0.06	0.74 ± 0.03	0.74 ± 0.03
Spleen	0.52 ± 0.06	0.54 ± 0.05	0.51 ± 0.06
Tibia	0.75 ± 0.13	0.75 ± 0.08	0.69 ± 0.03
Uterus	0.63 ± 0.13	0.59 ± 0.10	0.75 ± 0.31
Males			
Brain	1.65 ± 0.09	1.76 ± 0.13*	1.76 ± 0.09*
Heart	1.36 ± 0.09	1.35 ± 0.10	1.31 ± 0.19
L.kidney	1.31 ± 0.11	1.24 ±0.10	1.17 ± 0.10
Liver	12.34 ± 0.82	11.16 ± 0.88*	10.84 ± 1.00*
Lung	1.64 ± 0.21	1.53 ± 0.11	1.61 ± 0.14
R.kidney	1.35 ± 0.14	1.27 ± 0.11	1.21 ± 0.11
Spleen	0.77 ± 0.07	0.77 ± 0.05	0.79 ± 0.17
Tibia	1.04 ± 0.04	0.95 ± 0.08	1.03 ± 0.05

Notes: Means with asterisk are significantly different from the control at the 0.05 level. Means with different letters are significantly different at the 0.05 level. The relative organ weights (ROWs) were calculated by dividing the wet weight of each organ by the final body weight of the rat just before termination. ROWs have been multiplied by a factor of 10^2. Data are reported as mean values ± standard deviation

5.4 Discussion

The dioxins/furans and heavy metal levels found in Na-BENT were low. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) has placed a provisional tolerable daily intake (PTDI) values for dioxins of ~2.3 pg kg-bw^{-1} day^{-1} (FAO/WHO 2001) for humans. To reach the PTDI levels, and assuming bioavailability of the dioxins in the gastrointestinal tract, an average 70 kg person will have to consume about 2 kg of Na-BENT per day. Under realistic conditions, consumption of Na-BENT will be at lower levels that confer negligible risk of dioxin exposure. Similarly, according to the TDIs calculated for heavy metals such as As, Cd, Hg and Pb for a 70 kg person, the ingestion of 3 g of Na-BENT, which is maximum level of clay used in other studies to reduce AF bioavailability, does not seem to be a risk of exposure to these metals.

The mineral analyses for Na-BENT revealed similar characteristics expected for bentonites. The sample was mainly composed of montmorillonite but also contained mica, gypsum, feldspars and quartz minerals. Montmorillonite was the major mineral identified by XRD, FTIR and TEM of the clay fraction. Sand and silt fractions contained quartz, feldspars and mica particles as revealed by XRD and confirmed by SEM. The CEC obtained for Na-BENT was in the ranges reported for other natural smectites (Kannewischer et al. 2006; Marroquin-Cardona et al. 2011). Common CEC values for smectites fall between 80-150 cmol kg^{-1} (Sparks 1995; Deer et al. 1978). The particle morphology of sheets and rolls observed in TEM is a common feature observed for effective AF adsorbents, according to Mulder et al. (2008). The presence of Moiré

patterns in smectites, as in the present study, can be indicative of coarse particles composed of differently oriented plates (Singer et al. 1972).

Regarding the adsorption isotherms for AF Na-BENT depicted L-shape pattern at both pH levels. Specifically, L1 pattern was observed at pH 2 and L2 pattern at pH 6.5. L1 shape patterns are indicative of sorption processes in approximation of a plateau, while L2 pattern generally suggests that the plateau has been reached (Giles et al. 1960; Grant and Phillips et al. 1998). The differences in patterns may have accounted for the different Q_{max} values calculated at both pH levels. Ongoing experiments are focused on elucidating the specific effects of the pH on the behavior of montmorillonite and its sorption of AF.

The main purpose of the mineral characterization was to investigate its potential correlation with the safety/toxicity of this clay *in vivo*. In general, no dose-dependent effects resulted from the ingestion of Na-BENT for up to 3 months in rats. Nonetheless, it was apparent that the bentonite was able to increase Ca, Na, vitamin E, and to decrease K, in serum of males and females at both inclusion levels (0.25% and 2.0%). In the specific case of vitamin E, the effect of Na-BENT is not clear, but a possible protective effect of the clay on this vitamin has been suggested (Marroquin-Cardona et al. 2011).

Calcium was another parameter that was also significantly increased in the males of Na-BENT groups. Similar studies using SD rats have found significant increases in serum Ca levels in females ingesting 0.5 and 2.0% of NovaSil clay (Afriyie-Gywau et al. 2005) and in females and males ingesting 0.25 and 2% refined NovaSil clay in the diet (Marroquin-Cardona et al. 2011). In the present study, the females of 2% Na-BENT

had significantly increased levels of Ca but not the ones from the 0.25% group. The potential supplementation of Ca by clays has been previously suggested (Wiley and Katz 1998; Wilson 2003) and in the present study, based on the XRD patterns; the Ca sources in Na-BENT may include calcite, gypsum and montmorillonite.

On the contrary, concerning serum vitamin A levels, the males on 2% Na-BENT had significantly lower levels than the control group. Interactions of vitamin A and bentonites have been previously reported in studies using chickens and rats (Brigs and Fox 1956; Laughland and Phillips 1954), were these interactions occurred at high inclusion rates (≥ 3%) of Na-bentonites. Nonetheless, other studies in SD rats using 2% inclusion of similar clays (Ca-montmorillonite) have shown no significant changes in serum vitamin A levels, when compared to the control (Afriyie-Gyawu et al. 2005; Marroquin-Cardona et al. 2011).

Serum Zn was reduced in the males at both inclusion levels of bentonite, a trend previously observed for other bentonites (Marroquin-Cardona et al. 2011). Interactions of similar clays (e.g. hydrated sodium calcium aluminosilicates; HSCAS) with Zn have been reported by Chung and Baker (1990). In that study, the authors observed significantly ($p < 0.05$) lower Zn levels in the tibia of chickens fed 1% HSCAS in the diet. Zn tibia concentration is a more reliable indicator than serum Zn concentration of Zn levels in the body (Wedekind and Baker 1990). Nevertheless, serum and plasma Zn are useful to evaluate short-term changes in dietary zinc as it has been shown in studies feeding Zn deficient diets to SD rats (Baltaci et al. 2005; Sunnar et al. 2009). The results of the present study are similar to what we observed in a paired study using a refined

NovaSil clay (uniform particle size NovaSil; UPSN) (Marroquin-Cardona et al. 2011). In that study, a significant reduction of Zn ($P < 0.05$) was documented just in the males receiving the high dose of UPSN (2%). The difference between both studies is the type of bentonite used. For instance, UPSN is a montmorillonite rich Ca-bentonite with limited expansive behavior, while the bentonite in the present study is known to contain high amounts of Na and to display a high expansive behavior. Both bentonites are reported to have high CEC values and it has been previously proposed that these types of clays may retain cation exchange abilities in the gastrointestinal tract (Marroquin-Cardona et al. 2011). Together, these characteristics may potentially explain the reduction and increase of serum Zn and Na in males of Na-BENT groups, respectively. Nevertheless, the bentonite effects on Zn were not observed in the females and we do not have a clear explanation available.

Some differences from the control were observed, but no evidence of dose dependency was found and most parameters fell within ranges reported as normal physiological levels. Nonetheless, due to inherent cation exchange capacity of these types of clays, our findings with Ca, Na, K and Zn require further research.

VI. SUMMARY

Among the several natural toxic agents found in the food supply, mycotoxins are the most prevalent. Particularly, AFs are carcinogens with strong hepatotoxic, immunosuppressive and antinutritional effects that are commonly found in staple foods worldwide. Specifically, AFB_1 is the most toxic of four naturally occurring AFs (i.e., B_1, B_2, G_1 and G_2) and is considered a Group 1 carcinogen by the International Agency for Research on Cancer (IARC 2002). The exposure to AFs is unavoidable and strategies to ameliorate their toxicity in animals and humans are needed. Extensive research based on clay therapy has been developed to protect animals against this toxin (Phillips et al. 1988; Grant and Phillips 1998; Phillips 1999). Natural bentonites (rich in montmorillonite) are the common additives used for this purpose. The proposed mechanism for which montmorillonite reduces AF bioavailability is by adsorbing the molecule into the different clay surfaces (i.e. external, edges and interlayer spaces), being the interlayer space the most important site. As a consequence of the pioneering studies mentioned above, a variety of materials labeled as mycotoxin binders for use in feeds have been introduced in the market, many of them without detailed effectiveness and safety studies. Thus, the first objective in the present study was to elucidate the mineral characteristics, potential safety and AFB_1 adsorption capacities of edible clays marketed in Mexico and other countries as AF binders for inclusion in animal feeds. The commercial clays were compared to NSP, a montmorillonite rich calcium bentonite that has been extensively used as positive control for AF adsorption in many studies.

According to the AF sorption analyses, NovaSil, Mycoad, Milbond and Volclay showed the best sorption characteristics (i.e. L-shape sorption pattern at pH 2 and 6.5 and Q_{max} values above 0.2 mol kg^{-1}). Importantly, the most effective sorbents showed relatively higher levels of montmorillonite (according to the relative intensities recorded by XRD) when compared to the least effective ones. The differences in AF sorption capacity observed for the binders were attributed to the montmorillonite content, particle size distribution and/or presence of diluting agents. Regarding potential safety, the organoclay-based additives as well as the additive containing yeast cell walls were the only ones that induced a toxic response in the hydra assay. This emphasized the need of safety studies of organophilic clays that may use quaternary amines of lipids in their preparation. The effects of these components on the intestinal micro biota and mucosal cells may also be deleterious. The toxicity of yeast derived product was assumed to be related to the presence of living yeasts and their growth metabolites. Based on our work, the most effective AF-binding additives intended for animal feed had considerable high amounts of smectite (i.e., montmorillonite) and this presence of smectite was correlated with a favorable L-shape pattern of sorption.

After the success observed in several animal studies using NS clay to reduce AF bioavailability, the application of this technology in humans was of great importance in many developing countries with populations at elevated risk. In the Ashanti region in Ghana, a population at risk for AFs was identified by measuring AFM_1 in the urine and AFB_1-lysine albumin adducts in the blood. When investigating the cultural habits of this population, it was noted that geophagy was prevalent. Edible clays were available in the

market place for different intended purposes. Nevertheless, the mineralogy and potential AF sorption characteristics of these clays were unknown. Hence, the second objective in our study was to investigate the mineral composition, potential AF sorption capacity and preliminary safety of edible clays for humans. Geophagic clays from other countries and NS were also investigated for comparison purposes. Kaolinite, muscovite and quartz were the dominant minerals observed in the majority of the geophagic clays. The presence of smectite was evident in several edible clays and its presence was correlated with a favorable AF sorption pattern (i.e. L-shape, Langmuir). Nevertheless, most of the edible clays had low AF binding capacities (below 0.2 mol kg^{-1}) and were regarded as ineffective adsorbents for AF. Since none of the clays from Ghana had significant AF sorption capacity, NS clay was successfully used in a Phase IIa clinical intervention trial conducted in the Ashanti Region of Ghana.

On the other hand, because NS is derived from naturally-occurring bentonite deposits that vary from batch-to-batch in composition, particle size, non-framework trace metal content and dioxin levels, an alternative may be more appropriate for long-term studies in humans. To address this issue and improve consistency and uniformity, in this project, we utilized Uniform Particle Size NovaSil (UPSN), a refined material selected from NS parent clay that has a more uniform particle size distribution, ideal for long-term human application. The selection procedure at which UPSN was subjected improved its consistency and uniformity in composition between batches, hence favoring its use in clinical trials and as eventual therapy for human aflatoxicosis. As a consequence, as part of our third objective, the mineral and AF sorption characteristics

of parent NS and UPSN were investigated. Furthermore, since the safety of UPSN was unknown, a 3 month rodent study was designed to investigate its effects *in vivo*. Four-week-old male and female Sprague Dawley® rats were fed rations free of clay (control) and containing UPSN at low dose (0.25%) and high dose (2.0%) levels for 13 weeks. Growth parameters such as initial body weight, final body weight, feed conversion efficiency, total feed consumption and total body gain weight were determined. Serum and blood biochemical parameters were measured along with serum vitamin A and E, and iron and zinc. No major differences were noted in the mineral properties and AFB_1 sorption characteristics for both clays. Total body weight gain was unaffected in either sex at the doses tested. No UPSN-dependent differences in relative organ weights or gross appearance were observed. Isolated differences between UPSN groups and the control were observed for some biochemical parameters and selected vitamins and minerals. None of these differences were dose-dependent, and all parameters fell between ranges reported as normal for rats less than 6 month old. Na, Na/K ratio and vitamin E were the only parameters that were increased in both males and females in UPSN groups. Zinc levels in males of 2% UPSN group were lower compared to the control. Serum K levels were lower in the males of UPSN groups than in the control. Importantly, neither Na/K, K, nor Zn values fell outside ranges reported as normal, nor apparent dose dependency was observed. These results suggested that dietary inclusion of UPSN at levels as high as 2% (w/w) does not result in overt toxicity.

As the use of smectite clays, mainly Ca-montmorillonite, as strategy to decrease bioavailability of AFs became more popular, sustainability issues were apparent and

other sources of this material were investigated. According to the major cation present, dioctahedral smectites can be regarded as sodium (Na) or calcium (Ca) saturated. Na-bentonites (mainly composed by montmorillonite) have also been used to prevent AF's effects on dairy cattle (Diaz et al. 2004), but their potential interactions with other additives (Shryock et al. 1994), anticoccidials (Gray et al. 1998) and probable interactions with vitamin A (Briggs and Fox, 1956; Laughland and Phillips 1956) have been reported. Additionally, earlier investigations have reported incomplete protection against AF effects (Voss et al. 1993; Abdel et al. 1999). On the other hand, due to the natural origin of this clay, potential contamination with heavy metals and dioxins may also be present. Hence, safety and efficacy studies were warranted for this type of clay. Our objective five was to elucidate the mineral and AF sorption characteristics of a Na-bentonite and to determine its safety in a 13 weeks rodent model using Sprague Dawley rats. Na-montmorillonite (Na-BENT) was obtained from AMCOL Specialty Minerals (Hoffman Estates, IL). Levels of dioxins and selected heavy metals were performed by Analytical Perspectives (Wilmington, NC) using US Environmental Protection Agency (USEPA) methods. Low levels of both dioxins/furans and the selected heavy metals were found in Na-BENT. When the TEQs levels of dioxins/furans (0.081 pg g^{-1}) where compared to the PTDI (~2.3 pg kg-bw^{-1}) values reported by the JECFA, it was determined that 3 g of Na-BENT (the intended dose likely to be given to a human subject) confer negligible risk of dioxin exposure. Similarly, according to TDI values reported by JECFA, the exposure to heavy metals like As, Cd, Hg and Pb from 3 g of Na-BENT was minor.

In the mineralogical analyses, montmorillonite was confirmed as the main ingredient in Na-BENT, according to XRD and FTIR. Na-BENT also contained muscovite, quartz, gypsum, calcite and feldspars. The CEC and pH of Na-BENT were similar to the values expected for other montmorillonite rich bentonites. In the AF adsorption isotherms, the capacity of Na-BENT for AF sorption was also similar to observations for other bentonites (Q_{max} = 0.3 mol kg^{-1}). According to this, it is expected that Na-BENT may be effective as AF adsorbent *in vivo*.

No apparent toxicity effects due to consumption of bentonite at either dose were noticed throughout the rodent study. Major findings of the study included 1) the increased vitamin E levels observed in both females and males of Na-BENT groups, 2) the reduction in serum levels of Zn in the males of Na-BENT groups, 3) the increased serum Na and Ca levels in males of Na-BENT groups, and 4) reduction in serum vitamin A levels in males of 2% Na-BENT group. Similar results for Na, Ca, Zn and K serum levels have been observed in rats ingesting other bentonites (i.e. UPSN) (Marroquin-Cardona et al. 2011). In the same way, interactions of bentonites with vitamin A have also been previously reported in rats ingesting similar clays (Laughland and Phillips 1954). This may be specific for Na-BENT since other studies using SD rats and 2% inclusion in feed of Ca-bentonites have shown no significant changes in serum vitamin A levels (Afriyie-Gyawu et al. 2005; Marroquin-Cardona et al. 2011). More research is clearly warranted to explain these specific findings.

In summary, the presence of AFs in feeds seems to be a problem without a clear solution. Although there are many strategies to ameliorate the effects of AFs, none of

them is solely effective and a multidisciplinary approach must be the best selection. The use of clay as potential enterosorbent for AFs is a natural and safe alternative that is also cost effective and culturally accepted in some of the countries with the highest risk of exposure to AFs. Hopefully in the future, with all the scientific evidence of effectiveness and safety that has been accumulated from several animal and human studies, this technology can be successfully transferred to populations at risk. Thus contributing to a solution for the global health problems associated to AF exposure.

REFERENCES

Abdel-Wahhab MA, Nada SA, Amra HA. 1999. Effect of aluminosilicate and bentonite on AF-induced developmental toxicity in rats. J Appl Toxicol. 19:199-204.

Adamson AW. 1982. Physical chemistry of surfaces. New York (NY): Wiley. The solid-liquid interface adsorption from solution; p. 369-401.

Afriyie-Gyawu E, Ankrah NA, Huebner HJ, Ofosuhene M, Kumi J, Johnson NM, Tang L, Xu L, Ofori- Adjei D, Williams JH, Wang JS, Phillips TD. 2008a. NovaSil clay intervention in Ghanaians at high risk for aflatoxicosis: I. Study design and clinical outcomes. Food Addit Contam. 25:76-87.

Afriyie-Gyawu E, Mackie J, Dash B, Wiles M, Taylor J, Huebner H, Tang L, Guan H, Wang J-S, Phillips TD. 2005. Chronic toxicological evaluation of dietary NovaSil Clay in Sprague-Dawley rats. Food Addit Contam: Part A. 22:259-269.

Afriyie-Gyawu E, Wang Z, Ankrah N-A, Xu L, Johnson NM, Tang L, Guan H, Huebner HJ, Jolly PE, Ellis WO, Taylor R, Brattin B, Ofori-Adjei D, Williams JH, Wang J-S, Phillips TD. 2008b. NovaSil clay does not affect the concentrations of vitamins A and E and nutrient minerals in serum samples from Ghanaians at high risk for aflatoxicosis. Food Addit Contam: Part A. 25:872-884.

Ake CL, Mayura K, Huebner H, Bratton GR, Phillips TD. 2001. Development of porous clay based composites for the sorption of lead from water. J Toxicol Environ Health A. 63:459–475.

Alekseyev VA. 2007. Equations for the Dissolution Reaction Rates of Montmorillonite, Illite, and Chlorite. Geochemistry International. 45(8):770-780.

Almodovar-Cuevas C, Navarro-Ruiz A, Bastidas-Ramirez BE, Mora-Navarro MR, Garzon P. 1985. Valproic acid effects on leukocytes and platelets of Sprague-Dawley rats. Gen Pharmac. 16:423-426.

Amla I, Kamala CS, Gopalakrishna GS, Jayaraj AP, Sreehivasamurthy V, Parpia HAB. 1971. Cirrhosis in children from peanut meal contaminated by aflatoxin. Am J Clin Nutr. 24:609-614.

Baltaci AK, Mogulkoc R, Halifeoglu I. 2005. Effects of zinc deficiency and supplementation on plasma leptin levels in rats. Biological Trace Element Research. 104:41-46.

Bastianello SS, Nesbit JW, Williams MC, Lange AL. 1987. Pathological findings in a natural outbreak of aflatoxicosis in dogs. Onderstepoort J Vet Res. 54:635-640.

Battista JR, Marnnet LJ. 1985. Prostaglandine H synthase-dependent epoxidation of aflatoxin B_1. Carcinogenesis. 6:1227-1229.

Benham AJ, Brown TJ. 2007. African mineral production 2001-2005. Keywoth (Nottingham): British Geological Survey. p. 6.

Bhatnagar D, Yu J, Ehrlich KC. 2002. Toxins of filamentous fungi. Chem Immunology. 81:167-206.

Bigham JM, Fitzpatrick RW, Shulze DG. 2002. Soil mineralogy with environmental applications. Madison (WI): Soil Science Society of America Inc. Iron oxides; 323-366.

Bondy GS, Pestka JJ. 2000. Immunomodulation by fungal toxins. J Toxicol Env Healt B. 3:109-143.

Brady NC, Weil RR. 2002. The nature and properties of soils. 13th ed. Englewood Cliffs (NJ): Prentice-Hall. p. 123–124.

Briggs GM, Fox MR. 1956. Vitamin A deficiency in chicks produced by adding high levels of bentonite to synthetic diets. Poultry Sci. 35:570-576.

Buol SW, Hole FD, McCracken RJ. 1980. Soil genesis and classification. Ames (IA): Iowa State University Press. p. 66.

Busby WF, Wogan GN. 1984. Chemical Carcinogens. Washington: American Chemical Society. Aflatoxins; p. 945-1136.

Butler WH, Barnes JM. 1966. Carcinoma of the glandular stomach in rats given diets containing aflatoxin. Nature. 209:90.

CAST (Council for Agricultural Science and Technology). 2003. Mycotoxins: Risks in plant, animal, and human systems. CAST, Ames, IA. Task Force Report No. 139. pp. 13-85.

Carretero MI, Gomes CSF, Tateo F. 2006. Handbook of clay science. Oxford:Elsevier. Clays and human health; 717–741.

Cary JW, Linz JE, Bhatnagar D. 2000. Microbial foodborne diseases: mechanisms of pathogenesis and toxin synthesis. Lancaster (PA): Technomic Publishing Company Inc. Aflatoxins: Biological significance and regulation of biosynthesis; p. 317-361.

Castegnaro M, Hunt DC, Sansone EB, Schuller PL, Siriwardana MG, Telling GM, van Egmond HP, Walker EA.1980. Laboratory decontamination and destruction of aflatoxins B1, B2, G1, G2 in laboratory wastes. Lyon (France): IARC Press. IARC Scientific Publications No. 37.

Chen J. 1997. Dietary aflatoxin intake levels in china: data compilation (information submitted to WHO).

Choudary C, Rao MRKM. 1982. An outbreak of aflatoxicosis in commercial poultry farms. Poultry Adviser. 16:75-76.

Chung TK, Erdman Jr. JW, Baker DH. 1990. Hydrated sodium calcium aluminosilicate: Effects on zinc, manganese, vitamin A, and riboflavin utilization. Poultry Sci. 69: 1364-1370.

Cole RJ, Cox RH. 1981. Handbook of toxic fungal metabolites. 1st. ed. New York (NY): Academic Press. p. 1-903.

Coulombe RA. 1993. Biological action of mycotoxins. J Dairy Sci. 76:880-891.

Coulombe RA. 1994. The toxicology of aflatoxins: human health, veterinary, and agricultural significance. San Diego (California): Academic Press, Inc. Nonhepatic disposition and effects of aflatoxin B1; p. 89-101.

Cullen JM, Newberne PM 1994. The toxicology of aflatoxins. San Diego (CA): Academic Press. Acute hepatotoxicity of aflatoxins; p. 3-26.

Dakovic A, Tomasevic-Canovic M, Dondur V, Rottinghaus GE, Medakovic V, Zaric S. 2005. Adsorption of mycotoxins by organozeolites. Colloid Surf B Biointerfac. 46(1):20-25

Deer WA, Howie RA, Zussman J. 1978. An introduction to the rock forming minerals. Longman, London, UK.

Degen LP, Phillips SF. 1996. Variability of gastrointestinal transit in healthy women and men. Gut. 39:299-305.

Deng Y, Barrientos-Velázquez AL, Billes F, Dixon JB. 2010. Bonding mechanisms between aflatoxin B1 and smectite. Appl Clay Sci. 50(1):92-98.

Desheng Q, Fan L, Yanhu Y, Niya Z. 2005. Adsorption of aflatoxin B1 on montmorillonite. Poultry Sci 84:959-961.

Diamond JM. 1999. Dirty eating for healthy living. Nature. 400:120-121.

Diaz DE, Hagler Jr. WM, Blackwelder JT, Eve JA, Hopkins BA, Anderson KL, Jones FT, Whitlow LW. 2004. Aflatoxin binders II: reduction of aflatoxin M1 in milk by sequestering agents of cows consuming aflatoxin in feed. Mycopathologia. 157:233-241.

Diaz DE, Hagler Jr WM, Hopkins BA, Whitlow LW. 2002. Aflatoxin binders I: In vitro binding assay for aflatoxin B1 by several potential sequestering agents. Mycopathologia. 156:223-226.

Dixon JB, Kannewischer I, Tenorio-Arvide MG, Barrientos-Velazquez AL. 2008. Aflatoxin sequestration in animal feeds by quality-labeled smectite clays: An introductory plan. Appl Clay Sci. 40:201-208.

Dollear FG, Mann GE, Codifer LP, Gardener HK, Koltun Jr SP, Vix HLF. 1968. Elimination of aflatoxins from peanut meal. J Am Oil Chem Soc. 54:862-865.

Doner HE, Grossl PR. 2002. Carbonates and evaporites. Soil mineralogy with environmental applications. A. D. Warren. Madison, Soil Science Society of America 7: 199-228.

Dragan YP, Pitiot HC. 1994. The toxicology of aflatoxins: human health, veterinary and agricultural significance. New York (NY): Academic Press. Aflatoxin carcinogenesis in the context of the multistage nature of cancer; p. 179-206.

Dyer Z, Peltekian K, van Zanten SV. 2005. Review article: the changing epidemiology of hepatocellular carcinoma in Canada. Aliment Pharmacol Ther. 22:17-22.

Eaton DL, Ramsdell HS, Neal GE. 1994. The toxicology of aflatoxins: human health, veterinary, and agricultural significance. San Diego (CA): Academic Press Inc. Biotransformation of aflatoxins; p. 45-72.

European Food Safety Authority (EFSA). 2004. Opinion of the Scientific Panel on Contaminants in the Food Chain on a request from the Commission related to Aflatoxin B_1 as undesirable substance in animal feed. The EFSA Journal. 39:1-27.

Elder DP. 2005. Pharmaceutical applications of ion-exchange resins. J Chem Edu. 82: 575-587.

El-Serag HB, Davila JA, Petersen NJ, McGlynn KA. 2003. The continuing increase in the incidence of hepatocellular carcinoma in the United States: an update. Ann Intern Med. 139:817-823.

Electronic Code of Federal Regulations. Substances generally recognized as safe, 21 CFR 182.2729. Accesed July 22, 2010.

Epstein SM, Bartus B, Farber E. 1969. Renal epithelial neoplasms induced in male wistar rats by oral aflatoxin B1. Cancer Res. 29:1045-1050.

Essigmann JM, Croy RG, Nadzan AM, Busby WF Jr, Reinhold VN, Buchi G, Wogan GN. 1977. Structural identification of the major DNA adduct formed by aflatoxin B1 in vitro. Proc Natl Acad Sci. 74:1870-1874.

Essigmann JM, Green CL, Croy RG, Fowler KW, Buchi GH, Wogan GN. 1983. Interactions of aflatoxin B1 and alkylating agents with DNA: structural and functional studies. Cold Spring Harb Symp Quant Biol. 47**:**327-37.

FDA (Food and Drug Administration). 1997. http://www.fda.gov/AnimalVeterinary/Products/AnimalFoodFeeds/Contaminants/ucm050430.htm

FDA (Food and Drug Administration). 2011. http://www.fda.gov/ICECI/ComplianceManuals/CompliancePolicyGuidanceManual/ucm074482.htm. Accessed on March 3, 2011.

Foster PL, Eisenstadtt E, Millert JH. 1983. Genetics base substitution mutations induced by metabolically activated aflatoxin B1. Proc Natl Acad Sci. 80:2695-2698.

Galia E, Nicolaides E, Hörter D, Löbenberg R, Reppas C, Dressman JB. 1998. Evaluation of various siddolution media for predicting in vivo performance of class I and II drugs. Pharmaceut Res. 15:698-705.

Gallagher EP, Wienkers LC, Stapleton PL, Kunze KL, Eaton DL. 1994. Role of human microsomal and human complementary DNA-expressed cytochromes P4501A2 and P4503A4 in the bioactivation of aflatoxin B1. Cancer Res. 54:101-108.

García-Morales AR, Rosiles-Martínez R, Bautista-Ordoñez J, Avila-González E. 2004. In vitro binding ability of ochratoxin A by commercial mycotoxin binders available in Mexico. Vet Mex. 35(4):351–358.

Garland T, Reagor J. 2001. Mycotoxins and phycotoxins in perspective at the turn of the millennium. Wageningen: WJ de Koe. Chronic canine aflatoxicosis and management of an epidemic; p. 231-236.

Gelderblom WCA, Marasas WFO, Lebepe-Mazur S, Swanevelder S, Vessey CJ, Hall P de la M. 2002. Interaction of fumonisin B1 and aflatoxin B1 in a short-term carcinogenesis model in rat liver. Toxicol. 171:161-173.

Giles CH, MacEwan TH, Nakhwa SN, Smith D. 1960. Studies in Adsorption. Part XI. A system of classification of solution adsorption isotherms, and its use in diagnosis of

adsorption mechanisms and in measurement of specific areas of solids. J Chem Soc. 3973-3993.

Giles CH, Smith D, Huitson A. 1974a. A general treatment and classification of the solute adsorption isotherm I. Theoretical. J Colloid Interface Sci. 47:755-765.

Giles CH, D'Silva AP, Easton IA. 1974b. A general treatment and classification of the solute adsorption isotherm Part II. Experimental interpretation. J Colloid Interface Sci. 47:766-778.

Glintborg B, Weimann A, Kensler TW, Poulsen HE. 2006. Oltipraz chemoprevention trial in Qidong, People's Republic of China: Unaltered oxidative biomarkers. Free Radical Bio Med. 41:1010-1014.

Gopalakrishnan S, Harris TM, Stone MP. 1990. Intercalation of aflatoxin B1 in two oligodeoxynucleotide adducts: Comparative 1H NMR analysis of d(ATCAFBGAT) d(ATCGAT) and d(ATAFBGCAT)2. Biochemistry 29**:**10438-10448.

Grant PG, Phillips TD. 1998. Isothermal adsorption of aflatoxin B1 on HSCAS clay. J Agric Food Chem. 46:599-605.

Gray SJ, Ward TL, Southern LL, Ingram DR. 1998. Interactive effects of sodium bentonite and coccidiosis with monensin or salinomycin in chicks. Poult Sci. 77:600-604.

Groopman JD. 1994. The toxicology of aflatoxins: human health, veterinary, and agricultural significance. San Diego (CA): Academic Press, Inc. Molecular dosimetry methods for assessing human aflatoxin exposures; 259-279.

Groopman JD, Donahue PR, Zhu JQ, Chen JS, Wogan GN. 1985. Aflatoxin metabolism in humans: detection of metabolites and nucleic acid adducts in urine by affinity chromatography. Proc Natl Acad Sci USA. 82:6492-6496.

Guengerich FP, Johnson WW, Shimada T, Ueng YF, Yamazaki H, Langouët S. 1998. Activation and detoxication of aflatoxin B1. Mutat Res. 402:121–128.

Han SH, Jeon YJ, Yea SS, Yang KH. 1999. Suppression of the interleukin-2 gene expression by aflatoxin B_1 is mediated through the down-regulation of the NF-AT and AP-1 transcription factors. Toxicol Lett. 108:1-10.

Hartley RD, Nesbitt BF, O'Kelly J. 1963. Toxic metabolites of *Aspergillus flavus*. Nature 198:1056-1058.

Harvey RB, Kubena LF, Elissalde MH, Corrier DE, Phillips TD. 1994. Comparison of two hydrated sodium calcium aluminosilicates compounds to experimentally protect growing barrows from aflatoxicosis. J Vet Diag Invest. 6:88-92.

Harvey RB, Kubena LF, Elissalde MH, Phillips TD. 1993. Efficacy of zeolitic ore compounds on the toxicity of aflatoxin to growing broiler chickens. Avain Dis. 37(1):67-73.

Harvey RB, Phillips TD, Ellis JA, Kubena LF, Huff WE, Petersen HD. 1991. Effects of aflatoxin M_1 residues in milk by addition of hydrated sodium calcium aluminosilicate to aflatoxin-contaminated diets of dairy cows. Am J Vet Res. 52:1556-1559.

Hayes JD, Judah DJ, Neal GE. 1993. Resistance to aflatoxin B1 is associated with the expression of a novel aldo-keto reductase which has catalytic activity towards a cytotoxic aldehyde-containing metabolite of the toxin. Cancer Res. 53:3887–3894.

He C, Breuning-Madsen H, Awadzi TW. 2007. Mineralogy of dust deposited during the Harmattan season in Ghana. Danish Journal of Geography. 107(1):9-15.

Hendrickse RG, Maxwell SM. 1989. Aflatoxins and child health in the tropics. J Toxicol Toxin Rev. 8:31-41.

Henry SH, Bosch FX, Bowers JC. 2002. Aflatoxins, hepatitis, and worldwide liver cancer risks. Adv. Exp Med Biol. 504:229–233.

Hinton DM, Myers MJ, Raybourne RA, Francke-Carroll S, Sotomayor RE, Shaddock J, Warbritton A, Chou MW. 2003. Immunotoxicity of aflatoxins in rats: Effects on lymphocytes and the inflammatory response in a chronic intermittent dosing study. Tox Sci. 73:362-377.

Holeski CJ, Eaton DL, Monroe DH, Bellamy GM. 1987. Effects of phenobarbitol on the biliary excretion of aflatoxin P1-glucuronide and aflatoxin B1-*S*-glutathione in the rat. Xenobiotica. 17:139-153.

Hollstein M, Sidransky D, Vogelstein B, Harris CC. 1991. p53 mutations in human cancers. Science. 253:49-53.

Holmgren GGS, Juve RL, Geschwender RC. 1977. A mechanically controlled variable rate leaching device. Soil Sci Soc Am J. 41:1207-1208.

Hong WK, Lippman SM, Itri LM, Karp DD, Lee JS, Byers RM, Schantz SP, Kramer AM, Lotan R, Peters LJ, Dimery IW, Brown BW, Goepfert H. 1990. Prevention of second primary tumors with isotretinoin in squamous-cell carcinoma of the head and neck. N Engl J Med. 323:795-801.

Hooda PS, Henry CJ, Seyoum TA, Armstrong LD, Fowler MB. 2002. The potential impact of soil ingestion on human mineral nutrition. Environ Geochem Health. 24:305-319.

Horii Y, van Bavel B, Kannan K, Petrick G, Nachtigall K, Yamashita N. 2008. Novel evidence for natural formation of dioxins in ball clay. Chemosphere. 70:1280-1289.

Huang WH, Kiang WC. 1972. Laboratory dissolution of plagioclase feldspars in water and organic acids at room temperature. American Mineralogist 57:1849-1859.

Huebner HJ, Mayura K, Pallaroni L, Ake CL, Lemke SL, Herrera P, Phillips TD. 2000. Development and characterization of a carbon-based composite material for reducing patulin in apple juice. J Food Prot. 63:106-110.

Huwig A, Freimund S, Ka¨ ppeli O, Dutler H. 2001. Mycotoxin detoxication of animal feed by different adsorbents. Toxicol Lett. 122:179-188.

National Cancer Institute (NCI). 2010. http://www.cancer.gov/cancertopics/pdq/prevention/hepatocellular/Patient/page2.

International Agency for Research on Cancer (IARC). 2002. IARC monographs on the evaluation of the carcinogenic risk of chemicals to man: some traditional herbal medicines, some mycotoxins, naphthalene and styrene. Lyon (France) 82:169-300.

International Crops Research Institute for the Semi-Arid Tropics (ICRISAT). 2002. Aflatoxin-A deadly hazard.

International Agency for Research on Cancer (IARC). 1987. Overall evaluations of carcinogenicity. Monographs on the Evaluation of Carcinogenic Risks to Humans. Lyon (France). 7:440.

JECFA. 1998. Safety Evaluation of Certain Food Additives and Contaminants: Aflatoxins (WHO Food Additives Series 40), 49th Meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), Geneva, International Programme on Chemical Safety, World Health

Jiang Y, Jolly PE, Ellis WO, Wang J-S, Phillips TD, et al. 2005. Aflatoxin B1 albumin adduct levels and cellular immune status in Ghanaians. Int Immunol. 17:807-814.

Joint FAO/WHO Expert Committee on Food Additives (JECFA). 2007. Safety of certain food additives and contaminants. WHO food additives series 58. WHO, Geneva, 2007.

Joint FAO/WHO Expert Committee on Food Additives (JECFA). 2001. Summary and Conclusions of the Fifty-seventh Meeting. Evaluation of certain food additives and contaminants: fifty-seventh report of the Joint FAO/WHO Expert Committee on Food Additives.

Joint FAO/WHO Expert Committee on Food Additives (JEFCA). 2000. Safety evaluation of certain food additives / prepared by the forty-four meeting. Lead WHO Food additives Series 44.

Joint FAO/WHO Expert Committee on Food Additives (JECFA). 1982. Zinc (WHO Food Additives Series 17.

Jolly PE, Jiang Y, Ellis W, Awuah R, Nnedu O, Phillips TD, Wang JS, Afriyie-Gyawu E, Tang L, Person S, Williams J, Jolly C. 2006. Determinants of aflatoxin levels in Ghanaians: sociodemographic factors, knowledge of aflatoxin and food handling and consumption practices. Int J Hyg Environ Health. 209:345-58.

Johns T, Duquette M. 1991. Detoxification and mineral supplementation as functions of geophagy. Am J Clin Nutr. 53**:**448-456.

Jozefaciuk G, Bowanko G. 2002. Effect of acid and alkali treatments on surface areas and adsorption energies of selected minerals. Clay Clay Miner. 50:771-783.

Kannewischer I, Tenorio AMG, White GN, Dixon JB. 2006. Smectite clays as adsorbents of aflatoxin B1: initial steps. Clay Sci. 12:199-204.

Karntanut W, Pascoe D. 2002. The toxicity of copper, cadmium and zinc to four different Hydra (Cnidaria: Hydrozoa). Chemosphere. 47:1059–1064.

Keay J. 1993. Eating dirt in Venezuela, pp. 342–350. *in* J. Keay (ed). The Robinson Book of Exploration. Robinson, London. (Taken from Alexander von Humboldt: Personal Narrative of Travels to the Equinoctial Regions of America. George Routledge and Sons, London).

Kensler TW, Davis EF, Bolton MG 1994. The Toxicology of Aflatoxins: Human Health, Veterinary, and Agricultural Significance. San Diego (CA):Academic Press. Strategies for chemoprotection against aflatoxin-induced liver cancer; p. 281-306.

Kensler TW, Groopman JD, Eaton DL, Curphey TJ, Roebuck BD. 1992. Potent inhibition of aflatoxin-induced hepatic tumorigenesis by the monofunctional enzyme inducer 1,2-dithiole-3-thione. Carcinogenesis. 13:95-100.

Kimanya ME, De Meulenaer B, Tiisekwa B, Ndomondo-Sigonda M, Devlieghere F, Van Camp J, Kolsteren P. 2008. Co-occurrence of fumonisins with aflatoxins in

home-stored maize for human consumption in rural villages of Tanzania. Food Addit Contam: Part A. 25:1353-1364.

Kohn DF, Clifford CB. 2002. Laboratory Animal Medicine. 2 ed. San Diego (CA): Academic Press. Biology and diseases of rats; p. 128-131.

Kolars JC, Benedict P, Schmiedlin RP, Watkins PB. 1994. Aflatoxin B1-adduct formation in rat and human small bowel enterocytes. Gastroenterology. 106:433-439.

Krishnamachari KAVR, Bhat RV, Nagaraja V, Tilak TBG. 1975. Hepatitis due to aflatoxicosis: An outbreak in western India. Lancet. 1:1061-1063.

Kutz RE, Sampson JD, Pompeu LB, Ledoux DR, Spain JN, Vázquez-Añón M, Rottinghaus GE. 2009. Efficacy of Solis, Novasil Plus, and MTB-100 to reduce aflatoxin M1 levels in milk of early to mid lactation dairy cows fed aflatoxin B1. J Dairy Sci. 92:3959-3963.

Larsson P, Tjalve H. 1995. Extrahepatic bioactivation of aflatoxin B1 in fetal, infant and adult rats. Chem Biol Interact. 94:1-19.

Laughland DH, Phillips WE. 1954. The effect of sodium bentonite administration on vitamin A metabolism in the rat. Can J Biochem Physiol. 32(6):593-599.

Ledoux DR, Rottinghaus GE, Bermudez AJ, Alonso-Debolt M. 1999. Efficacy of a hydrated sodium calcium aluminosilicate to ameliorate the toxic effects of aflatoxin in broiler chicks. Poult Sci. 78:204-210.

Leeson S, Diaz GJ, Summers JD. 1995. Poultry metabolic disorders and mycotoxins. Guelph, (ON): University Books. p. 190-309.

Lemke SL, Grant PG, Phillips TD. 1998. Adsorption of zearalenone by organophilic montmorillonite clay. J Agric Food Chem. 46:3789-3796.

Lemke SL, Mayura K, Reeves WR, Wang N, Fickey C, Phillips TD. 2001a. Investigation of organophilic montmorillonite clay inclusion in zearalenone contaminated diets using the mouse uterine weight bioassay. J Toxicol Environ Hlth. 62:243-258.

Lemke SL, Ottinger SE, Mayura K, Ake CL, Pimpukdee K, Wang N, Phillips TD. 2001b. Development of a multitiered approach to the in vitro prescreening of clay-based enterosorbents. Anim Feed Sci Technol. 93:17-29.

Li F, Jiang Y, Yu L, Yang Z, Hou T, Sun S. 2005. Surface effect of natural zeolite (clinoptilolite) on the photocatalytic activity of TiO2. Appl Surf Sci. 252:1410-1416.

Lillie LE, Temple NJ, Florence LZ. 1996. Reference values for young normal Sprague-Dawley rats: weight gain, hematology and clinical chemistry. Hum Exp Toxicol. 15: 612-616.

Lin JK, Miller JA, Miller EC. 1977. 2,3-Dihydro-2-(guan-7-yl)-3-hydroxy-aflatoxin B_1, a major acid hydrolysis product of aflatoxin B1-DNA or -ribosomal RNA adducts formed in hepatic microsome-mediated reactions and in rat liver in vivo. Cancer Res. 37:4430-4438.

Liu L, Massey TE. 1992. Bioactivation of aflatoxin B1 by lipoxygenases, prostaglandin H synthase and cytochrome P450 monooxygenase in guinea-pig tissues. Carcinogenesis. 13:533-539.

Lopez C, Ramos L, Bulacio L, Ramadan S, Rodriguez F. 2002. Aflatoxin B1 content in patients with hepatic diseases. Medicina. 62:313-316.

Madejová J, Bujdák J, M. Janek M, Komadel P. 1998. Comparative FT-IR study of structural modifications during acid treatment of dioctahedral smectites and hectorite. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy. 54(10):1397-1406.

Márquez-Márquez RN, Tejada de Hernández I. 1995. Aflatoxin adsorbent capacity of two Mexican aluminosilicates in experimentally contaminated chick diets. Food Addit Contam: Part A. 12:431-433.

Marroquín-Cardona A, Deng Y, Taylor JF, Hallmark CT, Johnson NM, Phillips TD. 2009. In vitro and in vivo characterization of mycotoxin-binding additives used for animal feeds in Mexico. Food Addit Contam: Part A. 26:733-743.

Masimango N, Remacle J, Ramzut JL. 1978. The role of adsorption in the elimination of Aflatoxin B1 from contaminated media. Eur J Appl Microbiol Biotechnol. 6:101-105.

Mayura K, Abdel-Wahhab MA, McKensie KS, Sarr AB, Edwards JF, Naguib K, Phillips TD. 1998. Prevention of maternal and developmental toxicity in rats via dietary inclusion of common aflatoxin sorbents: potential for hidden risks. Toxicol Sci. 41:175–182.

Mayura K, Smith EE, Clement BA, Phillips TD. 1991. Evaluation of the developmental toxicity of chlorinated phenols utilizing Hydra attenuata and postimplantation rat embryos in culture. Toxicol Appl Pharmacol. 108:253-266.

Mckenzie KS, Kubena LF, Denvir AJ, Rogers TD, Hitchens GD. Bailey RH, Harvey RB, Buckley SA, Phillips TD. 1998. Aflatoxicosis in turkey poults is prevented by

treatment of naturally contaminated corn with ozone generated by electrolysis. Poultry Sci. 77:1094-1102.

McKenzie KS, Sarr AB, Mayura K, Bailey RH, Miller DR, Rogers TD, Norred WP, Voss KA, Plattner RD, Kubena LF, Phillips TD. 1997. Oxidative degradation and detoxification of mycotoxins using a novel source of ozone. Food Chem Toxicol. 35:807-820.

Milford MH. 1997. Introduction to Soils and Soil Science, Laboratory Exercises, fourth ed. Dubuque, Iowa.

Miller MD, Wilson MD. 1994. Toxicology of Aflatoxins. San Diego (CA): Academic Press. Veterinary diseases related to aflatoxins; p. 347-364.

Moon EY, Rhee DK, Pyo S. 1999. Inhibition of various functions in murine peritoneal macrophages by aflatoxin B1 exposure in vivo. Int J Immunopharmaco. 21:47-58.

Moss EJ. Neal GE. 1985. The metabolism of aflatoxin B1 in human liver. Biochem Pharmacol. 34:3193-3197.

Muench KF, Misra RP, Humayun MZ. 1983. Biochemistry sequence specificity in aflatoxin B1-DNA interactions. Proc Natl Acad Sci. 80:6-11.

Mulder I, Tenorio-Arvide MG, White GN, Dixon JB. 2008. Smectite clay sequestration of aflatoxin B1: Mineral dispersivity and morphology. Clays Clay Miner. 56: 559–571.

Murray HH, Keller WD. 1993. Kaolin genesis and utilization. Boulder (CO): Clay Mineral Society. Kaolins, kaolins and kaolins; p. 1-24.

Ngindu A, Johnson BK, Kenya PR, Ngira JA, Ocheng DM, Nandwa H, Omondi TN, Jansen AJ, Ngare W, Kaviti JN, Gatei D, Siongok TA. 1982. Outbreak of acute hepatitis caused by aflatoxin poisoning in Kenya. Lancet. 1:1346-1348.

Ominski KH, Marquardt RR, Sinha RN, Abramson D. 1994. Mycotoxins in Grain: Compounds other than aflatoxin. St. Paul (Minnesota): Eagan Press. Ecological aspects of growth of mycotoxin production by storage fungi; p. 287-312.

O'Neil MJ, Smith A, Heckelman PE. 2001. The merck index. 13th ed. Whitehouse Station (NJ): Merck & Co. p. 34-35.

Osweiler GD, Trampel DW. 1985. Aflatoxicosis in feedlot cattle. J Am Vet Med Assoc. 187:636-637.

Ottinger SE, Mayura K, Lemke SL, McKenzie KS, Wang N, Kubena LF, Phillips TD. 1999. Utilization of electrochemically generated ozone in the degradation and detoxication of benzo[a]pyrene. J Toxicol Environ Hlth. 57:565-583.

Özdemir M, Çetisli H. 2005. Extraction kinetics of alunite in sulfuric acid and hydrochloric acid. Hydrometallurgy 76:217-224.

Pasha TN, Farooq MU, Khattak FM, Jabbar MA, Khan AD. 2007. Effectiveness of sodium bentonite and two commercial products as aflatoxin absorbents in diets for broiler chickens. Anim Feed Sci Technol. 132:103-110.

Persson AM, Sokolowski A, Pettersson C. 2009. Correlation of in vitro dissolution rate and apparent solubility in buffered media using a miniaturized rotating disk equipment: Part I. Comparison with a traditional USP rotating disk apparatus. Drug Discov Ther. 3:104-113.

Phillips TD. 1999. Dietary clay in the chemoprevention of aflatoxin-induced disease. Tox Sci. 52:118-126.

Phillips TD, Afriyie-Gyawu E, Wang JS, Williams J, Huebner H. 2006. The mycotoxin factbook. Wageningen (Netherlands): Wageningen Academic. The potential of aflatoxin sequestering clay; p. 329-346.

Phillips TD, Clement BA, Kubena LF, Harvey RB. 1990. Detection and detoxification of aflatoxins: prevention of aflatoxicosis and aflatoxin residues with hydrated sodium calcium aluminosilicate. Vet Hum Toxicol. 32:15-19.

Phillips TD, Clement BA, Park DL. 1994. The toxicology of aflatoxins. San Diego (CA): Academic Press. Approaches to reduction of aflatoxins in foods and feeds; p. 383-406.

Phillips TD, Lemke SL, Grant PG. 2002. Mycotoxins and food safety: advances in experimental medicine and biology. New York (NY): Kluwer Academic/Plenum Publishers. Characterization of clay based enterosorbents for the prevention of aflatoxicosis; p. 157.

Phillips TD, Kubena LF, Harvey RB, Taylor DR, Heidelbaugh ND. 1988. Hydrated sodium calcium aluminosilicate: A high affinity sorbent for aflatoxin. Poult Sci. 67:243-247.

Phillips TD, Afriyie-Gyawu E, Williams J, Huebner H, Ankrah N-A, Ofori-Adjei DP, Jolly P, Johnson N, Taylor J, Marroquin-Cardona A, Xu L, Tang L, Wang, J-S. 2009. Reducing human exposure to aflatoxin through the use of clay: A review. Food Addit Contam: Part A. 25:134-145.

Phillips TD, Sarr AB, Grant PG. 1995. Selective chemisorption and detoxification of aflatoxins by phyllosilicate clay. Nat Toxins. 3:204-213.

Pier AC. 1986. Diagnosis of mycotoxicosis. Boston (MA): Martinus Nijhoff Publishers. Immunomodulation in aflatoxicosis; p. 143-148.

Pimpukdee K, Kubena LF, Bailey CA, Huebner HJ, Afriyie-Gyawu E, Phillips TD. 2004. Aflatoxin-induced toxicity and depletion of hepatic vitamin A in young broiler chicks: Protection of chicks in the presence of low levels of NovaSil PLUS in the diet. Poult Sci. 83:737-744.

Ramsdell HS, Eaton DL. 1990. Species susceptibility to aflatoxin B1 carcinogenesis: Comparative kinetics of microsomal biotransformation. Cancer Res. 50**:**615-620.

Reddy RV, Taylor MJ, Sharma RP. 1987. Studies of immune function of CD-1mice exposed to aflatoxinB1. Toxicology. 43:123-32.

Reid-Soukup DA, Ulery AL. 2002. Soil Mineralogy with environmental applications. Madison (WI): Soil Science Society Inc. Smectites; p. 467-499.

Richard J, Thurston JR, Pier AC. 1978. Toxins: animal, plant and microbial. New York (NY): Pergamon Press. Effects of mycotoxins on immunity; p. 801-817.

Rhoades JD, Clark M. 1978. Sampling procedures and chemical methods in use at the U.S. Salinity Laboratory for characterizing salt-affected soils and waters. U.S. Salinity Laboratory USDA, Riverside CA. p. 11-12.

Roebuck BD, Maxuitenko YY. 1994. The toxicology of aflatoxins. San Diego (CA): Academic Press. Biochemical mechanisms and biological implications of the toxicity of aflatoxins as related to aflatoxin carcinogenesis; p. 27.

Rupérez FJ, Mach M, Barbas C. 2004. Direct liquid chromatography method for retinol, α- and γ-tocopherols in rat plasma. J Chromatogr B. 800:225-230.

Sargeant K, Sheridan A, O'kelly J, Carnaghan RBA. 1961. Toxicity associated with certain samples of groundnuts. Nature 192:1096-1097.

Scientific Committee on Animal Nutrition (SCAN). 2000. Opinion of the Scientific Committee on Animal Nutrition on the Dioxin contamination of feedingstuffs and their contribution to the contamination of food of animal origin. Adopted 06 Nov 2000.

Schwertmann U, Taylor RM. 1989. Minerals in soil environments. Madison (WI): Soil Science Society of America. Iron oxides; p. 379-438.

Seifi B, Kadkhodaee M, Zahmatkesh M. Golab F, Bakhshi E. 2009. Changes in serum and renal vitamin E levels in deoxycorticosterone acetate–salt hypertensive rats. Transplantation P. 41:2910-2911.

Shank RC, Bourgeois CH, Keschamras N, Chandavimol P. 1971. Aflatoxin and autopsy specimens from Thai children with an acute disease of unknown aetiology. Food Cosmet Toxicol. 9:501-507.

Shotyk W, Nesbit HW. 1992. Incongruent and congruent dissolution of plagioclase feldspar:effect of feldspar composition and ligand complexation. Geoderma. 55: 55-78.

Shryock TR, Klink PR, Readnour RS, Tonkinson LV. 1994. Effect of bentonite incorporated in a feed ration with tilmicosin in the prevention of induced mycoplasma gallisepticum airsacculitis in broiler chickens. Avian Dis. 38:501-505.

Shupe T, Sell S. 2004. Low hepatic glutathione-S-transferase and increased hepatic DNA adduction contribute to tumorigenicity of aflatoxin B1 in newborn and partially hepatectomized mice. Toxicology Lett.148:1-9.

Singer A, Gal M, Banin A. 1972. Clay minerals in recent sediments of Lake Kinneret (Tiberias), Israel. Sediment Geol. 8:289-308.

Smela ME, Currier SS, Bailey EA, Essigmann JM. 2001. The chemistry and biology of aflatoxin B1: from mutational spectrometry to carcinogenesis. Carcinogenesis. 22:535–545.

Smith EE, Phillips TD, Ellis JA, Harvey RB, Kubena LF, Thompson J, Newton G. 1994. Dietary hydrated sodium calcium aluminosilicate reduction on aflatoxin M1 residue in dairy goat milk and effects on milk production and components. J Anim Sci. 72:677-682.

Soil Survey Staff. 1996. Soil survey laboratory methods manual. Soil Survey Investigations. Report No. 42. U. S. Govt. Printing Office, Washington, D. C.

Soukup DA, Buck BJ, Harris W. 2008. Methods of soil analysis, Part 5-Mineralogical Methods. Winsconsin: Soil Science Society of America Inc. Preparing soils for mineralogical analyses; p. 13-31.

Sparks DL. 1995. Environmental soil chemistry. San Diego (CA): Academic Press.

Stenske KA, Smith JR, Newman SJ, Newman LB, Kirk CA. 2006. Aflatoxicosis in dogs and dealing with suspected contaminated commercial foods. J Am Vet Med Assoc. 228:11.

Sun Z, Lu P, Gail MH, Pee D, Zhang Q, Ming L, Wang J, Liu G, Wu Y, Zhu Y. 1999. Increased risk of hepatocellular carcinoma in male hepatitis B surface antigen carriers with chronic hepatitis who have detectable urinary aflatoxin metabolite M1. Hepatology. 30:379-383.

Sunar F. Baltaci A. Ergene N, Mogulkoc R. 2009. Zinc deficiency and supplementation in ovariectomized rats: Their effect on serum estrogen and progesterone levels and their relation to calcium and phosphorus. Pak J Pharm Sci. 22(2):150-154.

Taylor JF, Robinson A, Johnson N, M arrowuin-Cardona A, Brattin B, Taylor R, Phillips TD. 2009. In vitro evaluation of ferrihydrite as an enterosorbent for arsenic from contaminated drinking water. Environ Sci Technol. 43(14): 5501–5506.

Tenorio-Arvide MG, Mulder I, Barrientos-Velazquez AL, Dixon JB. 2008. Smectite clay adsorption of aflatoxin vs. octahedral composition as indicated by FTIR. Clay clay Miner. 56:571-578.

Thompson ML, Ukrainczyk L. 2002. Soil Mineralogy with Environmental Applications. Madison (WI): Soil Sci Soc Am. Micas; p. 431-466.

Turner PC, Collinson AC, Cheung YB, Gong YY, Hall AJ, Prentice AM, Wild CP. 2007. Aflatoxin exposure in utero causes growth faltering in Gambian infants. Int J Epidemiol. 36:1119-1125.

Turner PC, Moore SE, Hall AJ, Prentice AM, Wild CP. 2003. Modification of immune function through exposure to dietary aflatoxin in Gambian children. Environ Health Persp. 111:217-220.

Ueno Y, Iijima K, Wang SD, Sugiura Y, Sekijima M, Tanaka T, Chen C, Yu SZ. 1997. Fumonisins as a possible contributory risk factor for primary liver cancer: A 3-year study of corn harvested in haimen, China, by HPLC and ELISA. Food Chem Toxicol. 35:1143-1150.

US Department of Agriculture, Natural Resources Conservation Service. 2007. National soil survey handbook. 430-VI. NSSH Part 618.38 Soil Properties and Qualities. Washington, DC: US Department of Agriculture. 15 March 2009. Available from: http://soils.usda.gov/technical/handbook/

US Pharmacopoeia. 2005. The official compendia of standards. USP 28 NF 23. Twinbrook Parkway: USP Convention Inc. p. 2961.

US Salinity Laboratory Staff. 1969. Diagnosis and improvement of saline and alkali soils (revised edition). Agriculture Handbook 60, USDA, U. S. Govt. Printing Office, Washington, D.C.

Vermeer DE, Ferrell RE. 1985. Nigerian geophagical clay: a traditional antidiarrheal pharmaceutical. Science. 227: 634–636.

Voss KA, Dorner JW, Cole RJ. 1993. Amelioration of aflatoxicosis in rats by Volclay NF-BC, microfine bentonite. J Food Prot. 56:595-598.

Wallwork JC. 1987. Appraisal of the methodology and applications for measurement of the zinc content of blood components as indicators of zinc status. Biol Trace Element Res. 12:335-350.

Wang JS, Shen X, He X, Zhu YR, Zhang BC, Wang JB, Qian GS, Kuang SY, Zarba A, Egner PA, Jacobson LP, Muñoz A, Helzlsouer KJ, Groopman JD, Kensler TW. 1999. Protective alterations of phase 1 and 2 metabolism of aflatoxin B1 by oltipraz in residents of Qidong, People's Republic of China. J Nat Cancer Inst. 91:347-354.

Wang P, Afriyie-Gyawu E, Tang Y, Johnson NM, Xu L, Tang L, Huebner HJ, Ankrah NA, Ofori-Adjei D, Ellis W, Jolly PE, Williams JH, Wang JS, Phillips TD. 2008. NovaSil clay intervention in Ghanaians at high risk for aflatoxicosis: II. Reduction in biomarkers of aflatoxin exposure in blood and urine. Food Addit Contam: Part A. 25:622-634.

Wedekind KJ, Baker DH. 1990. Zinc bioavallability in feed-grade sources of zinc. J Ani Sci. 68:684-689.

Weinmann ARM, Oliveira MS, Jorge SM, Martins AR. 1999. Simultaneous high-performance liquid chromatographic determination of retinol by fluorometry and of tocopherol by ultraviolet absorbance in the serum of newborns. J Chromatogr B 729: 231-236.

White GN, Dixon JB. Soil mineralogy with environmental applications. Madison (WI): Soil Science Society of America. Kaolin-Serpentine Minerals; p. 389-414.

Wilby OK. 1988. Proceedings of workshop organised by Association Francaise de Teratologie. The Hydra regeneration assay; p. 108–124.

Wild CP, Hall AJ. 2000. Primary prevention of hepatocellular carcinoma in developing countries. Mutat Res. 462:381-393.

Wild CP, Turner PC. 2002. The toxicology of aflatoxins as a basis for public health decisions. Mutagenesis. 17:471-481.

Wildman REC, Medeiros DM. 2000. Advanced human nutrition. Boca Raton (FL):CRC Press.

Wiley AS, Katz SH. 1998. Geophagy in pregnancy: a test of a hypothesis. Curr Anthropol. 39:532-545.

Williams JH, Phillips TD, Jolly PE, Stiles JK, Jolly CM, Aggarwal D. 2004. Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences, and interventions. Am J Clin Nutr. 80:1106-1122.

Wilson DM, Payne GA. 1994. The toxicology of aflatoxins: human health, veterinary, and agricultural significance. San Diego (CA): Academic Press, Inc. Factors affecting *Aspergillus flavus* group infection and aflatoxin contamination of crops; p. 309-325.

Wilson MJ. 2003. Clay mineralogical and related characteristics of geophagic materials. J Chem Ecol 29(7):1525-1547.

Wogan GN, Newberne PM. 1967. Dose-response characteristics of aflatoxin B1 carcinogenesis in rats. Cancer Res. 27:2370–2376.

Wolford ST, Schroer RA, Gohs FX, Gallo PP, Brodeck M, Falk HB, Ruhren R. 1986. Reference range data for serum chemistry and hematology values in laboratory animals. J Tox Env Health. 18:161-168.

Wong ZA, Hsieh DPH. 1980. The comparative metabolism and toxicokinetics of aflatoxin B1 in the monkey, rat, and mouse. Toxicol Appl Pharmacol. 55:115-125.

World Health Organization (WHO). 2005. Bentonite, kaolin, and selected clay minerals. Environmental health criteria 231. Geneva, Switzerland.

Yiannikouris A, Andre G, Poughon L, Francois J, Dussap CG, Jeminet G, Bertin G, Jouany JP. 2006. Chemical and conformational study of the interactions involved in mycotoxin complexation with ß-D-Glucans. Biomacromolecules. 7:1147-1155.

Zlotowski P, Correa AMR, Rozza DB, Driemeier D, Mallmann CA, Migliavacca FA. 2004. Swine aflatoxicosis outbreak in the state of Rio Grande do Sul, Brazil. Pesquisa Vet Brasil. 24:207-210.

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Marroquin-Cardona A, Deng Y, Garcia-Mazcorro JF, Johnson NM, Mitchell N, Tang L, Robinson A, Taylor JF, Wang JS, Phillips TD. 2011. Characterization and Safety of Uniform Particle Size NovaSil Clay as a Potential Aflatoxin Enterosorbent. Appl Clay Sci (submitted).

Marroquin-Cardona A, Deng Y, Garcia-Mazcorro JF, Johnson NM, Mitchell N, Tang L, Wang JS, Phillips TD. 2011. Toxicological Evaluation of Dietary Sodium Bentonite Clay as a Potential Aflatoxin Sorbent. In preparation.

Johnson NM, Qian G, Xu L, Tietze D, Marroquin-Cardona A, Robinson A, Rodriguez M, Kaufman L, Cunningham K, Wittmer J, Guerra F, Donnelly KC, Williams JH, Wang JS, Phillips TD. 2010. Aflatoxin and PAH exposure biomarkers in a U.S. population with a high incidence of hepatocellular carcinoma. Sci. Total Environ. 1; 408 (23): 6027-31.

Marroquin-Cardona A, Taylor JF, Hallmark CT, Deng Y, Johnson NM, Phillips TD. 2009. *In vitro* and *in vivo* characterization of mycotoxin-binding additives used for animal feeds in México. Food Addit Contam 26:733–743.

Johnson NM, Afriyie-Gyawu E, Huebner HJ, Marroquin-Cardona A, Robinson A, Tang L, Xu L, Ankrah NA, Ofori-Adjei D, Jolly PE, Williams JH, Wang JS, Phillips TD. 2009. PAH Exposure in a Ghanaian population at high risk for aflatoxicosis. Sci Total Environ 407:1886–1891.

Taylor JF, Robinson A, Johnson NM, Marroquin-Cardona A, Brattin B, Taylor R, Phillips TD. 2009. *In vitro* evaluation of ferrihydrite as an enterosorbent for arsenic from contaminated drinking water. Environ Sci Technol 43:5501–5506.

Phillips TD, Afriyie-Gyawu E, Williams JH, Huebner HJ, Ankrah NA, Ofori-Adjei D, Jolly PE, Johnson NM, Taylor JF, Marroquin-Cardona A, Xu L, Tang L, Wang JS. 2008. Reducing human exposure to aflatoxin through use of clay. Food Addit Contam 25:134–145.

PROFESIONAL AFILIATIONS: Society of Toxicology, American Association for the Advancement of Science