TEXAS BENTONITES AS AMENDMENTS OF AFLATOXIN-CONTAMINATED POULTRY FEED

A Thesis

by

ANA LUISA BARRIENTOS VELAZQUEZ

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

MASTER OF SCIENCE

May 2011

Major Subject: Soil Science

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Approved by:

Co-Chairs of Committee,	Youjun Deng
	Joe B. Dixon
Committee Member,	Christopher A. Bailey
Head of Department,	David Baltensperger

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ABSTRACT

Texas Bentonites as Amendments of
Aflatoxin-Contaminated Poultry Feed. (May 2011)
Ana Luisa Barrientos Velázquez, B.S., Autonomous University of Puebla, Mexico
Co-Chairs of Advisory Committee: Dr. Youjun Deng
Dr. Joe B. Dixon

Aflatoxins are toxic organic compounds produced by fungi in grains. Moderately contaminated grains that cannot be used as food are often directed to animal feed. Economically-feasible detoxification measures for contaminated feeds are needed. The objectives of this research were to identify effective bentonites as aflatoxin adsorbents and to evaluate the performance of the clays as aflatoxin amendments in feed for broiler chickens.

Five bentonite samples from Gonzales, Texas, USA were collected and analyzed against the published selection criteria for aflatoxin adsorbents: aflatoxin adsorption capacity, pH, cation exchange capacity (CEC), organic carbon, particle size distribution, and mineralogical and structural compositions. Two bentonites were identified as potentially good aflatoxin adsorbents based on the analyses. These two bentonites were selected for an in vivo poultry experiment where chickens were fed with aflatoxin-contaminated corn (1400 ppb) to test the detoxification efficacy of the clays. Detailed mineralogy analyses were conducted on these two samples (4TX and 1TX) after size fractionation. Clay 4TX and 1TX contained 87% and 65% clay, respectively. Smectite was the dominant mineral phase in both clay fractions. Quartz and feldspars were also present in both samples. These minerals are unlikely to cause harmful effects on the chickens. The presence of pyrite and heavy metals in 1TX raised concerns about its use in animal feed.

The clays were introduced into feed by mixing the dry bentonite powder with the feed for twelve minutes in a mechanical mixer. The body weight was increased by 21% with clay 4TX and 14% with clay 1TX in the aflatoxin diet. The concentration of total

aflatoxins in liver was reduced by 36% with the addition of clays. Liver visual appearance was also improved from pale red to a more reddish color resembling the healthy red liver. All chickens fed clean feed had significantly higher body weights than those fed with highly contaminated feed, suggesting that the clays did not completely eliminate aflatoxin toxicity.

The published aflatoxin binder selection criteria were useful for screening bentonites as aflatoxin amendments. The selected bentonites based on the criteria could effectively sequester aflatoxins in vivo. Yet direct mixing of bentonite as dry powder to highly contaminated poultry feed could not eliminate the toxicity of aflatoxins.

DEDICATION

To my parents, Dolores and Miguel

Your love and support made who I am today

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CHAPTER I
INTRODUCTION

It has been frequently reported that human and animal health was compromised by fungal contamination in food and feed. Those fungal species produce toxic organic molecules. *Mycotoxin* is the term designated for organic compounds produced by fungi that are toxic to humans and animals in small concentrations (Zain, 2011). The most common mycotoxins present in grains and cereals that represent a risk for animals and humans are: aflatoxins, deoxynivalenol (DON or vomitoxin), fumonisins, ochratoxin A (OTA), patulin, zearalenone (ZEA) (Basappa, 2009), and T2 toxins (Boudergue et al., 2009). Among these mycotoxins, aflatoxins represent the greatest toxicity for animal and humans. Intensive research on the characteristics and deleterious effects of aflatoxins on human and animal health started with the massive death of about 100, 000 turkeys in England in 1960. The first called "Turkey X" was later confirmed to be a result of ingestion of aflatoxin contaminated feed.

Warm and humid conditions promote the growth of *Aspergillus flavus* and *Aspergillus parasiticus* in corn and other grains (Eaton and Groopman, 1994). These fungal species produce aflatoxins (Heathcote and Hibbert, 1978). There are approximately 20 forms of aflatoxins (Basappa, 2009) and most of them are derivatives of the metabolism or transformation of aflatoxin B_1 (AfB_1). Aflatoxin B_1 represents the greatest concern due to its deleterious effects on animals and humans. It has the greatest toxicity among all aflatoxins, and therefore most research is focused on this mycotoxin. The International Agency for Research on Cancer (IARC) classified aflatoxins as Group I carcinogens to humans based on research that shows the potential of aflatoxins to induce liver cancer in humans and animals (Heinrich, 2003).

The Food and Drug Administration (FDA) has set regulatory levels to control exposure by establishing a maximum concentration of 20 ppb of aflatoxin in food.

This thesis follows the style of Soil Science Society of America Journal.

The FDA has also set regulatory levels of aflatoxins in feed (Rustom, 1997). The levels vary, depending on animals and age. Yet several investigations on different animals have shown that even at concentrations of 20 ppb, feed intake and milk production are reduced, and liver weight increased in beef cattle fed with 100 ppb aflatoxin in feed (Jouany and Diaz, 2005).

Several chemical, physical and biological techniques have been investigated to remove or degrade aflatoxins in grain. Addition of bentonite in aflatoxin contaminated feed had shown to be effective in reducing the impact of aflatoxin on animals. Bentonites adsorb aflatoxin molecules in the gastrointestinal tract (Phillips, 1999). There is extensive research on the adsorption effectiveness of bentonite in vivo and in vitro. However, results can be highly variable from in vitro to animal experiment, and from bentonite sources. Evaluation of several bentonite samples from different sources had shown a wide range in adsorption capacity attributed to the smectite clay properties (Kannewischer et al., 2006; Magnoli et al., 2008b). Yet there are not clear relationships in the bentonite properties that influence the aflatoxin adsorption capacity.

The purpose of this thesis is to provide a detailed investigation of the bentonite properties that can affect their aflatoxin adsorption capacity, and that can be used as a basis selecting effective in vivo adsorbents. The first study (Chapter III) focuses on the preliminary evaluation of bentonites from Texas collected at field sites as aflatoxin adsorbents according to published selection criteria. In this chapter the properties that influence the adsorption of aflatoxin are described. Two samples were selected for further studies based on the properties analyzed. The second study (Chapter IV) presents a detailed mineralogical characterization of the two selected samples from chapter III. The in vitro aflatoxin adsorption capacity was compared from the unfractionated bentonite and the clay fraction. Additionally it addresses the interlayer allocation of aflatoxin molecules and the stability of the aflatoxin-clay complex. The third study (chapter V) addresses the effectiveness of the two selected clays in detoxifying aflatoxins by evaluating chicken performance after adding the clays in aflatoxin contaminated diet.

CHAPTER II
LITERATURE REVIEW

Aflatoxin contamination is a common natural phenomenon that is difficult to avoid or control; and it can occur during pre and post harvest stages (Kabak et al., 2006). Late planting, crop spoilage by insects, heat stress and draught increase the risk of high levels of aflatoxins. Good management practices as early harvest, reduced humidity in grain, dry storage conditions, and insect management had shown to be effective in reducing the risk of aflatoxin contamination (Zain, 2011). Although the implementation of these techniques had shown to decrease the aflatoxin concentration, they cannot fully avoid contamination due to influence of environmental conditions and economical resources (Basappa, 2009).

Extensive research had been conducted in developing techniques to prevent crop contamination. A promising approach to control the aflatoxin contamination in the field is with strains of *Aspergillus flavus* that do not produce aflatoxins (atoxigenic) (Cotty and Bayman, 1993; Daigle and Cotty, 1995). The technique is based on competitive exclusion principle, where the nontoxigenic strains compete with indigenous toxic strains for crop substrates. The effectiveness of atoxigenic *Aspergillus* to displace toxic strains depends on the quantity and the delivery method (Dorner, 2004). Greenhouse experiments had shown significant reduction of aflatoxin production by the addition of atoxigenic *A. flavus* in cottonseeds (Cotty, 1990). It has been observed that timing of applying the aflatoxigenic strains is important: adding nontoxigenic strains was more effective in yielding low levels of aflatoxin when they were added one day before the addition of toxic strains or coinoculation. On the contrary, adding the atoxigenic strain after inoculation of toxic *Aspergillus* gave similar high aflatoxin concentrations as the toxigenic strains alone (Cotty, 1990). Field experiments had also been successful in controlling the levels of aflatoxins in maize (Brown et al., 1991). Brown et al., (1991) observed a range from 80 to 95% reduction in aflatoxin production by the addition of atoxigenic fungi. Their results were in agreement with those obtained by Cotty, (1990) in

controlled conditions. Furthermore they exposed the harvested grain to stress conditions during storage and found that effective preharvest suppression of aflatoxin also prevent high concentration during storage.

Reduction of aflatoxin in food and feed

Several biological, chemical, and physical techniques have been developed to either decontaminate or detoxify aflatoxins in food and feed. Decontamination methods remove toxins, while the purpose of detoxification is to inactivate, destroy, or degrade aflatoxins (Eaton and Groopman, 1994; Rustom, 1997; Basappa, 2009).

Biological detoxification

A wide range and extensive number of fungi, bacteria, and yeast had been investigated in their potential to degrade aflatoxins (Basappa, 2009; Boudergue et al., 2009). Some fungal species such as *Rhizopus, Aspergillus niger*, and protozoa *Tetrahymena pyriformis* can successfully degrade aflatoxin B1 (AfB1) (Mann and Rehm, 1975). These microorganisms are capable of transforming AfB1 to aflatoxicol (Teunisson et al., 1970), a compound that is 18 times less toxic than AfB1 (Doyle et al., 1982; Dorner and Cole, 1997). Despite the lower toxicity of aflatoxicol, its production may be undesirable because it can be highly toxic for some fish species like rainbow trout under acute toxicity. Additionally it can be as carcinogen as AfB1(Pawlowski et al., 1976) with about 70% of mutagenicity, but also aflatoxicol can be oxidized back to AfB1 (Eaton and Groopman, 1994).

A bacterium *Nocardia corynebacteroides* (formely *Flavobacterium aurantiacum*) effectively reduce the concentration of aflatoxin B1 in solution (Tejada-Castaneda et al., 2008). The in vivo experiment by Tejeda-Cataneda et al., (2008) showed that the feed treated with *N. corynebacteroide* had reduced aflatoxin concentration by 75%. Further ingestion of chickens fed with the bacterium-treated feed showed that the bacteria were not harmful to animals and that chicken´s performance was improved in comparison with chickens fed with the untreated aflatoxin diet. The

performance was not as good as chickens feed with aflatoxin-free diet. It has been reported that yeast *Saccharomyces cerevistae* were effective binder of aflatoxin B1 both in vivo and in vitro. Poultry experiments had shown that they improved the body and liver weights when live yeast was added at different concentrations (Boudergue et al., 2009).

Chemical detoxification

Certain chemicals such as oxidizing agents, acids, bases, and bisulfate can degrade aflatoxin molecules. While some authors consider these as the most practical approach (Bassapa, 2009), others emphasize that some methods can form toxic residues and alter the nutrient value in food and feed (Phillips et al., 1994). The effectiveness of any chemical treatment is achieve by the conversion of toxic compounds to less or non toxic ones without causing alterations of nutritious values of the products. Tthe majority of the chemicals tested are impractical and unsafe because they produce toxic residues, and also alter the flavor, odor or nutrient content.

Ammoniation of contaminated food has been extensively investigated. Several experiments on contaminated peanut, cottonseed meals and corn had demonstrated that the use of ammonium hydroxide or gaseous ammonia can reduce the concentration of aflatoxins up to 99% (Heathcote and Hibbert, 1978; Phillips et al., 1994; Basappa, 2009). The safety of the treated products had been demonstrated with chickens, trouts, and rats, and the impacts of aflatoxins where significantly reduced. All scientific evidences had led to the approval of this method by the Food and Drug Administration (FDA). The major disadvantages of this method are the need of infrastructure and the safety in handling the reactant (Bassapa, 2009).

Sodium bisulfate is commonly used to preserve food and beverages (Piva et al., 1995). It had shown to reduce the concentration of aflatoxin M1 in milk by 45% when added at 0.04g per 10 ml. It reacts with aflatoxin molecules to form an aflatoxin-sulfate complex (Bassapa, 2009). Yet, the toxicity of the complex has not been addressed (Phillips et al., 1994).

Physical detoxification

Thermal

A wide range of heating techniques had been investigated in their potential to reduce aflatoxin concentrations in a variety of products. Inconsistent results on the effectiveness of heating have been reported in the literature. Early studies on oilseed meals showed that up to 80 % decontamination was achieved by heating at 100°C for 2 hours at a moisture content of 2 % (Heathcote and Hibbert, 1978). Experiments on roasting procedures for peanut showed that a range from 43 to 83 % of reduction of aflatoxins can be achieved, and that the effectiveness was dependant on the temperature, time and moisture content (Heathcote and Hibbert, 1978). Moisture content was observed to have the greatest impact on the amount of degradation, high moisture was more effective in reducing aflatoxins concentrations (Doyle et al., 1982). On the other hand, common frying temperatures were not effective on decontaminating oils, because temperatures were below 200°C (Peers and Linsell, 1975). Aflatoxins are stable at most common cooking temperatures. Their melting point is 269°C, thus for some products high temperatures are required to decrease the aflatoxin concentration (Basappa, 2009). Autoclaving at temperature of 121°C showed a 95 % reduction in high humidity groundnut meal. The efficacy was tested in duckling by the evaluation of tissue damage and weight gain. On the contrary the similar autoclaving procedure in fruits and spices was not effective (Basappa, 2009).

Although some experiments had shown reduction of aflatoxins concentration in different products there is a concern of the quality alteration. Oils seed products could undergo oxidative degradation at high temperatures (Heathcote and Hibbert, 1978), and autoclaving can alter the nutrient value (Basappa, 2009).

Irradiation

Radiation with gamma rays and ultraviolet light had shown varied effectiveness in reduce aflatoxin levels in food. Aquino et al., (2005) showed that gamma irradiation on artificially contaminated maize samples, reduced the number of *Aspergillus flavus*

colonies and, therefore inhibited toxin production. With this method, high humidity was important to produce radicals that can interact with AfB1 molecules and form less toxic compounds (Aquino et al., 2005). On the other hand low dosage of gamma radiation had shown to increase the toxin production (Basappa, 2009). No alteration was observed in contaminated peanut meal after gamma radiation treatment (Phillips et al., 1994). Peanut meal exposed to 2.5 Mrad gamma radiation was not effective in protecting ducks of deleterious effects of aflatoxin. Similar results were obtained with UV light (Heathcote and Hibbert, 1978). Other studies suggested that UV was effective in reducing aflatoxin M1 concentrations in contaminated milk (Phillips et al., 1994).

Non-nutritive adsorbents

The addition of binders into aflatoxin contaminated feed to adsorb the toxic molecules had caused an increasing attention from scientists over the years. The adsorbents are added to the diet of exposed animals to prevent uptake and metabolismof the toxins (Phillips et al., 2002). Bentonites, zeolites and activated carbon are the most studied aflatoxin adsorbents.

Bentonite is the terminology used to describe the clay rock material composed mainly of montmorillonite clay (Eisenhour and Brown, 2009) with the presence of common impurities such as quartz, feldspars and other minerals. Montmorillonite is a phyllosilicate mineral belonging to the smectite group (Reid-Soukup and Ulery, 2002). Smectites have a structural layer formed by two tetrahedral sheets and one octahedral sheet that was sandwiched by the tetrahedral sheets. Montmorillonite contains predominantely Al^{3+} in the octahedral positions with some substitution by Fe^{3+} and Mg^{2+}. The tetrahedral sheets are dominated by Si^{4+}. The unbalance charges that arise from the isomorphic substitutions give an overall negative charge to montmorillonites. The charge is compensated by exchangeable cations between the layers. This interlayer space is highly expandable depending on hydration and cation valence. The high surface area (~800 m^2/g) and the expandable structure are important properties in smectites that allow for a wide range of applications.

Montmorillonite can adsorb high concentrations of aflatoxins from aqueous solutions. Adsorption isotherms are used to determine the maximum binding capacity of the clays (Phillips et al., 1988). Phillips et al., (1988) showed the strong stability of the adsorbed aflatoxin. Less than 10% of the bound aflatoxin was removed after washing with water, acetonitrile, methanol, acetone, chloroform, benzene, or toluene.

The in vitro adsorption capacity serves as a basis to predict whether a binder can be effective when introduced in animal experiments. Several poultry experiments had shown the efficacy of bentonite clays (Marquez Marquez, 1995; Rosa et al., 2001; Miazzo et al., 2005b; Magnoli et al., 2008a; Magnoli et al., 2008b; Kermanshashi et al., 2009) and a commercial HSCAS (Novasil) (Davidson et al., 1987; Kubena et al., 1992; Kubena et al., 1993b; Southern et al., 1994; Phillips et al., 1995; Edrington et al., 1996; Bailey et al., 1998; Kubena et al., 1998; Bailey et al., 2006) in reducing the negative effects of aflatoxins. The efficacy of the binders was reflected on the improvement in body weight, feed conversion ratio, and relative organ weight. Additionally some researches had evaluated blood chemistry and histological tissue alteration. The adsorbents do not significantly interfere with nutrient utilization. For example, vitamin A, riboflavin and zinc levels are not affected (Phillips et al., 2002). In similar experiments by Phillips et al., (2002) it was demonstrated that a 0.5% of clay addition was sufficient to improve animal performance.

Zeolites are aluminosilicate minerals with a three dimensional framework structure where the channels and cages are interconnected. Their structure is formed of primary units of silica (SiO_4^{4-}) and aluminum (AlO_4^{5-}) tetrahedra. The different arrangement of these tetrahedra causes differences in channel size and structure that form the different types of zeolites (Boettinger and Ming, 2002). These minerals are characteristic of high cation exchange capacity (CEC) (ranging from 220 cmol/kg to 420 cmol/kg) that arises from the substitutions of $Si4^+$ by Al^{3+}. The charge deficiency is compensated by cations held in the channels and cages. Unlike smecties, zeolites cannot expand substantially upon hydration. The channels have defined dimension that are not affected by hydration or exchangeable cations. Natural and synthetic zeolites had been

used widely for many purposes from removal of organic compound from waste water, adsorption of radioactive elements, and in animal feed to improve body weight, feed utilization (Boettinger and Ming, 2002).

The most commonly used zeolite is clinoptilolite. Tomasevic-Canovic et al., (1994) showed that this mineral can absorb up to 80% of aflatoxin B1 from aqueous solution. In their experiment the adsorption was measured at different time spanning from 5 min to 48 hours. Their observation suggests that the initial adsorption reaction fast but slowed down later. Additionally they conclude that the aflatoxin-clinoptilolite complex was stable and that less than 10% of aflatoxin could be desorbed. Similar results were confirmed later (Dakovic et al., 2000).

The addition of clinoptilolite to aflatoxin contaminated feed of chickens had shown effectiveness in alleviating the negative impact of aflatoxins (Scheideler, 1993). Ozguz et al., (2000) also observed that chickens fed aflatoxin (2.5 mg of aflatoxin/kg of feed) plus clinoptilolite (1.5 and 2.5 %) diet improved the inorganic P level in which was severely reduced by the aflatoxin. Additionally, applying clinoptilolite in the aflatoxin-contaminated diet recovered serum uric acid levels to the control group. Similar results for serum uric acid were obtained by Harvey et al., (1993). In that experiment clinoptilolite was added at 0.5% to a 3.5 mg of aflatoxin/ kg of feed diet. The addition of zeolites to the aflatoxin feed improved the body weight. The efficacy of the samples was variable. One sample showed body weight improvement of 41% which is a modest adsorption compared with the high efficiency (>60%) observed by a hydrated calcium sodium alumino silicate (Harvey et al., 1993). An important observation was that there was a correlation in the adsorption efficacy from in vitro to in vivo experiments.

Organozeolites, clinoptilolite treated with surfactant octadecyldimethylbenzyl ammonium (ODMBA), effectively adsorbed mycotoxins as zearalenone and ochratoxin A but not aflatoxins (Dakovic et al., 2005). As the clinoptilolite surface became more saturated with the organic cations, the adsorption of aflatoxin decreased. This indicated that the major adsorption sites in zeolites are at the external and not in the channels. This is in agreement with the prediction that aflatoxins molecules cannot be adsorbed in the

channels due to their larger size (Dakovic et al., 2005). Boettinger and Ming, (2002) had reported that common channel dimensions for clinoptilolite are 4.1x4.7 Å, 4.4x7.2 Å, and 4.0x5.5 Å. The aflatoxin B1 molecules size is 5.18 Å (Bassapa, 2009).

Even though the incorporation of binders, such as montmorillonite in feed, had significantly reduced the deleterious effects of aflatoxicosis in several animal experiments, the adsorbent did not have a complete protective effect because animal performance did not recovered to the same level as the control groups fed with aflatoxin-free diets. This could be due to the high levels of aflatoxins needed in the feed to compromise the animal health to measure the indicators as body weight, feed conversion ratio, and relative organ weight.

CHAPTER III
PRELIMINARY SCREENING OF FIVE NATURAL TEXAS BENTONITES AS AFLATOXIN ADSORBENTS

Introduction

Aflatoxins are the most commonly occurring mycotoxins in grains. This mycotoxin group is the most widely studied as it represents a health hazard to animals and humans. Many animal experiments have shown that they were capable of inducing liver cancer and compromising the immune system (among other effects) (Dvorackova, 1990).

Detoxification techniques of aflatoxin-contaminated grains include several physical, chemical, and biological methods. Adding clays to animal feeds is and effective and low cost procedure in reducing the bioavailability of aflatoxins in grains. The reduction of aflatoxin impact on animals suggested that adsorption of toxins by the clay did occur in the gastrointestinal tract (Phillips et al., 1988).

Over the last 20 years, several animal experiments have been performed to test the efficacy of commercial and natural bentonites in binding aflatoxin through the evaluation of animal performance (Ramos et al., 1996; Bailey et al., 2006). The experiments have consistently shown reduction of aflatoxin bioavailability to animals by including montmorillonite in the diet. Even though animal experiment results had demonstrated the efficacy of montmorillonite, there was also large variation in the in vivo protection capacity among samples (Pimpukdee et al., 2004; Magnoli et al., 2008b).

Kannewicher etal., (2006) investigated the in vitro aflatoxin adsorption capacity of 20 bentonite samples. They observed that their AfB_1 adsorption capacity varied from 1.8 to 21.1 % by mass. It has been demonstrated that the major site for aflatoxin adsorption is the interlayer space in smectites (Grant and Phillips, 1998; Kannewischer et al., 2006; Deng et al., 2010) and, the accessibility of aflatoxin molecules can be affected by many physical and chemical properties of the samples. In the study by Kannewicher etal., (2006), the adsorption capacity was not linearly correlated to one

single property such as cation exchange capacity (CEC), pH, coefficient of linear extensibility (COLE), or organic carbon content. Their observations were in agreement with others. (Vekiru et al., 2007) had tested a larger number of samples for aflatoxin adsorption and had observed the diversity in their adsorption capacity. They also observed the clay properties such as smectite content, CEC or structural composition did not show any correlation with aflatoxin binding capacities.

(Mulder et al., 2008) showed a linear correlation (R^2 = 0.73) between particle size distribution measured by laser diffraction among 12 bentonites with their aflatoxin adsorption capacity. This indicates that particle size can partially explain the differences in adsorption effectiveness. High clay content is preferable as the adsorbing montmorillonite accumulates in this fraction. Magnoli et al., (2008) proposed that there is an influence of the charge density from the isomorphic substitutions in the montmorillonite structure with the adsorption of aflatoxin.

Despite the lack of strong correlations among the investigated montmorillonite characteristics and aflatoxin B_1 adsorption capacity, several physical, chemical, and mineralogical properties had been recognized in influencing the amount of mycotoxin that can be adsorbed. Dixon et al., (2008) proposed bentonite selection criteria as aflatoxin binders, which were based on the investigation of a set of 20 bentonites. The selection criteria use seven properties to determine if the sample has the potential to be a good adsorbent when introduced in animal feed.

The main objective of the present study was to identify potential effective Texas bentonites as aflatoxin adsorbents based on the selection criteria. Specific objectives were 1) to compare physical, chemical and mineralogical properties of the bentonites and correlate them with their adsorption efficacy, and 2) to fine tune the methods proposed in the selection criteria.

Materials and methods

Five samples were collected from commercial bentonite pits of Southern clay Products Inc. in Fayette and Gonzales counties, Texas. The samples were labeled as follows based on color and hardness that used in field classifications: 1TX (blue clay), 2TX (white soft clay), 3TX (pink-white clay), 4TX (yellow clay) and 5TX (brown clay). Color and hardness were used as primary classification criteria according to field engineers from Southern Clay Products, Inc. Samples 4TX and 5TX were taken from the same mine site; sample 5TX was a clay layer above sample 4TX. Samples 2TX and 3TX were from the same pit but they mainly differ in hardness, sample 2TX is softer than sample 3TX.

The samples were air dried and mechanically ground to pass a 2 mm sieve. No other treatment was performed on these samples. Unfractionated materials were used for all analyses. According to the selection criteria proposed by Dixon et al., (2008), seven properties were analyzed to evaluate the potential of the bentonites as aflatoxin adsorbents.

Aflatoxin adsorption isotherms

We followed the procedure described by Kannewischer et al., (2006) to determine aflatoxin adsorption isotherms. Clay suspensions were prepared by mixing 0.01 g of bentonite sample with 5 ml of distilled water and then vortexing for 2 minutes. An 8 ppm aflatoxin solution was prepared by diluting a 1000 ppm aflatoxin B_1 /acetonitrile stock solution with deionized water. The aflatoxin was purchased from Sigma Chemical. A series of working aflatoxin solutions, with concentrations varied as 0.0, 0.4, 1.6, 3.2, 4.8, 6.4, and 8.0 ppm, were prepared by mixing deionized water with the 8 ppm aflatoxin solution with desired volume ratios. An aliquot of 50 µl bentonite suspension, containing 0.1 mg bentonite, and one of the working aflatoxin solutions were added to a 15-mL polypropylene centrifuge tube. After ~24-hr shaking on a rotary shaker at 200 motions per minute, the samples were centrifuged at 4500 rpm (5443.2 g) for 57 min with IEC PR-7000 centrifuge. Aflatoxin concentration in the supernatant was

quantified based on the absorbance at 365 nm with a Beckman Coulter DU 800 UV-visible spectrophotometer. The isotherms were fitted to Langmuir equations using optimization program LMMpro (v.1.6) by Alfisol, LLC.

Cation exchange capacity (CEC), organic carbon content and pH

These analyses were conducted according to the procedure described in the Soil Survey Laboratory Manual (Staff, 2004). Each sample was duplicated during the analysis and the averaged values were reported.

Mineralogical composition by X-Ray diffraction (XRD)

The samples were ground in an agate mortar to obtain a uniform particle size and then front loaded in sample holders. The patterns were recorded from 3 to 70 degrees two-theta using a D8 BRUKER ADVANCE diffractometer. The CuKα radiation and LynxEye detector were used during the recording.

Smectite octahedral composition by Fourier Transform infrared (FTIR)

About 0.01 g of each sample and 0.3 g of KBr (reagent grade) was mixed for 30 seconds using a Wig-L-Bug mixer. The FTIR spectra were recorded with diffuse reflectance infrared transform (DRIFT) accessory on a Perkin-Elmer Spectrum 100 spectrometer. An average of 128 scans at a resolution of 4 cm^{-1} was used for each sample spectrum.

Particle size by Laser Diffraction Particle Size Analysis (LDPSA)

About 0.1 g sample was dispersed in 10 ml sodium hexametaphosphate solution (Na-hmp) by overnight shaking. Particle size distribution histograms were obtained using a Small Volume model LS230 particle size analyzer from Beckman Coulter. The sample suspension was sonicated inside the instrument chamber and a particle size distribution histogram was collected. The sonication and histogram collection were

repeated three times for each suspension. The averaged histogram was used as one measurement. Each sample was measured twice on the particle size analyzer.

Results and discussion

Aflatoxin adsorption capacity

The adsorption isotherms (Figure 3.1) of the aflatoxin-smectite adsorbent reaction had the L2 shape described by Grant and Phillips (1998). They described the Langmuir model as monolayer adsorption of aflatoxin on smectite surfaces. The class L2 indicates that the adsorption has reached a maximum where all the adsorption sites are saturated.

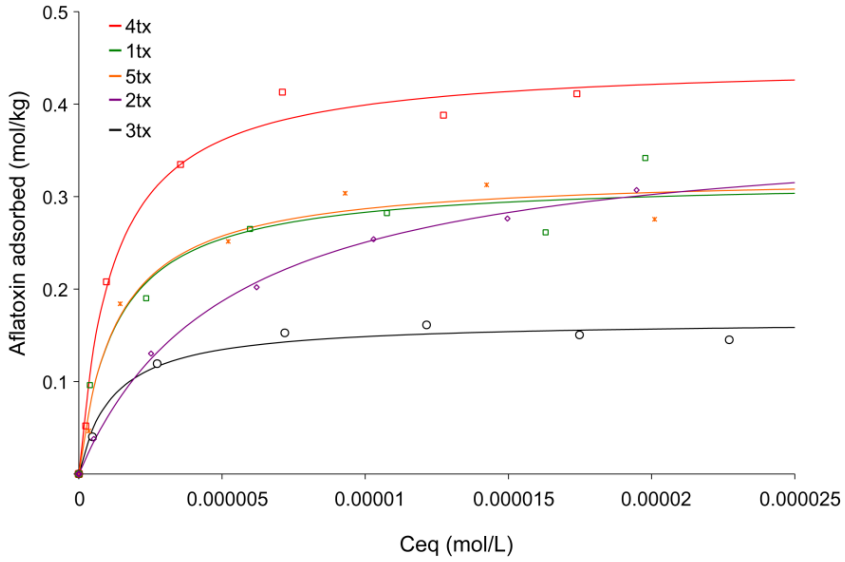

Figure 3.1 AfB_1 adsorption isotherms. Solid lines represent the Langmuir fitted data and symbols represent experimental data.

The maximum aflatoxin adsorption capacity (Qmax) of the five bentonites varied from 0.16 to 0.45 mol/kg (Table 3.1). Sample 4TX showed the highest adsorption capacity (13.9 % by mass). Samples 1TX, 5TX and 3TX were close in their adsorption capacities as illustrated in the adsorption isotherms (Figure 3.1). Numerically sample 1TX and 5TX adsorbed about 10% of aflatoxin by mass, and 2TX had slightly higher adsorption (Table 3.1). The poorest performance was by sample 3TX that adsorbed 4.5 % by mass.

Table 3.1. AfB_1 maximum adsorption capacity.

Sample	**K_d**	**AfB_1 (mol/kg)**	**η^2**	**AfB_1 adsorbed (% wt)**
4TX	8.54×10^5	0.45	0.980	13.9
1TX	7.85×10^5	0.32	0.897	9.9
5TX	7.69×10^5	0.32	0.956	10.1
2TX	1.93×10^5	0.38	0.992	11.8
3TX	8.57×10^5	0.16	0.955	4.5

The high adsorption capacity of sample 4TX was supported by the affinity value (K_d) (Table 3.1). Similar case was observed for samples 1TX and 5TX where the lower adsorption capacity, compared with sample 4TX, was also described by lower K_d values. However, in sample 2TX the affinity was the lowest indicating that interlayer adsorption was more restricted. This difference was well described by the adsorption isotherms (Figure 3.1). On the other hand, sample 3TX showed the lowest adsorption capacity but the affinity value was close to the best adsorbent of this set of samples. The low adsorption capacity can be a result of the smectite dilution due to presence of other minerals in the bentonite. The sample could be improved by concentrating the smectite.

According to the selection criteria by Dixon et al., (2008) the samples with a maximum adsorption capacity greater than 0.30 mol/kg (9.4 % by wt) can be consider

as good potential adsorbents of aflatoxin. Four of the samples in this group have met this criterion (Table 3.1).

One commercial hydrated sodium calcium aluminosilicate NovaSil has been extensively studied and shown to be effective both in vitro and in vivo. The maximum adsorption capacity reported for this sample was 0.461 mol/kg (Grant and Phillips, 1998), and sample 4TX's adsorption capacity was close to this value.

Physicochemical characterization

Cation exchange capacities (Table 3.2) of the samples fell in the typical values ranged from 70 to 130 cmol/kg of smectites (Bergaya et al., 2006). Sample 4TX and 2TX had the highest CEC values of 81.4 and 80.9 cmol/kg, respectively. Sample 1TX had a CEC of 74.5 cmol/kg followed by 5TX (70.9 cmol/kg). The lowest CEC was observed for sample 3TX (66.9 cmol/kg). The CEC values were used as an indication of the amount of smectite in the samples. Other properties such as presence of other minerals influence the CEC too. Sample 4TX had the highest CEC, an indication of the highest content of smectite in the samples, which was supported by its high aflatoxin B1 adsorption capacity result. On the contrary, the low CEC in sample 3TX could be explained as smectite being diluted by the presence of other minerals. According to the selection criteria, the CEC of high-performance bentonites should be about 70 cmol/ kg, This value was selected because Ca-bentonites are preferred for aflatoxin adsorbents over Na-montmorillonites (Dixon et al., 2008) and Ca-bentonites had CEC ranging from 40 to 70 cmol/kg (Murray, 2006) and Na-bentonites usually have CEC in the range from 80 to 130 cmol/ kg.

Table 3.2 Chemical characterization data.

Sample	CEC (cmol (+)/100g)	Org C (%)	pH
4TX	81.4	0.07	6.3
1TX	74.5	0.13	5.6
5TX	70.9	0.07	6.5
2TX	80.9	0.08	4.6
3TX	66.9	0.05	5.1

The organic carbon contents in the samples were very low (Table 3.2). The low content of organics in the samples suggests that the CEC of the samples were mainly due to smectite, and therefore, the CEC could be used as an estimation of smectites. Also, previous research had shown that high-organic samples performed poorly as aflatoxin adsorbents (Kannewischer et al., 2006). This was likely that the organic compounds can compete with aflatoxin molecules for adsorption sites.

Samples 4TX and 5TX, which came from the same mine site, had similar slightly acidic pH. The acidity in sample 1TX was expected due to the presence of pyrite. Acidic pH was observed for samples 2TX and 3TX, and sample 2TX was the most acidic specimen. The selection criteria suggest that strong acidity in the samples is a negative factor in aflatoxin adsorption as it promotes Al^{+3} mobilization and hydroxyl formation to adsorption sites making them unavailable for aflatoxin molecules (Dixon et al., 2008).

Mineralogical composition

The strong peaks in the XRD patterns at ~15 Å (Figure 3.2) of the untreated samples at room humidity (70%) suggest that the smectites had two layers of water in the interlayer space, an indicator of Ca occupancy at the exchange sites. If the exchange sites were occupied by Na, the smectite would have shown a lower d-spacing at ~12 Å. Other XRD peaks of smectite were observed at 5.1 Å (003), 4.47 Å (020), 2.98 Å (005), 2.56 Å (130) and 1.69 Å (009). The peak at 1.49 Å (060) suggests that the smectites were dioctahedral smectite in which Al^{+3} is the major cation in the octahedral sheet.

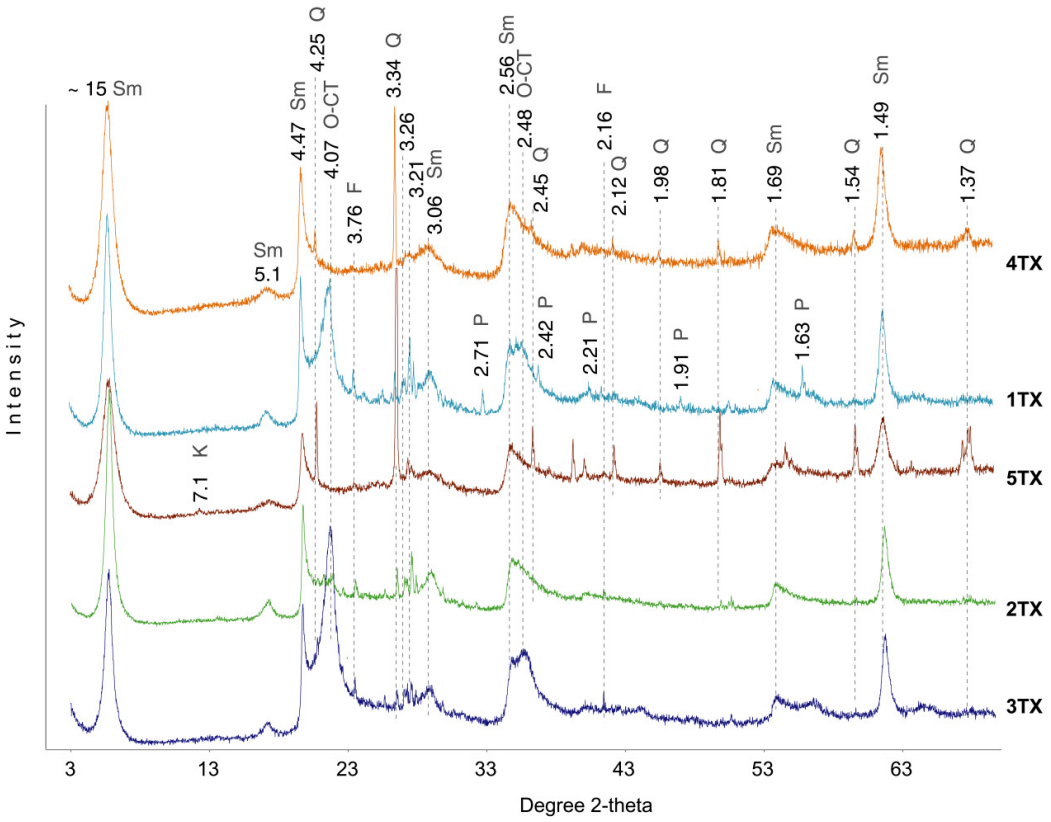

Figure 3.2: XRD diffraction patterns of the five samples arranged in decreasing adsorption capacity. Sm:smectite; Q:quartz; F: feldspars; O-CT: opal-CT; P: pyrite.

Quartz was a common diluent in all samples. Sharp reflections of quartz at d-spacing of 3.33, 4.25, 2.45, 2.12, 1.98, 1.81, 1.54 and 1.37 Å were observed in samples 4TX and 5TX. Similar reflections with weaker intensity were observed in samples 1TX and 3TX. Quartz peaks in sample 2TX was low. The strong peak at 4.07 Å indicates the presences of opal-CT in samples 1TX and 3TX (Chipera and Bish, 2001). Less amount of opal-CT was present in sample 2TX as well. The samples also contained feldspars. The sharp reflections at 3.26, 3.21 and 2.16 Å were attributed to sanidine. The peaks at 3.26 -3.13 Å were common for several feldspars but the small sharp reflection at 3.46 Å confirmed the presence of albite in samples 2TX and 1TX. Sample 1TX also contained pyrite as indicated by the reflections at 2.71, 2.42, 2.21, 1.91 and 1.63 Å. In the XRD

pattern of sample 5TX, the small sharp reflection at 7.1 Å suggested the presence of kaolinite.

The presence of quartz, feldspars, and opal-CT in the samples reduced the concentration of the reactive montmorillonite, thus reduced the aflatoxin adsorption capacity of each sample. For example 4TX had the greatest adsorption of aflatoxin and the sample contained a small amount of quartz. In contrast 3TX, which had low aflatoxin adsorption, had much more abundant opal-CT and feldspars.

Octahedral sheet cation composition

Octahedral cation composition in smectites can be distinguished by their infrared band positions since the vibrations were influenced by the cations in their structures. In montmorillonite, Al^{3+} is the dominant ion in the octahedral sheet with substituting Mg^{2+}, Fe^{3+} can also occur in the octahedral sheet. The tetrahedral sheet cations are Si^{4+} and Al^{3+} mostly (Reid-Soukup and Ulery, 2002).

All samples showed a band at 3628 cm^{-1} attributed to the stretching vibrations of the structural OH groups. The band position was determined by the major cations in the octahedral sheet. In montmorillonite, this band position indicates the dominance of Al^{+3}. Another band also produced by all five samples at 917cm^{-1} represents the bending vibrations of AlAl-OH (figure 3.3), supporting the dominant aluminum occupancy in the octahedral (Madejova and Komadel, 2001).

Sample 5TX had an additional shoulder (3699 cm-1) in the OH stretching region that was attributed to kaolinite in the sample (Madejova and Komadel, 2001). This was in agreement with the XRD data that had a small peak at 7.1 Å (figure 3.2). The band was due to the in-phase vibration of inner surface OH groups hydrogen bonded to the tetrahedral oxygen from the next layer (Madejová, 2003).

The five samples had an 840 cm^{-1} band (figure 3.3), which was attributed to the bending vibration of MgAlOH caused by Mg^{2+} isomorphic substitutions, and the intensity was different among the samples. Samples 1TX and 2TX had the greatest intensity of this band indicating that the amount of Mg^{2+} in the octahedral sheet is greater

in comparison with less intense bands in 4TX and 5TX (Gates, 2005). The major difference in terms of octahedral structural composition was the presence of Fe^{3+} in samples 4TX and 5TX. These two samples had the band at 880 cm^{-1} due to the bending vibration of $Al^{3+}Fe^{3+}$ – OH (figure 3.3). This band was absent in the other three samples. Tenorio et al., (2008) observed that bentonite samples with high adsorption capacity had structural Fe^{3+}, which was attributed to the weathering stage of the clays. This was suggested to be an indicator of good performing aflatoxin adsorbents (Dixon et al., 2008). Magnoli et al., (2008) quantified the octahedral iron and magnesium content in three bentonite samples that differed in aflatoxin adsorption. They observed that the samples with more octahedral Fe^{+3} were the better adsorbents. Also, they reported that surface charge could be related to adsorption. The high CEC for those samples can be a negative factor as highly charged montmorillonites and vermiculite had shown low aflatoxin adsorption (Tenorio Arvide et al., 2008). Contrary observations have been reported too. Vekiru et al., (2007) screened 61 bentonite samples from different sources and concluded that there was no relationship between the octahedral substitutions and the adsorption capacity. The correlation between octahedral iron content and the aflatoxin adsorption need to be studied further.

The infrared spectra of samples 1TX, 2T X and 3TX showed bands at 792 cm^{-1}, which was attributed to non crystalline silica (figure 3.3). This band and the 628 cm^{-1} band indicate the presence of opal-CT (Madejova and Komadel, 2001) which was supported by broad XRD peaks at 4.07 Å. Moreover, samples 1TX and 3TX had stronger opal-CT reflections at 4.07 Å on the XRD patterns than 2TX, and a similar intensity trend in infrared band of opal-CT was observed among the three samples. .

For samples 4TX and 5TX, the bands at 798 and 778 cm^{-1} were due to quartz, along with its weak band at 695 cm^{-1} (figure 3.3). These two samples showed a broad band at 621 cm^{-1} attributed to coupled Al-O and Si-O (Madejova and Komadel, 2001).

The band at 521 cm^{-1} originated from bending of the tetrahedral and octahedral cations (Si -O- Al). The predominance of Si^{+4} in the tetrahedral sheets produced a bending vibration at 466 cm^{-1} (Madejová, 2003).

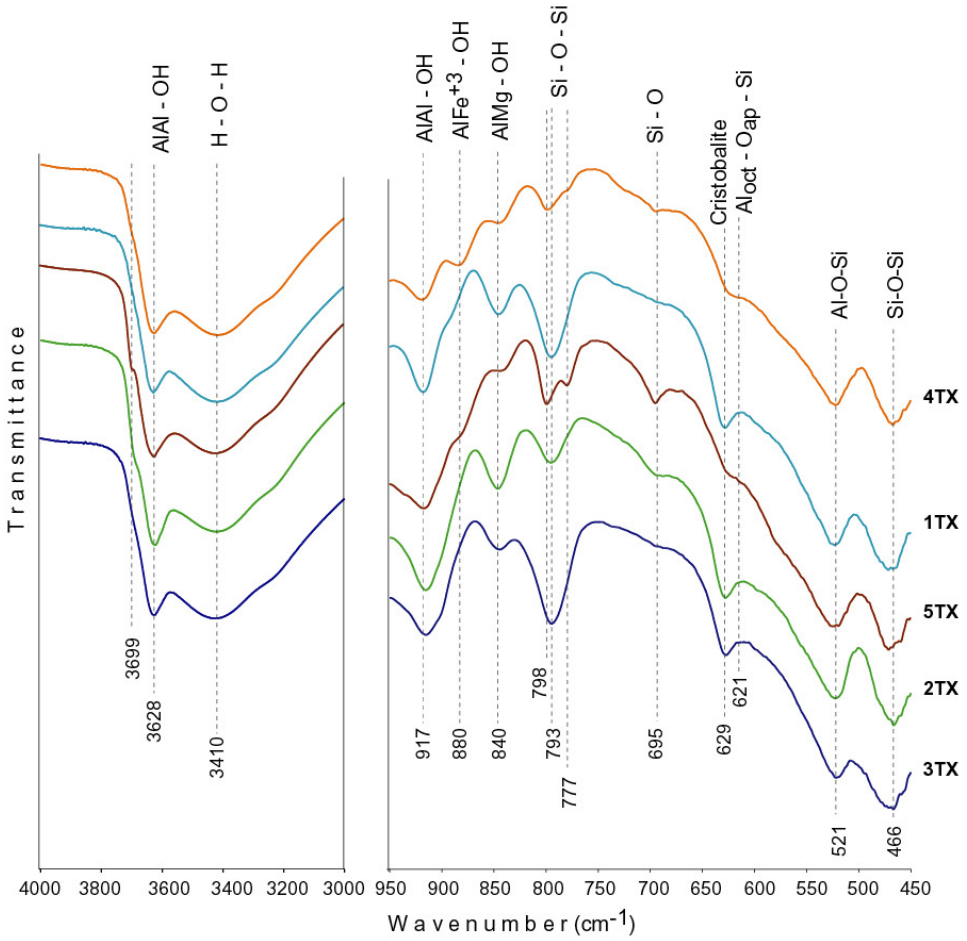

Figure 3.3: FTIR spectra of the samples showing bands for montmorillonite and diluents.

Particle size distribution

Particle size distribution measurements were strongly influenced by the dispersion status of the samples. Samples mixed with Na-hmp solution showed similar low percentages of clay fraction but large difference in the contents of sand particles (Figure 3.4a). Additional mechanical dispersion with ultrasonic probe improved dispersion, increasing the clay particles content in samples 4TX and 5TX (Figure 3.4b). The mechanical fractionation did not change the histograms of samples 1TX, 2TX or 3TX. This could indicate strongly aggregated particles and/or higher coarse particle content.

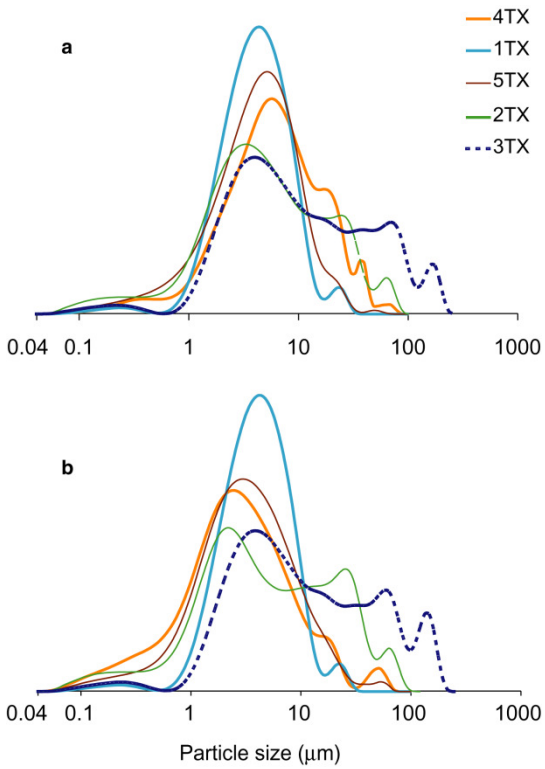

Figure 3.4: Particle size distribution of samples dispersed in a) Na-hmp solution and b) Na-hmp solution with mechanical dispersion.

Strong aggregation was more likely for samples 1TX and 2TX based on the CEC data and their high adsorption capacities, which indicate high smectite content. On the other hand, sample 3TX may be composed of coarse material because CEC was slightly lower and it did not perform as well as an adsorbent of aflatoxin.

Table 3.3 Particle size distribution data from histograms of figure 3.4. Mode indicates the particle size most frequent in the histograms.

Sample	Range of particle size		Mode (µm)	< 2 µm (%)	2-20 µ m (%)
	min (µm)	max (µm)			
4TX	0.05	80	2.5	38.4	57.4
1TX	0.06	35	4.4	15.6	82.5
5TX	0.06	80	3.0	31.1	65.7
2TX	0.05	116	2.3	27.8	52.4
3TX	0.05	825	4.4	8.6	47.5

Sample 4TX had the highest clay content, about 38% being the major particle size at 2.5 µm. The next sample high in clay content was 5TX with approximately 31 % but the greatest amount of particle size was at 3 µm. Sample 2TX showed clay content of 27.8% and similar particle size frequency to 4TX (Table 3.3). The high clay content appears to be related to the adsorption capacity of the samples. The exception was 1TX with low clay content (15.6 %) in comparison with the other 3 previous samples. Sample 3TX was the lowest in clay with only 8.6 %, which relates to the low adsorption capacity and low CEC.

The particle size distribution measured by laser diffraction had the advantage of being a fast method that requires small amounts of sample in comparison with traditional gravimetric methods. However, Eshel et al., (2004) stated that both techniques had limitations and results gravimetric and laser diffraction techniques differ. The morphology of the samples influences the determination of the particle frequency by the laser diffraction technique (Eshel et al., 2004). Samples that contain montmorillonite with platy morphology shape affect the mathematical functions on which the laser data are calculated because it considers the particles as spheres. The morphology can cause an overestimation of the particle size. Also, the particle size distribution histograms can be shifted by a combination of aggregation and underestimation due to particle shape.

Conclusions

The interaction of AfB_1 molecules with smectite clays was influenced by multiple bentonites properties. In the present set of samples particle size and montmorillonite concentration were the major properties that were correlated with the adsorption capacity.

Current and previous observations on bentonites demonstrate that even though all bentonites samples contained predominantly smectites their aflatoxin adsorption capacities differed substantially. This emphasizes the importance of evaluating the properties of the clays to assure adequate effectiveness before they are introduced in animal feed.

This study suggests that there are potential bentonites clays in Texas that can effectively adsorb aflatoxin. These sources are near to the areas where high levels of aflatoxin contamination in corn occur. These bentonites can be used to protect animals exposed to contaminated feed thus to reduce the risk of decreasing productivity and aflatoxin residues in feed and food products. State approval of amendment to feed is feasible and can be an effective means of reducing animal production costs, of saving grain that currently may be destroyed, and of improving animal's performance after feeding the contaminated diet.

Based on these evaluations, two samples 4TX and 1TX that showed high adsorption capacity and contrasting properties were selected for further detailed characterization and evaluation in a poultry experiment.

CHAPTER IV
CHARACTERIZATION OF SELECTED COMMERCIAL BENTONITE SAMPLES FOR AFLATOXIN ADSORBENTS

Introduction

Natural bentonites and various biological and organic commercial products have been tested in aflatoxin binding experiments. Due to the cost and maintenance of animal feed experiments, most of these products were tested only in vitro and their performance in vivo was uncertain. Detailed mineralogical and chemical characterization of these aflatoxin binders can help to understand the variation in adsorption effectiveness observed in animal experiments (Pasha et al., 2007) and therefore, to further narrow down the selections of screened binding candidates for animal feed experiments. It is montmorillonite that plays the most important role in binding aflatoxin by bentonites. Natural and commercially processed bentonites may contain ingredients such as organic compounds that can interfere the adsorption of aflatoxins, or reduce the effectiveness of montmorillonite by dilution effect.

The evaluation of five field samples from Gonzalez, Texas as aflatoxin adsorbents (Chapter III) using the selection criteria proposed by Dixon et al., (2008) led the selection of two samples (4TX and 1TX) for a poultry experiment. These two samples showed high adsorption capacity in vitro, and 4TX had higher adsorption capacity than 1TX. To explain the differences in their aflatoxin adsorption potentials, and their possible adverse health effects, the mineralogy of these two selected samples was characterized in detail.

The specific objectives of this study were: (1) to perform detailed characterization of the mineral components in the samples (2) to confirm the interlayer adsorption of aflatoxin in the smectites, (3) to quantify the stability of the adsorbed aflatoxin by desorption measurements, and (4) determine the properties of the samples that could influence their adsorption capacity.

Considering the fact that commercial bentonites used in feeding industry are dried and ground to fine powders, we obtained two commercial bentonites from Southern Clay Products Inc. (Gonzales, Texas). The two commercial samples originated from the same pits as the field sampled bentonites 4TX and 1TX reported in Chapter III)., the processed commercial products were more uniform in color and in fineness. As they came from the same pits as 4TX and 1TX, they were still labeled as 4TX and 1TX.

Materials and methods

Preliminary evaluation of unfractionated samples

Moisture contents of the samples were measured by determining the mass loss after drying approximately 1 g of samples at 110˚C overnight. The moisture contents were used to calculate the oven dried mass of the samples in the later size fractionation and other analyses. A few drops of diluted HCl solution (1 M) were added to about 0.5 g of each sample to test for carbonate minerals. A solution of 30% H_2O_2 was added to the wetted samples to check the presence of oxidizing/reducing compounds such as sulfides, manganese oxides, and organic matter. A magnetic stir bar was used to test the presence of magnetic minerals.

Presence of evaporite minerals (halides, sulfates, nitrates and borates) was evaluated by the electric conductivity of supernatant of 9.98 g of each sample and 50 ml of distilled water mixture after 30 min shaking, and centrifuging. Gypsum was tested by checking if white precipitates formed when 2 ml of acetone and 2 ml of the supernatant were mixed. The pH of the supernatant was also measured.

Size fractionation

The 9.98 g samples used in the preliminary evaluation were treated with pH5 sodium acetate solution three times to accomplish Na^+ saturation. Sample 4TX was treated with 30% H_2O_2 to remove any organic matter, but sample 1TX was not so that pyrite could be preserved in it.

Particle dispersion was accomplished by repeatedly adding 50 ml of pH 10 sodium carbonate solution, shaking and centrifuging at 2000 rpm for 10 min with IECPR-700 until a cloudy supernatant resulted in after centrifugation. The sand fraction was separated using a 53 μm sieve, washed with distilled water, recovered in a pre-weighed aluminum dish, and dried overnight at 105˚C. After drying, the mass of the sands was recorded.

The dispersion containing the silt and clay fractions was placed in 250 ml Nalgene centrifuge bottles previously marked at heights 1 to 9 cm from the bottom. The distances were used to calculate centrifugation time for clay separation. To disperse the samples pH 10 Na_2CO_3 solution was added to the 9 cm mark. The dispersion was shaken and then centrifuged at 750 rpm for 3.2 min. The clay-containing supernatant was collected after centrifugation. The procedure was repeated until the supernatant was clear. The sediment particles constitute the silt fraction and were recovered in a pre-weighed aluminum dish, dried at 105 ˚C and the dry mass was recorded.

The collected clay suspension was flocculated by adding NaCl powder. The clays were dialyzed to remove the excess of salts. Water was changed repeatedly until the EC of the water was less than 5 μS. The clay fraction was stored as a suspension and clay percentage in the suspension was calculated from the mass of oven dried clay in 1 ml aliquot of the dialyzed clay suspension. .

The total mass of clay in the sample was computed based on the clay percentage and the total mass of the dialyzed clay suspension. Part of the dialyzed clay suspension was saved and the rest was dried at 60 ˚C.

Aflatoxin adsorption isotherms

Aflatoxin adsorption isotherms were conducted on both unfractionated bulk samples and on the clay fraction of the samples. The adsorption capacities of the unfractionated samples were analyzed following the procedure described by Kannewischeret et al., (2006). Diluted bentonite suspensions were prepared by dispersing 0.01 g of sample in 5 ml of distilled water. Fifty microliters of suspension,

containing 0.1 mg bentonite, were e transferred to a series 15 mL polypropylene centrifuge tubes. An 8-ppm aflatoxin solution was prepared by diluting a stock solution (1000 ppm AfB_1 in acetronitrile), and then desired amounts of the 8-ppm aflatoxin solutions were added to the 15 mL to achieve the following aflatoxin concentrations: 0.0, 0.4, 1.6, 3.2, 4.8, 6.4, and 8.0 ppm. Each isotherm was duplicated. After overnight shaking at 200 motions per minute, the samples were centrifuged at 4500 rpm (5443.2 g) for 57 min. The aflatoxin concentration left in solution was analyzed using a Beckman Coulter DU 800 UV-spectrophotometer. The maximum adsorption was calculated using Langmuir isotherms.

The clay fractions (<2 μm) were also analyzed for adsorption capacity following a slightly modified procedure. The mass of the clay and volumes of the solutions were increased 10 times so there was enough clay (1mg) for XRD and FTIR analyses after the aflatoxin adsorption experiment. Clay suspension was prepared by diluting the dialyzed clay suspension to reach a final clay content of 1 mg per 5 mL. Five mL of the diluted clay suspension were added to 50 mL polypropylene centrifuge tubes. After the aflatoxin adsorption using only two concentrations per sample, the clays were washed four times with distilled water. The supernatant after each washing was recovered and analyzed for aflatoxin concentration in order to measure desorption. And the clays were used for XRD and FTIR analyses.

X-ray diffraction analysis

The sand and silt fractions were ground to pass a 140 mesh sieve. Bulk sample, sand and silt powders XRD patterns were recorded from 4 to 70 degrees two-theta using a D8 BRUKER ADVANCE diffractometer with Cu Kα radiation, 30 rpm spin rate, and 0.017 step size. A 1-D position sensitive detector LynxEye was used during XRD analyses. Mineral identification was done using the search/match function of software EVA (Bruker).

Each clay fraction was saturated with Mg^{+2} and with K^{+} to facilitate the identification of phyllosilicates as their d-spacing can shift, depending on interlayer

cations, swelling, and heat treatments. Approximately 60 mg of each clay sample was obtained by taking suitable amounts (3 ml for sample 4TX and 4 ml of sample 1TX) of clay stock suspensions. The samples were saturated with 0.5M $MgCl_2$ or 1M KCl solutions. The detailed procedure is described in the Soil Mineralogy Lab Manual (Deng et al., 2009). The suspensions of Mg- or K-saturated clays were air dried on glass discs. The XRD patterns of magnesium saturated clays were recorded at room humidity and after glycerol solvation. The K treatment patterns were recorded at room temperature, after 1-h heating at 330°C and 550°C.

To confirm the aflatoxin intercalation into smectite, basal spacings of the clays before and after aflatoxin adsorption were measured by XRD at room humidity and 0% humidity. Each clay suspension was air dried on a zero-background quartz slide and the slide was placed in an XRD 900 reactor chamber (Anton Paar). One XRD pattern was recorded at room humidity (40%) at 30°C, and a second pattern was recorded at nearly 0% humidity but the same temperature. The 0% humidity was achieved by alternative N_2 flushing and evacuating the chamber for 20 min. During XRD recording at 0% humidity, the chamber was filled with dry N_2.

Fourier transform infrared (FTIR) analysis

The FTIR spectra for the unfractionated powder samples were recorded using a Perkin Elmer Spectrum 100 with a DRIFT accessory. The samples were directly loaded into the sample holders without dilution during the analysis. Thirty-two scans from 7800 to 450 cm^{-1} at a 4 cm^{-1} resolution were recorded and averaged to obtain a spectrum. The spectra of the clay fractions were recorded using DRIFT accessory but the samples were diluted by mixing 0.01 g of sample with 0.3 g of KBr. Sixty-four scans from 4000 to 450 cm^{-1} at a resolution of 4 cm^{-1} were collected for each spectrum. The clays saturated with aflatoxin and washed with distilled water were placed on ZnS windows and air dried. The spectra were recorded using 32 scans and 1 cm^{-1} resolution, under dry conditions (N_2 purge).

Scanning electron micrographs (SEM) - Silt fraction

The silt fractions were mounted on conductive double adhesive SEM tabs on SEM stubs and carbon-coated before they were examined with a JEOL 6400 SEM. Energy dispersive X-ray spectrum (EDS) was used for chemical composition.

Transmission electron micrographs (TEM) - clay fraction

A few drops of the dialyzed clay suspension were diluted with distilled water in a glass vial until a slightly turbid suspension was achieved. A drop of diluted suspension was transferred onto a holey C-support membrane (Lacey Formvar/Carbon, 300mesh Copper Grids No. 01883-F), and dried under a 250-W heating-lamp. Clay particles were analyzed using a JEOL 2010 TEM.

Results and discussion

General characterization

The moisture contents were 10.4 % for 4TX and 9.8% for 1TX (Table 4.1). Carbonate minerals were absent in the samples based on the HCl test. Also no magnetic minerals were observed. Sample 1TX did not react vigorously with H_2O_2 despite the presence of pyrite detected by XRD. Acetone test did not reveal gypsum in the samples, yet the electrical conductivity (EC) of sample 1TX was as high as 1856 μS/cm, which indicates the presence of evaporites in the sample. The XRD analysis suggests the presence of gypsum. The gypsum test was repeated for sample 1TX and only a little precipitates was observed. The lack of precipitation of gypsum in the acetone test could be attributed to kinetic inhibitions.

Table 4.1: Sample evaluation results.

Sample	Moisture %	pH	EC (µS/cm)
4TX	10.4	6.97	99.6
1TX	9.8	7.04	1846

Size fractionation

The oven dried mass of 9.98 g 4TX was 8.94 g and of 1TX was 8.99 g. The mass of the sand and silt fractions were directly measured after recovered particles were oven dried. Both samples contained approximately the same percentage of sands (about 4%); sample 1TX had a higher percentage of silts (30.4%) than sample 4TX (8.8%). The percentage of clay was greater in 4TX (87.6 %) than in 1TX (65.7%) (Figure 4.1). Montmorillonite is assumed to be responsible for aflatoxin adsorption and it was concentrated in the clay fraction (as shown by XRD). Thus, other minerals are considered as diluents, and it is expected that high-clay content samples should have higher aflatoxin adsorption capacity. The fact that 4TX had higher aflatoxin adsorption capacity than 1TX could be partially explained by the higher amount of clay in sample 4TX.

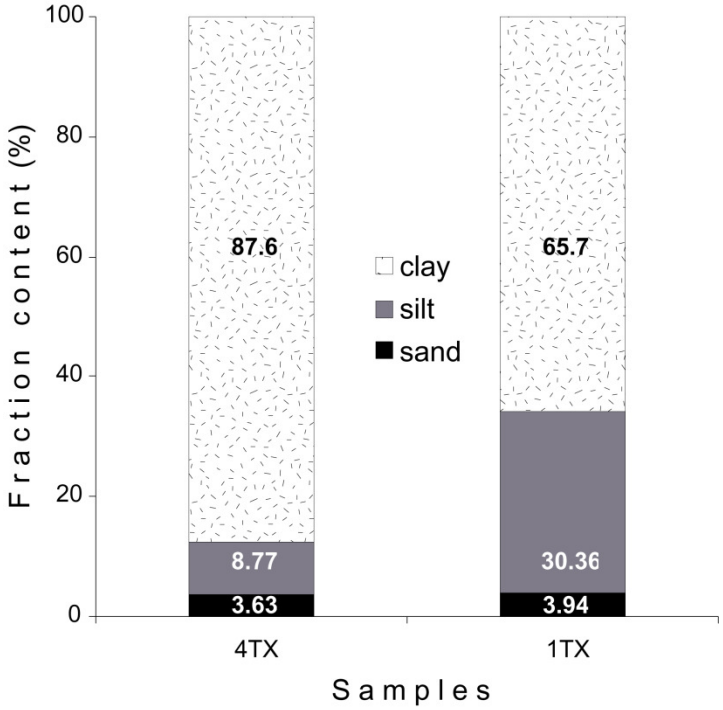

Figure 4.1: Percentage of sand, silt and clay fractions of samples 4TX and 1TX.

Aflatoxin adsorption isotherms

The adsorption data from the unfractionated materials fitted the Langmuir isotherm described in chapter II. Sample 4TX showed a high adsorption capacity (17.8% by wt), while sample 1TX had moderate aflatoxin adsorption (7.7% by wt). An interesting observation was that the results of the affinity values (K_d) were opposite of the adsorption maxima. Sample 1TX, which show less than half percentage of adsorption capacity than sample 4TX, had a K_d value higher than 4TX (Table 4.2). This indicates that 1TX was a potential good adsorbent, which adsorption capacity was decreased by dilution (Figure 4.1).

The clay fractions demonstrated a nearly linear isotherm curves (Figure 4.2), which resulted in high adsorption capacities values that were not realistic (Table 4.2). The isotherms did not reach a plateau at the concentrations tested due to incomplete

saturation of the adsorption sites on the clay. Moreover the affinity values indicated that aflatoxin accessibility to the interlayer was restricted. The drastically difference in adsorption capacities between the unfractionated samples and the clay fractions was a result of the exchangeable cation. The unfractionated materials were dominated by Ca whereas the clays were mostly Na saturated due to the fractionation treatments. The influence of the exchangeable cation on the aflatoxin-smectite reaction mechanism was addressed by Deng et al., (2010). Their FTIR experiments showed that divalent and monovalent cations caused major shifts in the aflatoxin bands. These observations led to recent unpublished experiments which demonstrated that the cation valence and hydration energy affects the adsorption efficiency of the smectite clays.

Comparing the adsorption capacities of the materials collected at the field (chapter III), the commercially processed product showed a higher adsorption capacity than the material collected at the field. This difference could be explained by the concentration of the clay material during the process. Contrary the commercial product of sample 1TX showed lower adsorption effectiveness than the material from the field. This illustrated the need of aflatoxin adsorption test before using them and the variability among batches.

Table 4.2: Aflatoxin adsorption isotherm values for unfractionated sample and clay fraction.

Sample	**K_d**	**Qmax (mol/kg)**	**η^2**
4TX unfractionated	2.94×10^5	0.5784	0.987
1TX unfractionated	4.39×10^5	0.2483	0.972
4TX clay	3.10×10^4	0.9717	0.993
1TX clay	0.8064	0.8073	0.979

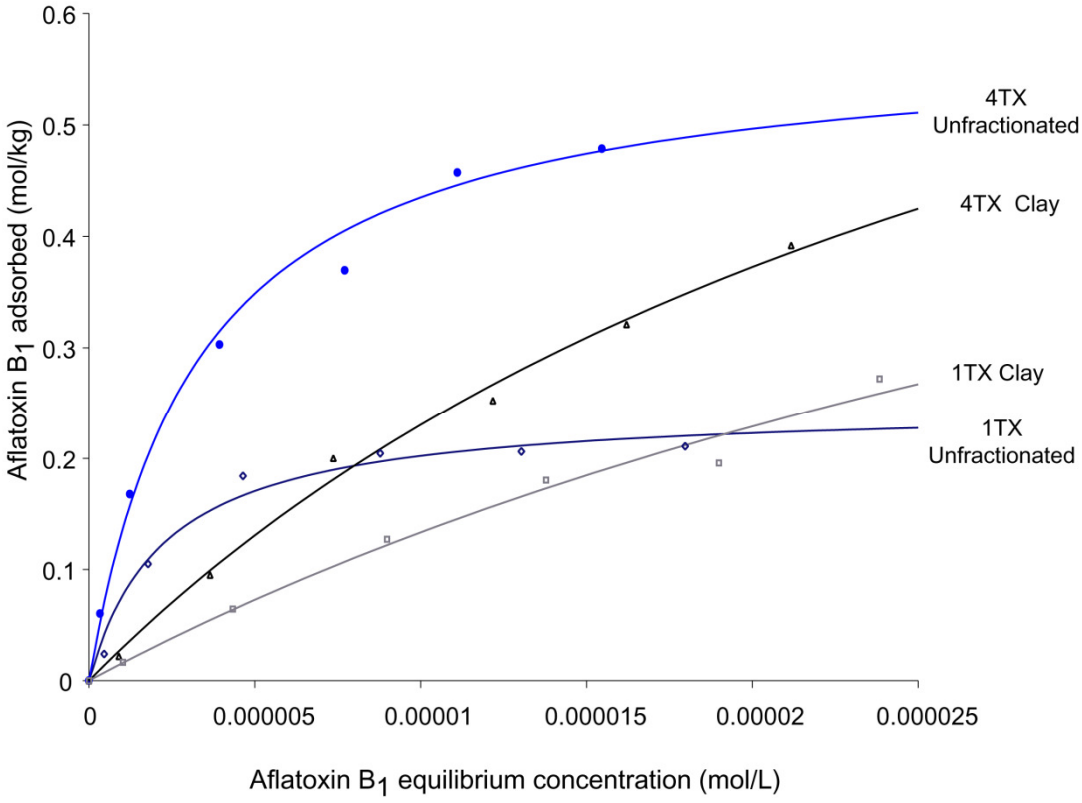

Figure 4.2: Adsorption isotherms of samples 4TX and 1TX.

Only two points of the isotherm were selected for the desorption experiment. Although different isotherm concentration points were used for each sample the data show that the clays even adsorbed more aflatoxin during the first wash, and no or negligible (<0.5%) desorption occurred in subsequent three washes. This desorption study suggested that the adsorbed aflatoxin is stable in the smectites.

Table 4.3: Desorption percentages after first wash.

Sample	Initial concentration (ppm)	Concentration after adsorption (mol/L)	Desorption (%)			
			1st	2nd	3rd	4th
4TX	1.6	0.0931	-14.1	0.37	0	0.094
	3.2	0.1923	-10.5	0.14	0	0.047
1TX	0.4	0.0161	-21.8	0.18	0.047	0
	4.8	0.1731	-14.9	0.54	0	0

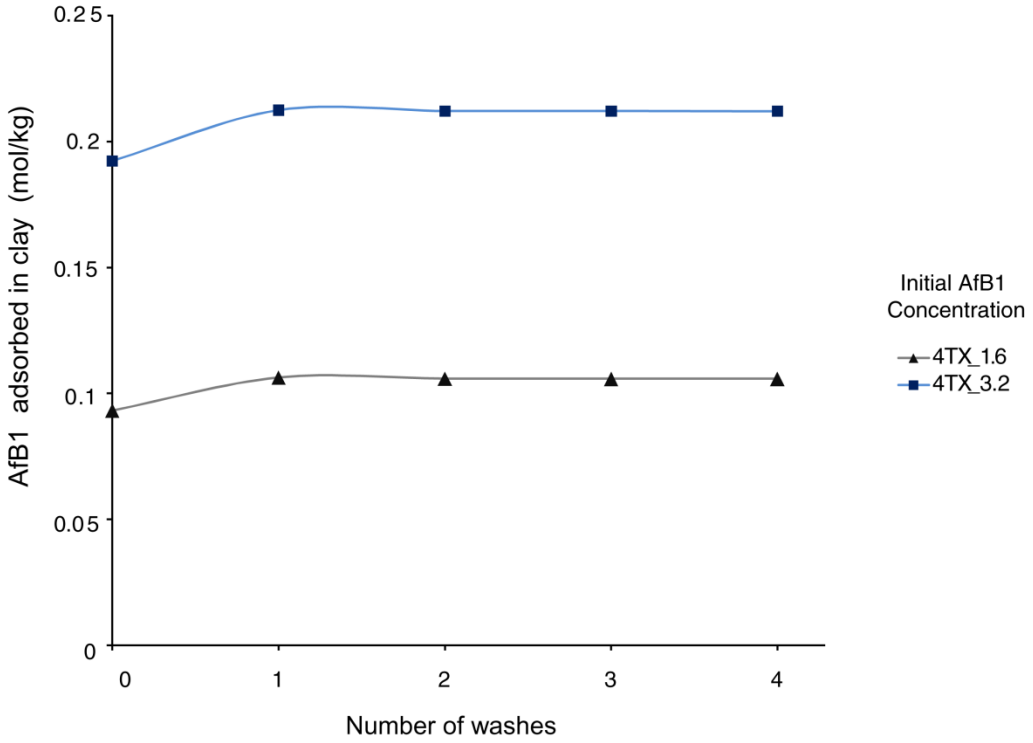

Figure 4.3: Desorption of aflatoxin after washing the AfB_1- clay fraction complex of 4TX at two points of the adsorption isotherm.

Both samples of each clay showed more than 10% of aflatoxin readsorbed from the solution (Table 4.3, Figures 4.3 & 4.4). The resistance of aflatoxin molecules to desorption was also confirmed by the constant concentration after the second to third wash (Figures 4.3 & 4.4).

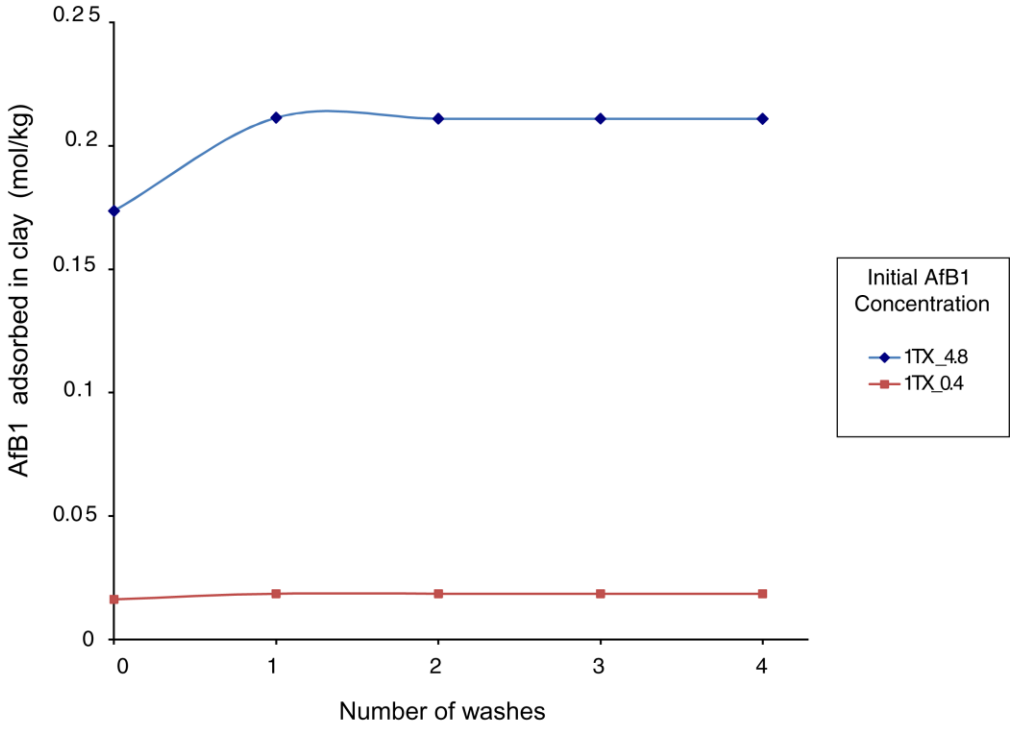

Figure 4.4: Desorption of aflatoxin after washing the AfB_1- clay fraction complex of 1TX at two points of the adsorption isotherm.

Occurrence of interlayer aflatoxin adsorption

Comparison of changes in the basal spacing of smectite was used to confirm the interlayer allocation of aflatoxin molecules. The Na-saturated clays without aflatoxin showed a basal spacing of ~12.0 Å at room humidity (66%) and a reduced ~10.0 Å at ~0% humidity (N_2 purge) (Figure 4.5 & 4.6). Clays adsorbed aflatoxin showed an expansion of the smectite basal spacing to ~ 14.0 Å at room humidity (66%), and to ~ 13.0 Å under 0% humidity (Figure 4.5 & 4.6). The aflatoxin adsorbed clays did not

collapse to 10 Å after repeated N_2 purge. The higher basal spacing in the aflatoxin adsorbed clays confirmed that aflatoxin molecules were held in the interlayer space of these two samples.

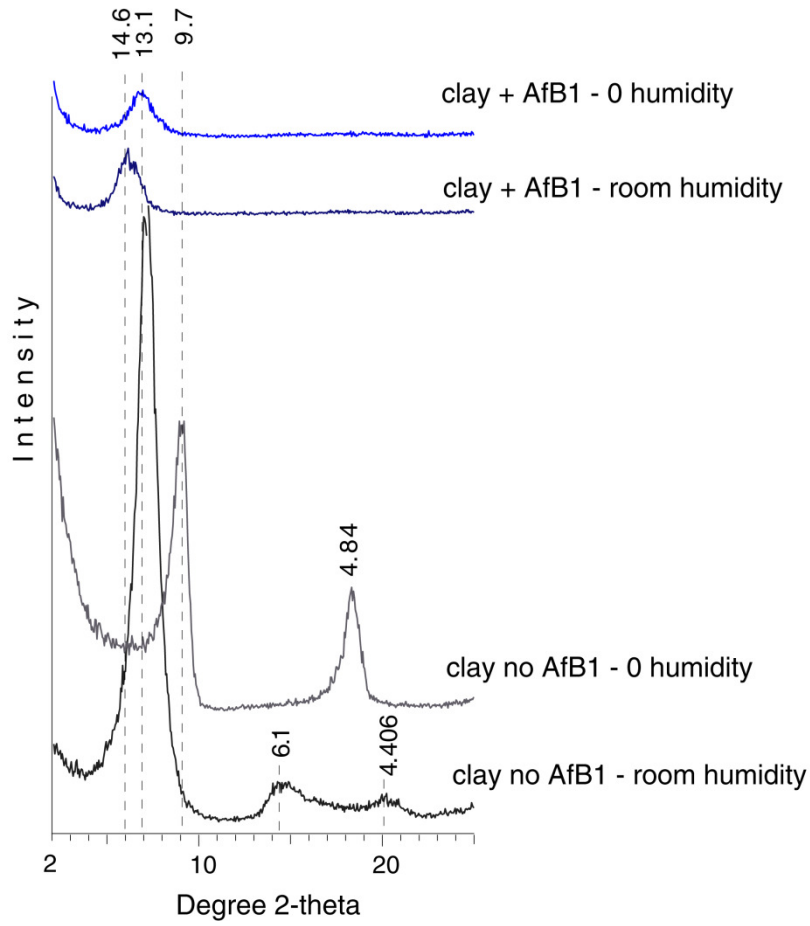

Figure 4.5: XRD patterns of clay from sample 4TX Na- saturated with and without AfB_1 at room and zero humidity.

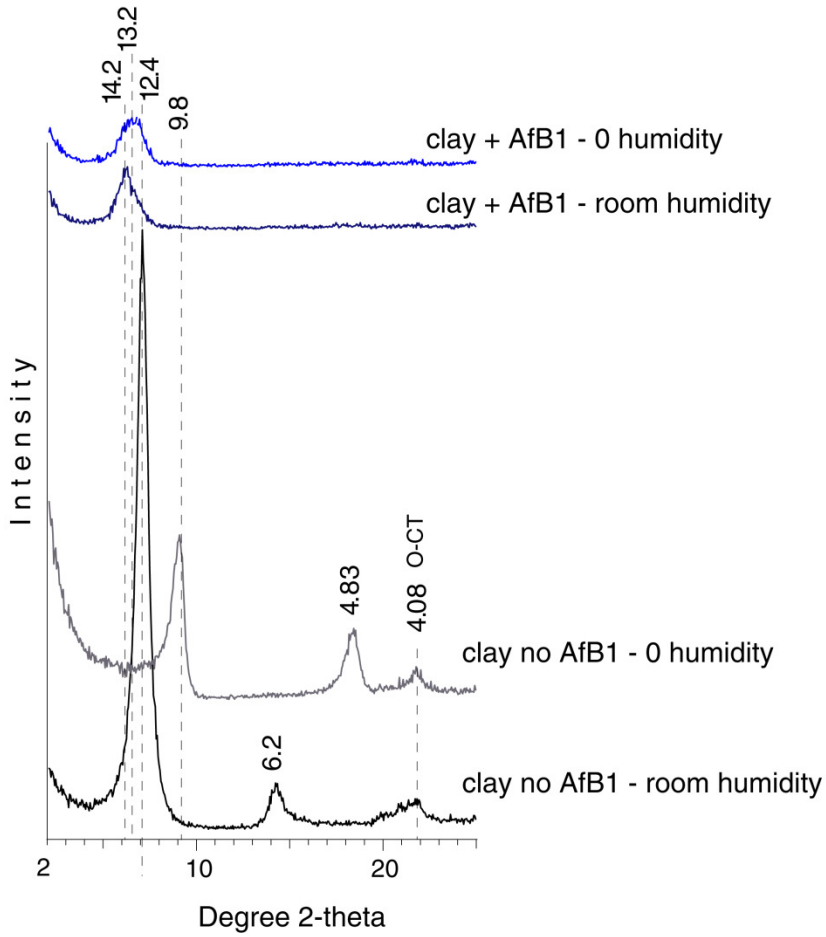

Figure 4.6: XRD patterns of clay from sample 1TX Na-saturated with and without AfB_1 at room and zero humidity.

Mineral identification by X-ray diffraction

The mineral composition of the unfractionated samples was dominantly smectite. The XRD patterns showed the characteristic reflection of Ca-smectite at 15.0 Å in both samples. The dioctahedral character was represented by the (060) reflections at 1.49 Å (Figures 4.7 & 4.8). The difference in the smectite (001) reflection intensity among the bulk, the sand and the silt indicates that smectite was mostly concentrated in the clay fraction. The broad reflections at ~ 12.0 Å in the sand and silt fractions were due to Na-saturation of smectite by sodium acetate during sample treatment. These reflections were more prominent in sample 1TX, which indicates more clay aggregates in this sample than in sample 4TX.

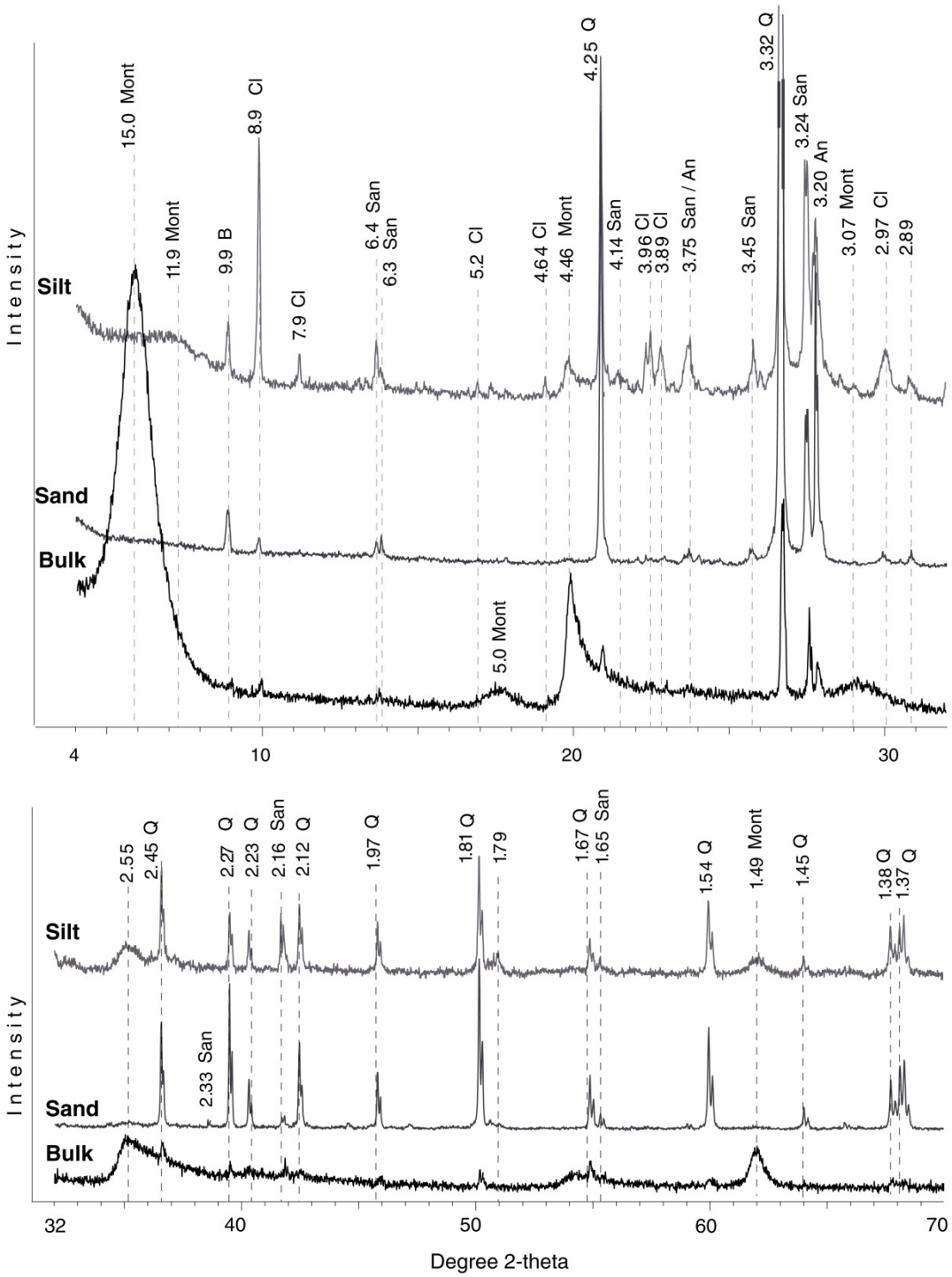

Figure 4.7: XRD pattern of whole (predominantly Ca saturated), sand and silt fractions (Na-saturated) of sample 4TX. Abbreviations: An: anorthite; B: biotite; Cl: clinoptilolite; Mont: montmorillonite; Q: quartz; San: sanidine.

The strong sharp reflections at 3.33 and 4.26 in the unfractionated pattern were due to quartz in both samples, which was mainly concentrated in the sand and silt fractions (Figures 4.7 & 4.8). In those fractions the region from 32 to 70 degree two-theta was dominated by the reflections of quartz.

Feldspars were also identified in both samples. Alkali feldspars, K-feldspar and albite occurred in sample 4TX, while plagioclase (labradorite?) and orthoclase (?) were present in sample 1TX. Biotite was the mineral identified by the presence of ~10.0 Å (001) reflection and the lack of (002) reflection at 5.00 Å in the sand and silt fractions of both samples. Minor amount of muscovite was identified in the silt fraction of 4TX by its weak 5.00 Å reflection. Clinoptilolite was present in sample 4TX, the XRD patterns showed a 8.9 Å reflection. This mineral was concentrated in silt fraction as the 8.9 Å reflection was intense and other reflection of clinoptilolite were observed (Figure 4.7).

The unfractionated 1TX showed 7.5 Å, and 4.28 Å XRD reflections, indicating the presence of gypsum, which was consistent with the high EC reading. This reflection disappeared in the sand and silt as gypsum was dissolved during the sample treatment (Figure 4.8). Sample 1TX also contained pyrite, identified in the unfractionated material and concentrated in the silt fraction, which gave 2.71, 2.42, 2.21, 1.91, and 1.63 Å reflections. This reflection became sharper in the silt fraction and almost absent in the sands fraction indicating the concentration of this mineral in the silts.

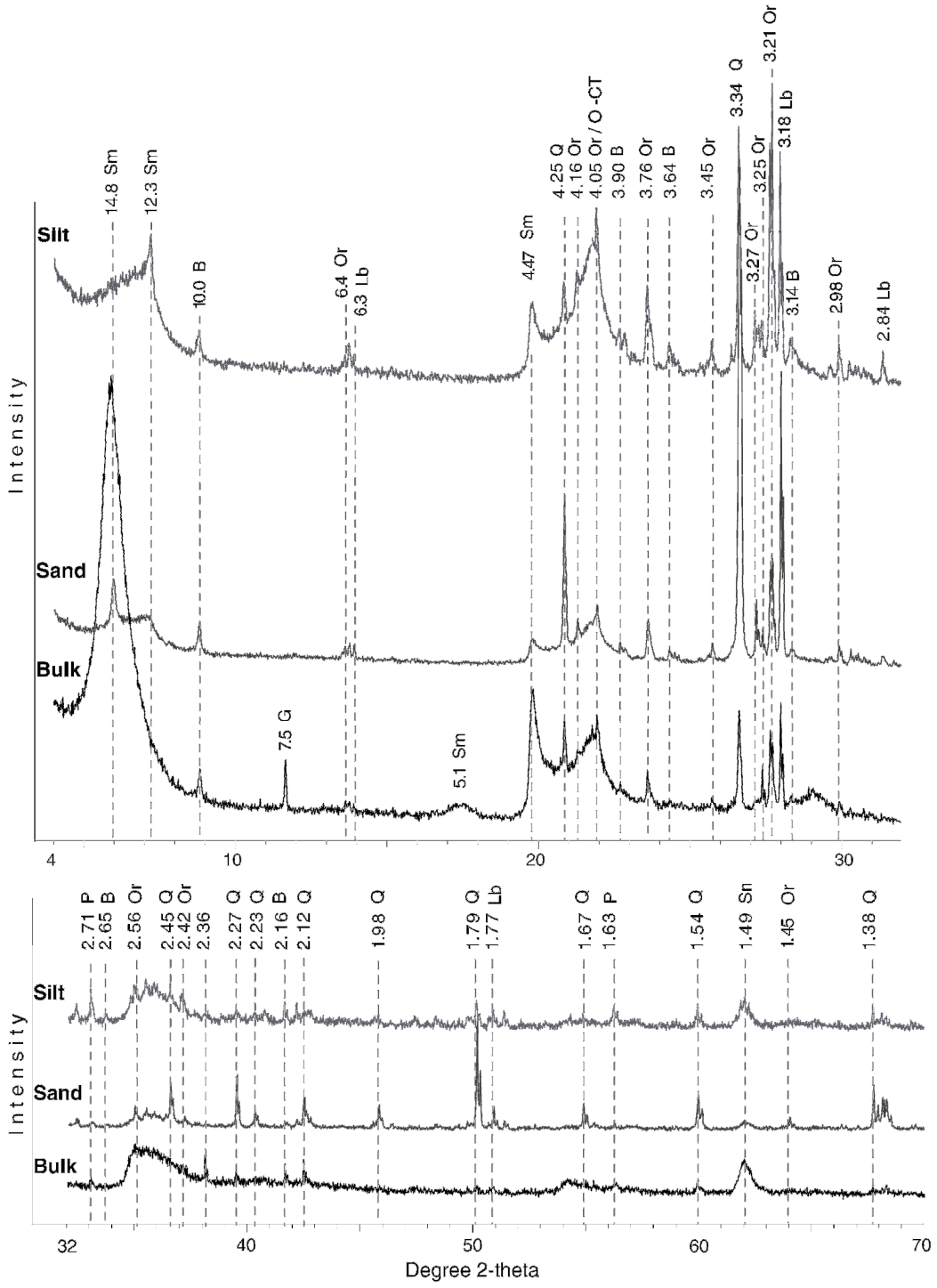

Figure 4.8: XRD patterns of whole (predominantly Ca saturated), sand and silt fractions (Na saturated) of sample 1TX. Abbreviations: B: biotite; G: gypsum; Lb: labradorite; O-CT: opal-CT; Or: orthoclase; P: pyrite; Sm:smectite; Q: quartz.

XRD patterns of the clay fractions

Saturation of the clay fractions with Mg in both samples had peaks at ~15 Å that expanded to ~18 Å upon glycerol solvation. The K saturated clays had reflections at ~12 Å that collapsed to 10 Å when heated (Figures 4.9 & 4.10). These trends of reflections shifting indicated that smectite was present in the clay fraction. The other reflections from the oriented specimens were high order reflections from the (001) planes of smectites. No other phyllosilicate minerals were found in the clay fractions. In sample 1TX the XRD patterns consistently had broad reflections at 4.06 Å that was not changed by solvation or heat treatment. This reflection originated from opal-CT (Figure 4.10), No distinct opal-CT reflections were observed in sample 4TX (Figure 4.9). The dilution effect of opal-CT could account for the lower adsorption capacity of 1TX compared with 4TX.

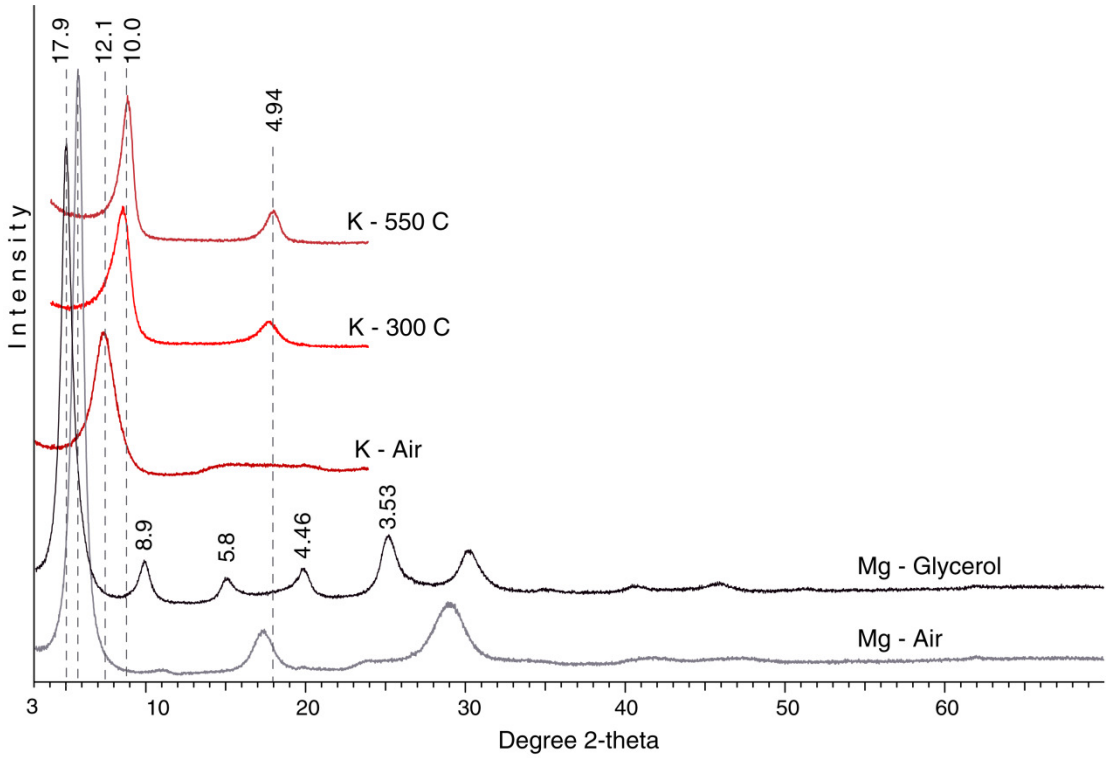

Figure 4.9: Clay fraction of sample 4TX.

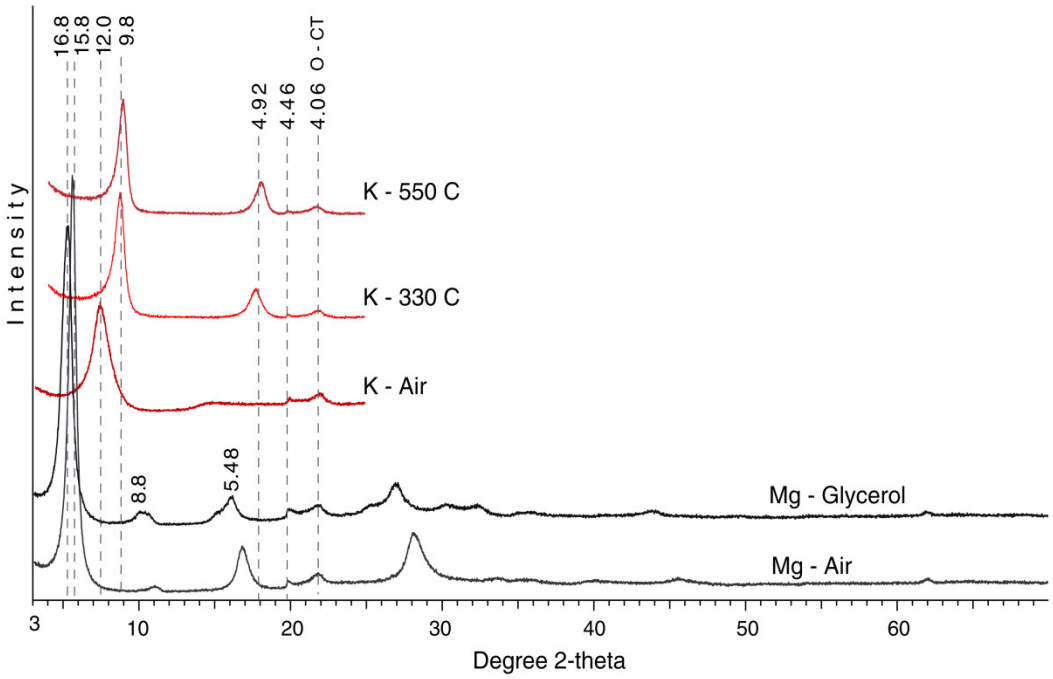

Figure 4.10: Clay fraction of sample 1TX. O-CT: opal-CT.

Structural composition by Fourier transform infrared spectroscopy

The bands in near infrared region from 7800 to 4000 cm^{-1} are mostly due to overtones and combinations of fundamental vibrations. The water vibrations were similar in both samples (Figure 4.11). The band at 7078 cm^{-1} was due to the overtones of octahedral OH-stretching vibrations (2*v*OH) of smectite and of water (2*vw*) bonded to the oxygens at the surfaces of the tetrahedral sheets (Madejova and Komadel, 2001). The vibration of OH in water molecules strongly bonded by H-bonding gave an overtone at 6836 cm^{-1} (2*vw*), approximately two times of the 3442 cm^{-1} band in sample 4TX. The band 5260 cm^{-1} was due to the combination of stretching and bending vibrations of adsorbed water molecules.

The band at 4532 cm^{-1} in both samples was a combination of the stretching vibration of octahedral OH (3626 cm^{-1}) and the bending vibration of octahedral AlAl-OH (919 cm^{-1}).. The major difference near this band was a shoulder at 4470 cm^{-1} in

4TX, this band was due to the isomorphic Fe-Al substitutions in the montmorillonite. The presence of Fe^{+3} in octahedral resulted in the shoulder at 4470 cm^{-1} in 4TX. The band was a combination of νOH and δAlFe-OH. This shoulder was absent in sample 1TX (Figure 4.12). The presence of Fe in octahedral sheet was further supported by bending band at 885 cm^{-1} from AlFe-OH in the mid FTIR spectrum of sample 4TX.

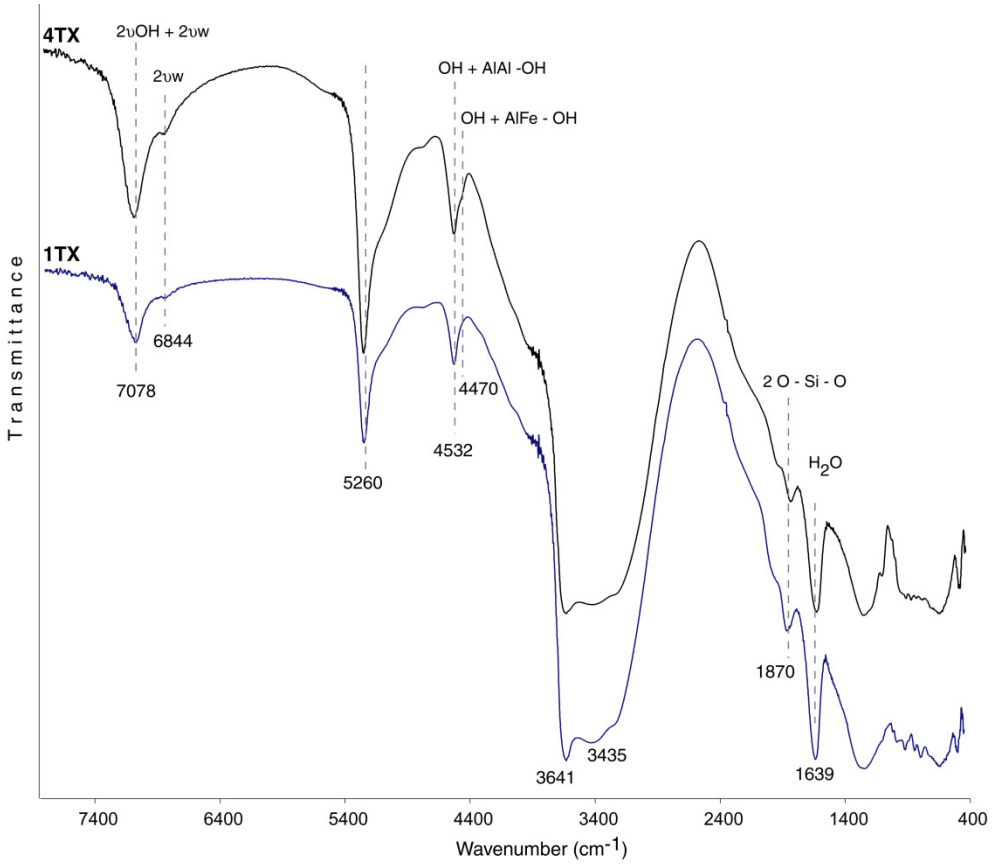

Figure 4.11: ATR-FTIR spectra of unfractionated powder samples 4TX and 1TX, showing bands in near and mid infrared regions.

On the FTIR spectra of both bulk samples there were well defined bands at 1846 cm^{-1} and shoulders at 2000 cm^{-1}. Nguyen et al., (1991) discussed that overtones for quartz occur in this region, yet other silica minerals have weak bands in these region too. Quartz was present in both bulk samples but it was absent in the clay fractions as

indicated by XRD analysis (Figure 4.9 & 4.10), the broad bands (1996 and 1861 cm^{-1}) in the clay fraction of 1TX were probably due to opal-CT.

Adsorbed water in the interlayer of smectite was evident by strong bands at 1) 3440 cm^{-1} that originated from O-H stretching vibrations (OH groups related to H-bonding of water to water) and 2) at 1630 cm^{-1} from the O-H bending vibration of water. The shoulder at 3239 cm^{-1} was also related to loosely adsorbed water (van der Marel and Beutelspacher, 1976; Madejova et al., 1994).

The strong band at 3626 cm^{-1} was attributed to the stretching vibration of OH groups in the octahedral sheets of montmorillonite. The band position was is influenced by the cation in the octahedra (Madejová, 2003). The position of the band at 3626 cm^{-1} indicates that Al^{3+} was the dominant cation in the octahedral sheets, thus both samples are mainly dioctahedral. For dioctahedral smectites the isomorphic substitution resulted in OH bending bands in the range from 950 to 800 cm^{-1}. The bands at 919 (4TX) and 917(1TX) cm^{-1} were designated to the AlAl-OH bending vibrations. The band at ~885 cm^{-1} was due to $Al^{3+}Fe^{3+}$-OH (Gates, 2005). In sample 4TX, the 885 cm^{-1} band was well defined while in sample 1TX it occurred only as a shoulder, which indicated more Fe present in the octahedral sheet of smectite in sample 4TX. The ~845 cm^{-1} band was designated to the $Al^{3+}Mg^{2+}$-OH bending vibration. The intensity of this band suggested that, in contrast to Fe, the Mg content is higher in sample 1TX than in 4TX.

Gates et al, 2005 designate a weak, broad band at 750-800 cm^{-1} to the $Fe^{3+}Mg^{2+}$-OH bending vibration of smectite. Silica cristobalite and tridymite have their Si-O stretching bands in this range too (Madejova and Komadel, 2001). The broad 794 and 618 cm^{-1} bands in 4TX could suggest the presence of cristobalite. But the XRD patterns of the clay fraction did not show any distinct peak at 4.05 Å, which suggest either the content or the crystallinity of opal-C was low in 4TX. The 618-628 cm^{-1} bands could be the coupled Si-O and Al-O out-plane bending vibration in aluminous smectites, or the Si-O stretching of the cristobalite. The 845 cm^{-1} band was due to AlMg-OH bending vibration of the octahedral sheets in montmorillonite. The more intense of the AlAl-OH band at 919 cm-1 and AlMg-OH band at 845 in 1TX than 4TX suggest the presence of

aluminous smectite in 1TX. When Mg and Al contents decreased as in 4TX, the coupled Si-O and Al-O bending band at 618 cm-1 became broader and less intense.

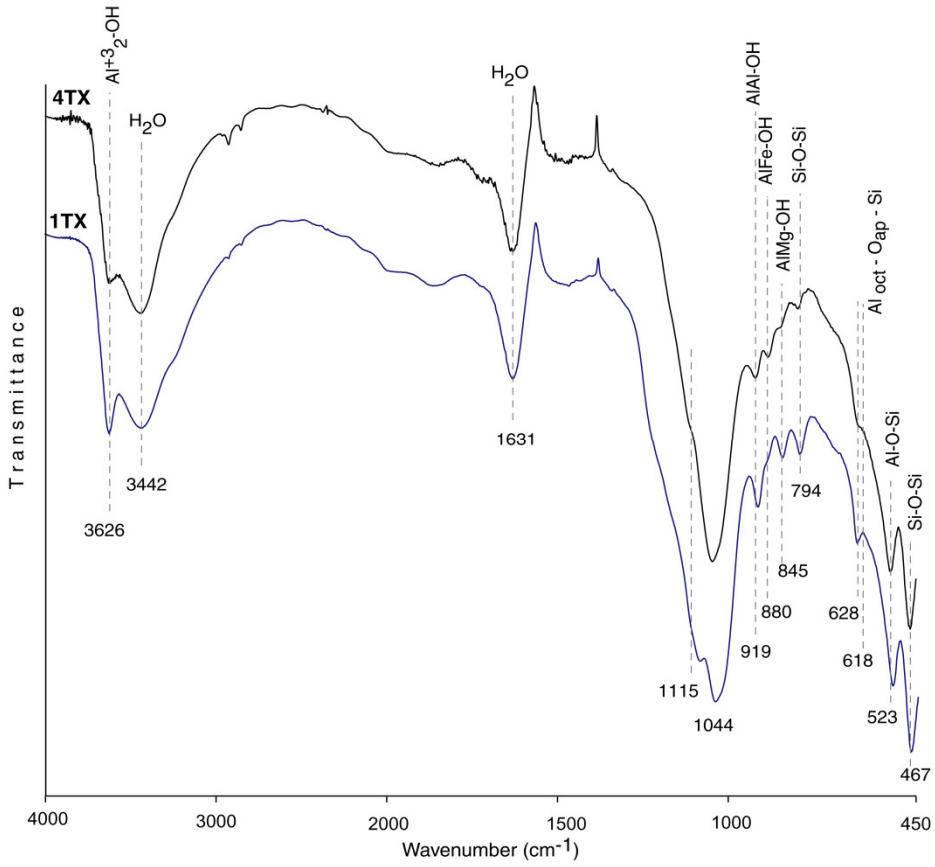

Figure 4.12: DRIFT-FTIR spectra of clay fractions of samples 4TX and 1TX, showing bands in near infrared region.

In sample 1TX the strong bands at 794 cm^{-1} and 628 cm^{-1} suggested the presence of cristobalite, which was confirmed by the XRD diffraction analysis of the clay fraction. A broad peak at 4.05 Å was due to cristobalite, it was not affected by the cation exchange, glycerol solvation, or heating treatments (Figures 4.9 & 4.10. In both samples the bands at ~523 and ~467 cm^{-1} were attributed to Al-O-Si deformation vibration and Si-O-Si deformation vibration respectively (Madejova and Komadel, 2001) .

Scanning electron micrographs (SEM) - silt fraction

Quartz, feldspars, biotite and clinoptilolite composed the sand and silt fractions of sample 4TX as indicated by the XRD patterns. Major feldspar particles observed were sanidine (K-feldspar) (Figure 4.13 a, c2, c3, d1, d2, d4, e1 & f1) and some albite particles too (Figure 4.13 d3). The chemical composition by EDS was used to identify the particles. Quartz particles are shown in Figure 4.13 c1 & f2. The identification of biotite in the XRD patterns indicated a Ti-rich mineral; this was confirmed with the chemical composition of an observed particle (Figure 4.13 e2).

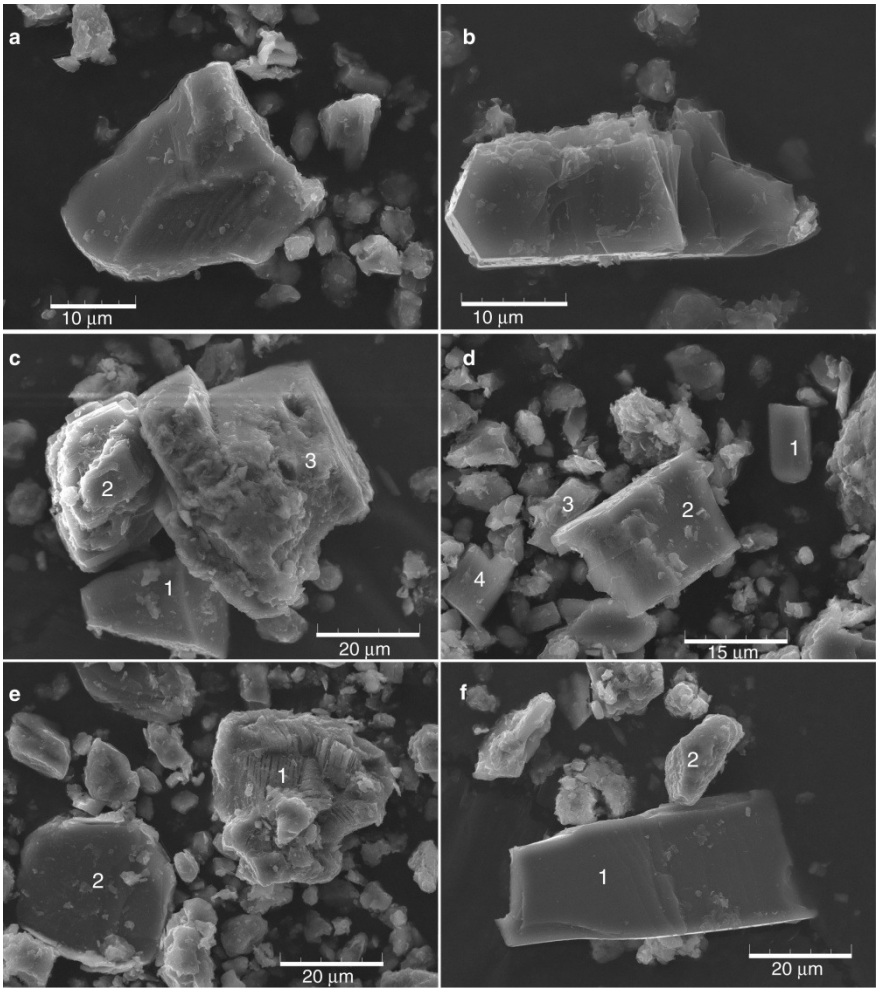

Figure 4.13: Particles in silt fraction of sample 4TX.

Quartz and feldspars were also present in the sand and silt fractions of sample 1TX. The major difference in comparison with 4TX was the presence of pyrite in 1TX. Several pyrite particles were observed in the silt fraction differing in size and morphology (Figure 4.14 a, b & c). Feldspar particles were also observed: labradorite (Figure 4.14 d2 & e2) and orthoclase (d3 & e1) were identified.

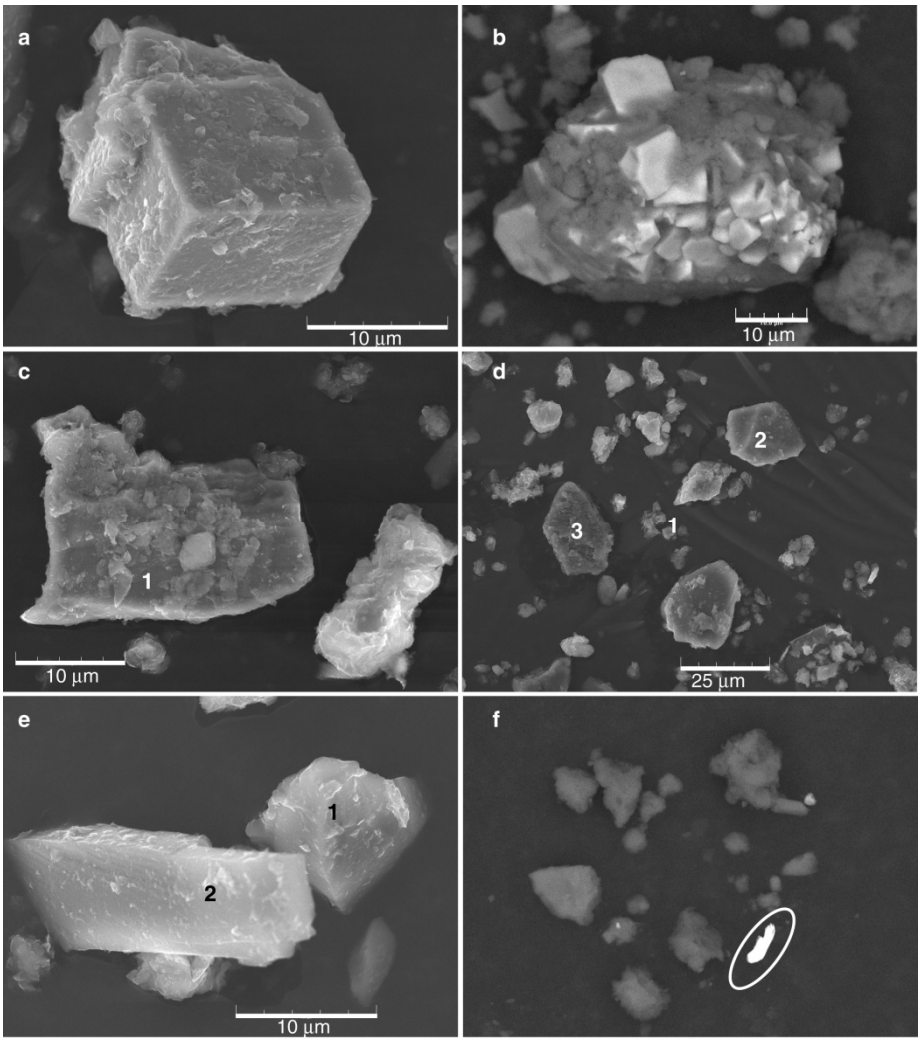

Figure 4.14: Particles in silt fraction of sample 1TX.

Bentonites are composed of montmorillonite but other minerals are associated with it too. It is important to know the composition of the clays that are introduced to the animal feed in order to identify or avoid the inclusion of minerals or elements that can interfere with the animal health. Quartz and feldspars are mostly inert minerals and their chemical composition does not represent a risk for animals. These are common minerals found in soils and it has been reported that several animal species tend to eat soil. Additionally the acid conditions in the stomach and the short residence time may not alter the stability of these minerals. In sample 1TX the presence of pyrite could be a concern but the reduced conditions of the intestinal tract favor its stability. The presence of heavy metals can be a negative factor in sample 1TX.

Transmission electron micrographs (TEM) - clay fraction

The clay fraction showed typical thin platy morphology for smectites (Figure 4.15a & 4.16c). Complex folding were observed also in both samples and more dominant in sample 1TX (Figure 4.15b & 4.16a,b,c). There were not well defined lattice fringes due to smectite in both samples when folds were observed at higher magnification (Figures 4.15c,d & 4.16 c,d).

In sample 4TX aggregated dense particles were attributed to iron oxides because a higher magnification image showed well defined lattice fringed with d-spacing was close to goethite (Figure 4.15 e & f). Aggregated small particles were observed in sample 1TX, which could be opal-CT particles or salts. Similar aggregates were observed by Mulder etal., (2008) that were attributed to a globular smectite like morphology.

The morphology of the smectites in samples 4TX and 1TX appear similar to the good bentonites adsorbents presented by Mulder et al., (2008). Also the thin and translucent particles seem to be accessible for aflatoxin rather than thick opaque particles (Dixon et al., 2010 in review).

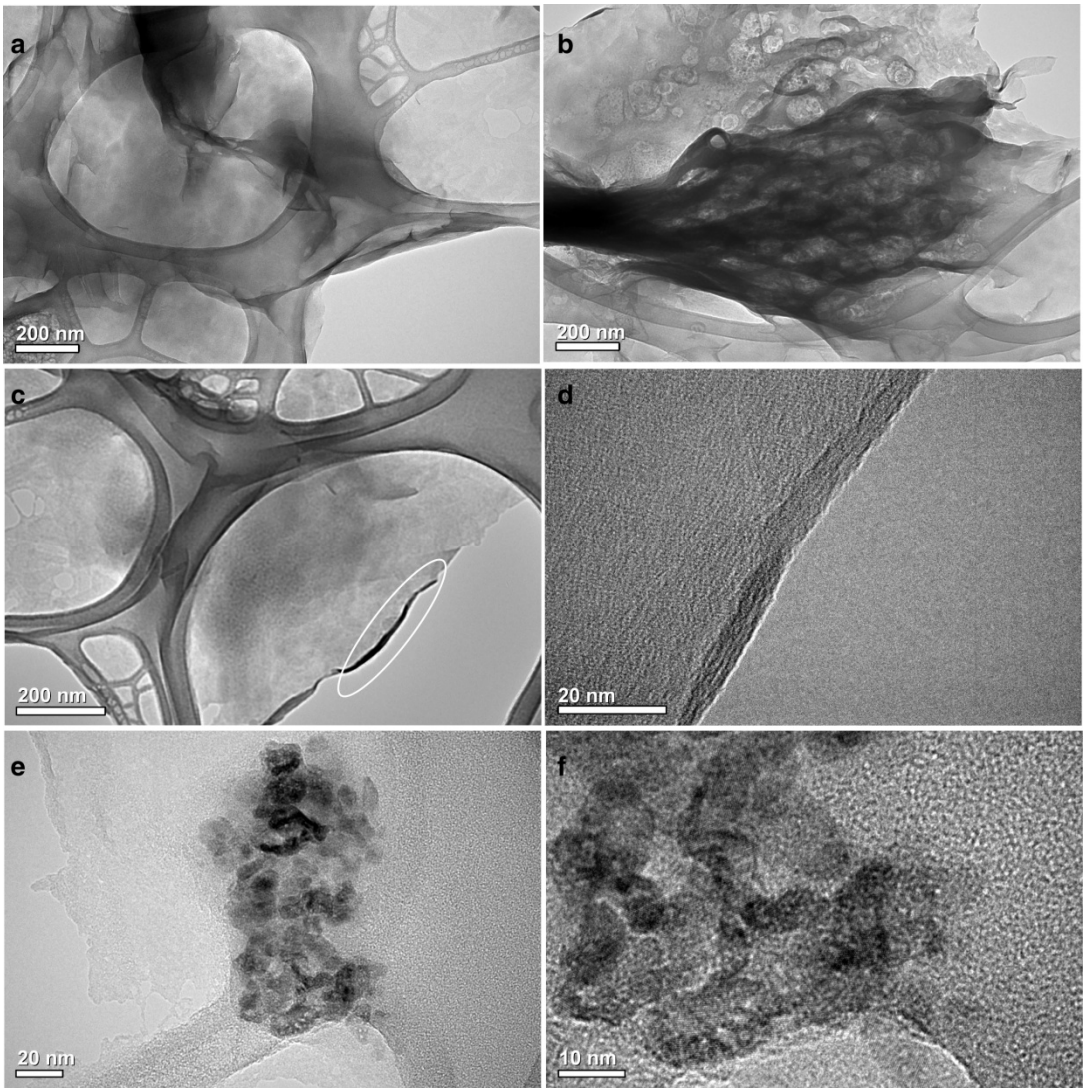

Figure 4.15: Transmission electron micrographs of clay fraction of sample 4TX.

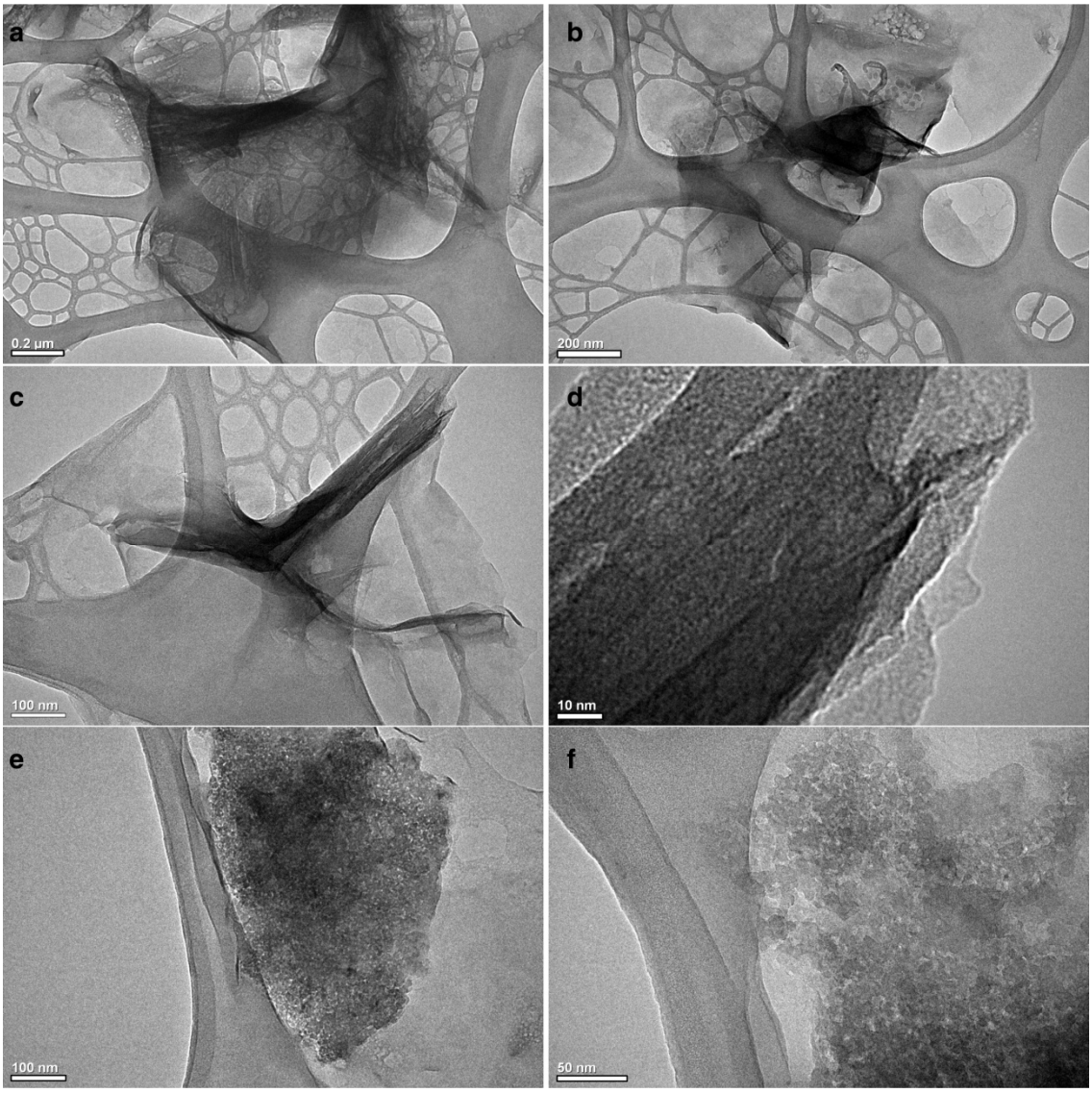

Figure 4.16: Transmission electron micrographs of clay fraction of sample 1TX.

Conclusions

The high clay content dominated by montmorillonite explains the high aflatoxin adsorption capacity of the bentonites. The different adsorption values can be attributed partially to the greater dilution of the smectite by the presence of other minerals in the sand and silt fraction but also in the clay.

The diluent minerals (quartz, feldspars, mica) are common in soils and are unlikely to cause adverse effects on animals. Detailed characterization of the samples can reveal the presence of mineral and/or heavy metals that can interfere with the animal health.

Analysis of the adsorption capacity of the clay fraction confirmed the strong interlayer adsorption of aflatoxin molecules, which was resistant to washing. Yet the amount of aflatoxin that can be adsorbed was influenced by the dominant exchangeable cation. The divalent cation as Ca^{2+} in the unfractionated material offered better conditions for aflatoxin adsorption than Na^{+} in the clay fraction.

The mineralogical properties observed in these bentonites were suitable for high in vitro adsorption capacity of aflatoxin. To confirm that this result can be extrapolated for further samples, these two effective bentonites are subjected to animal experiments to confirm in vitro effectiveness.

CHAPTER V
EFFICACY OF SELECTED COMMERCIAL BENTONITE SAMPLES AS AFLATOXIN ADSORBENTS IN BROILER CHICKENS

Introduction

Grain that contains more than 20 ppb aflatoxin level cannot be used in food but can be directed to feed with certain limitations made by the Food and Drug Administration (FDA). For young animals and dairy cows the aflatoxin concentration cannot exceed the same level as for human consumption (20 ppb). This is because younger animals are more susceptible to the effects of aflatoxins, and also due to presence of aflatoxin M_1 in milk. Although the aflatoxin levels from 100 to 300 ppb may not cause acute toxicity to certain animals like ruminants, but their performance can be compromised as indicated by a reduction in milk production (Jouany and Diaz, 2005). A review of effects of aflatoxin at different levels showed that even low concentrations (6 to 14 ppb) produce significant negative impact on the health of chickens, which impact the economical value (Devegowda and Murthy, 2005).

There are many proposed methods to inactivate, degrade, or remove aflatoxin in food and feed (Phillips et al., 1994). Among the decontamination techniques, incorporation of adsorbents in the diet of animals exposed to aflatoxin has been extensively investigated. Due to their low cost and wide availability, use of adsorbents is the one of the most economically feasible techniques that can be used to protect animals from the deleterious effects of aflatoxins. Additionally it complies with the recommended characteristics for an adequate detoxification technique (Diaz and Smith, 2005): 1) removes aflatoxin by adsorption, 2) no toxic residues are produced, 3) maintains the nutrient value of the feed, and 4) it is inexpensive and technologically feasible that does not increase product cost.

A wide range of animals had been tested in evaluate the effectiveness of adsorbents for aflatoxins. Pigs (Lindemann et al., 1993; Thieu et al., 2008) and turkeys (Ramos et al., 1996) had shown improvement in body weight and reduce mortality by

clay incorporation in the diet. Adding bentonite to feed also reduced the concentration of other less toxic residue forms of aflatoxin in milk in a dairy cows study (Stroud et al., 2006) and in goats (Ramos et al., 1996). The bentonite adsorbs aflatoxin in the gastrointestinal track before it can be assimilated and metabolized by the organism (Phillips et al., 1987).

Poultry are highly susceptible to aflatoxins and their susceptibility is only, after ducks and turkeys (Arafa et al., 1981; Dalvi, 1986; Leeson et al., 1995). Due to the economic significance and rapid growth, chickens are usually selected for animal trials to test exposure effects to different levels of aflatoxins or to evaluate the efficacy of aflatoxin binders. Addition of a hydrated sodium calcium alumino silicate (HSCAS) and bentonites in the aflatoxin diet of chicken had shown to significantly improve body weight (Phillips et al., 1988; Kubena et al., 1990; Rosa et al., 2001; Pimpukdee et al., 2004; Bailey et al., 2006; Phillips et al., 2008; Kermanshashi et al., 2009). Yet, as discussed in previous chapters, the adsorption capacity of bentonites can be highly variable, which may result in different effectiveness in detoxifying aflatoxins in vivo.

Aflatoxin adsorption capacity of smectites is usually evaluated in vitro by batch isotherms. A specific amount of clay is exposed to gradual aflatoxin concentrations, the concentration of aflatoxin remained in suspension is used to calculate the amount that was adsorbed. The data is evaluated using the Langmuir mathematical model to obtain the maximum adsorption capacity of the clays (Phillips et al., 1988; Kannewischer et al., 2006). The in vitro capacity of the clays needs to be tested in animal experiments to corroborate efficacy under more complex environment like the digestive system. Inconsistent in vitro and in vivo effectiveness have been reported. In some cases a poor adsorbent evaluated in vivo can show significant adsorption capacity for aflatoxins in vitro (Scheideler, 1993).

The objectives of this study were 1) to evaluate the efficacy of selected clays (chapter III) as amendments of aflatoxin contaminated feed of broiler chickens, and 2) to address the safety of the clays when incorporated in the diet.

Materials and methods

Feed preparation

Two sources of aflatoxin contaminated corn were used to achieve the desired aflatoxin level in the final poultry feed. One corn contained 803 ppb aflatoxin was provided by Dr. Tom Isakeit. The other corn was inoculated with *Aspergillus parasiticus* cultures (from Dr. Deepak Bhatnagar – ARS-USDA, New Orleans) under high humidity and under warm conditions. The inoculated corn contained ~6250 ppb aflatoxin. Aflatoxin quantification was performed by the Office of the Texas State Chemist using high-performance liquid chromatography (HPLC). The corn were ground and mixed to form a single aflatoxin contaminated corn source for the feed experiment.

Aflatoxin-feed and clean-feed were prepared at the same ratios of corn, soybean and nutrients. The mixture was homogenized using horizontal rotary mixer for 12 minutes. The clay powders were incorporated into the feed during the mixing. Clays were added at 0.5% level (weight of the feed).

The final concentrations of aflatoxins in the feed were analyzed by the Office of the Texas State Chemist. The aflatoxin concentration in the control feed (clean corn) was <20 ppb and, aflatoxin feed contained 1400 ppb. High aflatoxin concentration in the feed (>1000 ppb) is needed to cause significant alteration that can be detected by differences in body-weight (Aletor et al., 1981; Ostrowski-Meissner, 1984).

Feeding experiment

The poultry experiment design is illustrated in Table 5.1, which consisted of two major groups: 1) clean feed-group (<20 ppb aflatoxin) and 2) aflatoxin contaminated feed-group (1400ppb). The clean feed group or control group was used to identify any potential negative effects caused by the clays in the feed. Meanwhile the aflatoxin group evaluated the efficacy of selected clays to adsorb aflatoxin before it can be assimilated by the organism.

Table 5.1: Poultry experiment design.

Treatment	Treatment identification	No. Replicas (pens)	Chickens per replicate	Total chicken per treatment
Clean feed + No clay	CN	8	5	40
Clean feed + Clay 4TX	CA	8	5	40
Clean feed + Clay 1TX	CB	8	5	40
Aflatoxin feed + No clay	AN	8	5	40
Aflatoxin feed + Clay 4TX	AA	8	5	40
Aflatoxin feed + Clay 1TX	AB	8	5	40

Total chickens : 240

One-day-old broiler chickens were subjected to an aflatoxin-feed diet for 21 days. They were placed in batteries under controlled temperature and, artificial light. Their growth and health status were monitored daily for this three weeks feeding experiment. Mortality was recorded daily. At 1, 7 and 14 days the feed offered was recorded and at days 7, 14 and 21 the feed retained was recorded too. Body weight by pen (or replica), which is the average weight of the number of birds at the recording day, was obtained at days 7 and 14. At the end of the experiment (21 days) all of the individual weights of the surviving chickens were recorded.

At day 21 all the chickens were sacrificed and organs were collected. Liver, kidney, spleen and heart weights were recorded. Liver, kidney and spleen are reported to show weight change due to aflatoxins (Tessari et al., 2006). The liver is the most affected organ by aflatoxins that shows physical alterations as color and weight changes (Aletor et al., 1981; Phillips et al., 1988).

Five liver samples from each of the six treatments were collected and frozen for aflatoxin quantification by the Veterinary Diagnostic Laboratory at Iowa State University. Concentrations of AfB_1, AfB_2, AG_1, AG_2 and AfM_1 were analyzed with HPLC. The efficacy of the clays was evaluated based body weight, feed conversion ratio, weight gain and aflatoxin concentrations in liver between the aflatoxin feed group and the aflatoxin feed plus clays groups. The clean-feed group was also statistically

evaluated to observe differences between clean feed with no clay and the clean feed plus clays in order to address the safety of the clay addition in feed.

Results and discussion

Statistical data analysis of the indicator parameters as body weight and feed conversion ratio were analyzed using the average data per pen of each treatment with 8 replicas. The statistical analysis did not show significant differences for the control or aflatoxin group (Table 5.2).

$$\text{Body weight} = \frac{\text{Final weight per pen}}{\text{Number of birds (survived)}}$$

$$\text{Feed conversion ratio (FCR)} = \frac{\text{Feed offered} - \text{Feed retained}}{(\text{Final body weight} + \text{Mortality weight}) + \text{Mortality per week}}$$

In the control group the result demonstrated that the clays did not cause any negative effects in the weight of the chickens. On the other hand, no statistical differences at a $p<0.05$ between the aflatoxin no clay and aflatoxin plus clay treatments were observed, indicating that at this level the efficiency of the clays was not significant. The coefficient of variation in the experiment was large (>20%), which decrease the precision of getting significant differences between the body weight treatment means. This error is caused by the different response of animals to the treatments, a common observation in animal experiments that can be reduced by increasing the number of repetitions in each group. Thus, statistical analyses of body weight and organ/body weight ration were done using the individual chicken weights that survive the 21 days of treatment. The data analysis using individual weights does not allow accounting for FCR parameter.

Table 5.2 Treatments, body weight per bird and FCR means comparison.

Treatment	No. birds	Body weight per bird (g)	Feed conversion ratio (FRC)
		Aflatoxin feed group	
Clay 4TX	31	379 ± 5^{a}	$0.600^{a} \pm 0.033$
Clay 1TX	30	348 ± 12^{ab}	$0.628^{a} \pm 0.020$
No clay	30	308 ± 14^{b}	$0.536^{a} \pm 0.037$
		Clean feed group	
No clay	40	724 ± 15^{a}	$0.562^{a} \pm 0.026$
Clay 4TX	40	677 ± 24^{a}	$0.525^{a} \pm 0.013$
Clay 1TX	40	675 ± 22^{a}	$0.508^{a} \pm 0.025$

Data in a group with different letters ($^{a\ b}$) are significantly different at $p < 0.05$

Body weight of individual chickens

To compare the average body weight after 3 weeks, an ANOVA F-test was used following a complete randomized design (CRD) data analysis. The average body weights of chickens in <20 ppb aflatoxin treatment did not show significant differences. Although statistically it was not relevant, a slight decrease in average body weight in the clay treatments in comparison with no clay treatment was observed. This represents about 6.4 and 6.6 percent of reduction in clay 4TX and clay 1TX treatments, respectively. Similar weight reduction under clay treatments had been observed in other animal experiments (Kubena et al., 1993a; Bailey et al., 2006) but the explanation for this result was not addressed. The 0.5% addition of clay to the diet represents a same percentage of reduction in nutrient density, which can affect body weight. Additionally, due to the high adsorptive capacity of smectites there is a potential of the clays adsorbing some essential nutrients, which will be further tested. In the literature Zn, Mn, vitamin A and riboflavin had been evaluated as indicators of nutrient utilization, showing that at 0.5% clay addition did not cause significant impact (Phillips et al., 1995).

The difference in body weight of chickens under aflatoxin without clay diet was statistically different from the chickens subjected to aflatoxin plus clay treatments (Table 5.3). The addition of clay in the diet showed an increase in body weight, which reflects a 21% and 14% improvement with clay 4TX and clay 1TX, respectively. There was no significant difference among the clay treatments, suggesting that the clay effect was similar. The higher body weight in chickens under aflatoxin plus clay was an indirect indicator that the clays protected the animals from the toxic effects of the toxin.

Table 5.3 Treatments and body weight means comparison.

Treatment	No. birds	Body weight (g)	Treatment comparison (α=0.05)	
		Aflatoxin feed group		*Improvement (%)*
Clay 4TX	31	371 ±12	A	21
Clay 1TX	30	351 ±12	B	14
No clay	30	307 ±11	B	control
		Clean feed group		*Reduction (%)*
No clay	40	724 ±15	A	control
Clay 4TX	40	678 ±25	B	6.4
Clay 1TX	40	675 ±23	B	6.6

Data in a group with different letters (A B) are significantly different at $p < 0.05$

The large variation among individuals in each aflatoxin group was demonstrated by a box plot (Figure 5.1) which illustrates the data range. For example, aflatoxin with clay 4TX treatment had a difference of 210 g from the lowest bird weight to the highest. This variation was more pronounced in the aflatoxin with clay 1TX treatment. The differences in response of individuals could be an effect of non-homogenous distribution of the clay or hot spots of aflatoxin in the feed.

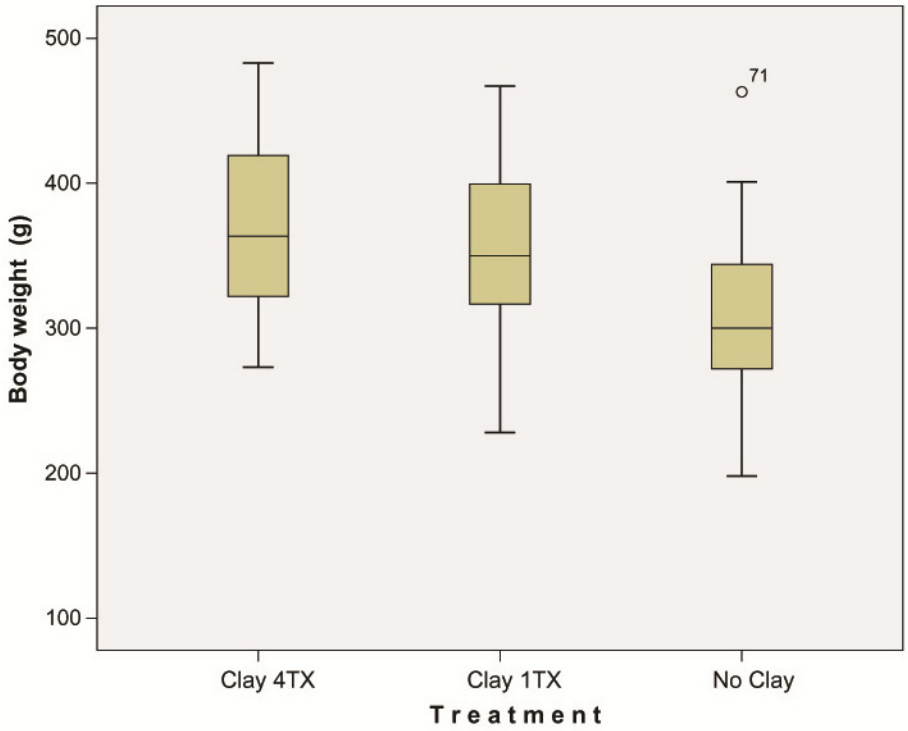

Figure 5.1: Box plot of individual body weight of chickens in the aflatoxin feed group that survived at 21 days.

The in vitro experiments showed that both clays were effective in adsorbing aflatoxin as also observed in the animal experiment. Moreover the experimental data showed that bentonite 4TX had a higher aflatoxin adsorption capacity than 1TX. This trend was also observed in the body weight of the chickens under aflatoxin plus clay feed. Even though there was no statistical difference between the average body-weight among the clay treatments, sample 4TX showed an increase in body weight of 21% while 1TX only increased the body weight by 14%. In the present study the in vitro result are in agreement with the in vivo performance in confirming the high binding capacity of the samples but also in the difference in adsorption. The characterization of the selection criteria is based only in bentonites samples and may not apply to other type of adsorbents.

Relative organ weight

The <20 ppb aflatoxin group showed no differences in any of the organ/body weight ratios, this observation indicates that the addition of clays in the diet did not cause significant secondary effects (Table 5.4).

One of the most characteristic signs of aflatoxicosis is the deterioration or alteration in size and color of the liver, as a result of continuous exposure due to metabolic transformation of the aflatoxin molecules. In animal experiments with broiler chickens researcher observed an increase in relative liver weight of the animals on aflatoxin diet in comparison with the control group (Aletor et al., 1981; Huff et al., 1992; Jaraprakash et al., 1992; Bailey et al., 2006; Tessari et al., 2006).

However, in the present experiment, the liver/body weight ratio was not significantly different among treatments for the aflatoxin group. The similar ratios among the aflatoxin treatments could be a result of the high dose of aflatoxin in the diet. This indicates that the relative liver weight was not a sensitive parameter to show the protection effect of the clays, at $p<0.05$. Similar results were obtained by Bailey et al., (2006), where the addition of 0.5% of HSCA clay to a feed containing ~ 3600 ppb of aflatoxin did not show significant differences in relative liver weight.

Researchers have observed that besides the liver other organs as heart, kidney, and spleen can be affected by aflatoxicosis, mainly by an increase in relative weight (Huff et al., 1992; Bailey et al., 1998; Quezada et al., 2000). Heart, kidney, and spleen weights were not significantly different among groups, indicating that these are not sensitive parameter to evaluate efficacy of the clays at a 5% level. This is in agreement with other animal experiments (Santurio, 1999). Tessari et al., (2006) observed differences in heart relative weight of animals subjected to different aflatoxin concentration; but no changes were observed in spleen relative weight.

Table 5.4: Organ /body weight ratios differences by group.

Treatment	No. birds	Organ /body weight ratio			
		Liver	Kidney	Spleen	Heart
		Aflatoxin feed group			
Clay 4TX	31	0.0464[ab]	0.0195[a]	0.00234[a]	0.00970[a]
Clay 1TX	30	0.0489[a]	0.0186[a]	0.00230[a]	0.00926[a]
No clay	30	0.0444[b]	0.0179[a]	0.00224[a]	0.00970[a]
		Clean feed group			
Clay 4TX	40	0.0327[a]	0.0086[a]	0.0011[a]	0.0064[a]
Clay 1TX	40	0.0323[a]	0.0086[a]	0.0010[a]	0.0066[a]
No clay	40	0.0310[a]	0.0079[a]	0.0010[a]	0.0064[a]

[ab] Data in a column with different superscripts are significantly different at $p < 0.05$

Liver appearance

Livers from animals subjected to < 20 ppb aflatoxin diet with and without clays had a similar dark red color and minimal size difference. In contrast, the high-aflatoxin exposure produced a significant change in the color of the livers with and without clay. Representative livers from animals under high aflatoxin without clay treatment showed pale yellow livers (Figure 5.2). The color improvement in the livers of animals feed with high aflatoxin plus clay treatment; indicating that less aflatoxin was gastro intestinally adsorbed. This indicates that the clays had a protective effect on the chickens by reducing the exposure.

The observations in this experiment concerning color differences in livers were in agreement with other animal experiments (Aletor et al., 1981; Phillips et al., 1988; Leeson et al., 1995; Miazzo et al., 2005a). Aletor et al., (1981) had reported that the relative liver weight tends to increase in size due to the fat accumulation.

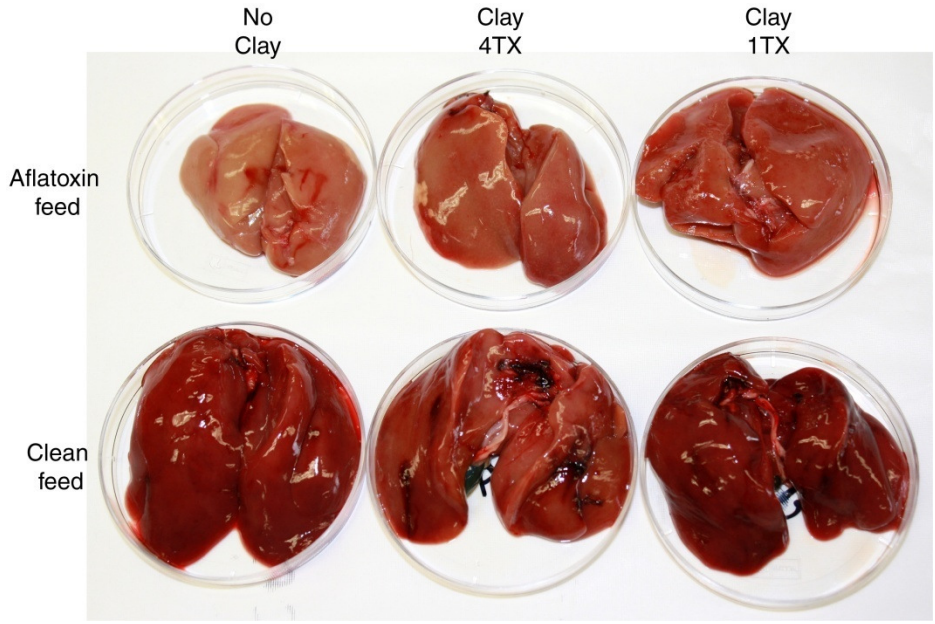

Figure 5.2: Representative chicken livers from each treatment, aflatoxin vs clean feed.

Aflatoxins in liver

No aflatoxin was detected in livers from the < 20 ppb aflatoxin clean feed treatment. Concentrations for the five aflatoxins tested were observed in the tissue samples on 1400 ppb aflatoxin contaminated feed (Table 5.5). AfB1 and AfG1 were present in the highest concentrations. These two are also the major forms present in the feed, and are metabolized to AfB2, AfG2 and AfM1. The metabolites are easily excreted, which explains the low concentrations observed (Chen et al., 1984). On the other hand the high levels of AfB1 and AfG1 were also indicators of overexposure because the livers were not able to metabolize all the mycotoxins absorbed.

Only AfB1 and AfG1 showed significant differences between the no clay and clay treatments. Considering the concentrations of all five aflatoxins, the total aflatoxin showed noticeable difference between the treatments (Table 5.5). The tissues from chicken subjected to an aflatoxin diet without clay addition showed higher concentration of total aflatoxin. There was a significant reduction (~36%) in the total aflatoxins concentration by the addition of clay in the diet. The total aflatoxin concentration was similar for both clay treatments.

Table 5.5: Aflatoxins concentration in livers from chickens in the high aflatoxin contaminated feed treatments.

	Concentration in liver (ppb)					
Treatment	AfB_1	AfG_1	AfB_2	AfG_2	AfM_1	Total aflatoxins
No clay	18.8^{a}	16.4^{a}	0.30^{a}	1.18^{a}	1.34^{a}	38.02^{a}
Clay 4TX	14.6^{ab}	7.6^{b}	0.46^{a}	0.46^{a}	1.20^{a}	24.32^{b}
Clay 1TX	10.0^{b}	11.0^{ab}	0.18^{a}	2.00^{a}	0.86^{a}	24.04^{b}

[ab] Data in a column with different superscripts are significantly different at $p < 0.05$

An important observation regarding the influence of AfB1 metabolism and high concentration in the liver was made by Cheng et al, (1984). They conducted a similar experiment using high concentration of AfB1 (2057 ppb) but the levels in liver tissue was less than the reported in the present experiment. Based on the observation from previous experiments, they attribute that there is influence of the presence of AfG1 in the metabolism of AfB1 which affects the concentrations in the liver. This indicates that the source of aflatoxin in the feed can also affect the variability of the results, an important consideration for further studies.

Conclusions

The in vivo experiments showed that both clays were effective in adsorbing aflatoxin. Bentonite 4TX increased more the body weight and both bentonites reduced concentrations of aflatoxins in liver. The better performance of clay 4TX is in agreement with the in vitro experiment presented in previous chapters.

Additionally, chickens subjected to an aflatoxin plus clay diet showed an improvement in liver color. Despite the improvements, the chickens fed bentonite did not have a 100% recovery from aflatoxin toxicity.

Furthermore, the addition of clays to aflatoxin-free diet did not cause statistically differences in chicken performance, yet further studies need to be conducted to address the interaction of clays with nutrients.

CHAPTER VI
SUMMARY

The properties evaluated according to the published selection criteria were useful in identifying potential aflatoxin adsorbents. Smectite-rich bentonites were effective in adsorbing aflatoxin according to lab experiments and corroborated with animal performance. This indicates that smectite is the most important mineral for adsorption of aflatoxin. Furthermore, a detailed screening of the mineral content in bentonites is important to identify possible health hazard minerals or heavy metals.

Laboratory experiments must be conducted to test the adsorption capacity of any bentonite added to feed and that claims to adsorb aflatoxins. As it was observed from the presented research the high adsorption capacity obtained in vitro correlated with the results in animal experiments. Although some researchers have reported that some clays did not perform as good in vivo as in vitro, it is more likely that a poor performance in vitro is not going to show a high performance in vivo.

This study corroborates the effectiveness of bentonites as aflatoxin adsorbent by increasing the animal performance. However there were several identified limitations or issues that have to be considered for further animal experiments. It is suspected that the large variations in animal performance can be a result of the uneven distribution of the aflatoxin in the feed, as well as the distribution of the clay. In terms of the clay, further studies will be conducted to address mechanism to provide a better incorporation of the clay in the feed. More tests will be conducted to improve dispersion of clay in the gastro intestinal tract. The competition of vitamins and essential nutrients with aflatoxin for adsorption sites had been recognized, yet there are no data that address the direct interaction of vitamins or nutrients with bentonites. There is a need of more sensitive biomarkers that can allow using aflatoxin concentrations at more realistic levels encounter in the field and storage.

REFERENCES

Aletor, V.A., O.B. Kasali, and B.L. Fetuga. 1981. Effects of sublethal levels of dietary aflatoxins in broiler chickens. Zentralblatt für Veterinärmedizin Reihe A 28:774-781.

Aquino, S., F. Ferreira, D.H.B. Ribeiro, B. Correa, R. Greiner, and A. Villavicencio. 2005. Evaluation of viability of *Aspergillus flavus* and aflatoxins degradation in irradiated samples of maize. Brazilian Journal of Microbiology 36:352--356.

Arafa, A.S., R.J. Bloomer, H.R. Wilson, C.F. Simpson, and R.H. Harms. 1981. Susceptibility of various poultry species to dietary aflatoxin. British Poultry Science 22:431--436.

Bailey, C.A., G.W. Latimer, A.C. Barr, W.L. Wigle, A.U. Haq, J.E. Balthrop, and L.F. Kubena. 2006. Efficacy of montmorillonite clay (NovaSil PLUS) for protecting full-term broilers from aflatoxicosis. Journal of Applied Poultry Research 15:198--206.

Bailey, R., L. Kubena, R. Harvey, S. Buckley, and G. Rottinghaus. 1998. Efficacy of various inorganic sorbents to reduce the toxicity of aflatoxin and T-2 toxin in broiler chickens. Poultry Science 77:1623-1630.

Basappa, S.C. 2009. Aflatoxins formation, analysis and control. Alpha Science International Ltd., Oxford.

Bergaya, F., G. Lagaly, M. Vayer, B.K.G.T. Faïza Bergaya, and L. Gerhard. 2006. Cation and anion exchange, p. 979-1001, *In* F. Bergaya and B. K. G. Theng, eds. Developments in clay science, Vol. 1. Elsevier, Amsterdam.

Boettinger, J.L., and D.W. Ming. 2002. Zeolites, p. 585-605, *In* J. B. Dixon and D. G. Schulze, eds. Soil mineralogy with environmental applications. Soil Science Society of America, Inc, Madison, WI.

Boudergue, C., C. Burel, S. Dragacci, M.-C. Favrot, J.-M. Fremy, C. Massimi, P. Prigent, P. Debongnie, L. Pussemiern, H. Boudra, D. Morgavi, I. Oswald, A. Perez, and G. Avantaggiato. 2009. Review of mycotoxin-detoxifiying agents used as feed additives: mode of action, efficacy and feed/food safety. Agense Francaise de Securite Sanitaire des Aliements, Centre d'etude et de recherches veterinaires et agrochimiques, Institut National de la Recherche Agronomique, Institute de Recerca i Tecnologia Agroalimentari, Instituto di Scienze delle Produzioni Alimentari. .

Brown, R.L., P.J. Cotty, and T.E. Cleveland. 1991. Reduction in aflatoxin content of maize by atoxigenic strains of *Aspergillus flavus*. Journal of Food Protection 54:623-626.

Cotty, P.J. 1990. Effect of atoxigenic strains of *Aspergillus flavus* on aflatoxin contamination of developing cottonseed. Plant Disease 74:233-235.

Cotty, P.J., and P. Bayman. 1993. Competitive exclusion of a toxigenic strain of *Aspergillus flavus* by an atoxigenic strain. Phytopathology 83:1283-1287.

Chen, C., A.M. Pearson, T.H. Coleman, J.I. Gray, J.J. Pestka, and S.D. Aust. 1984. Tissue deposition and clearance of aflatoxins from broiler chickens fed a contaminated diet. Food and Chemical Toxicology 22:447-451.

Chipera, S.J., and D.L. Bish. 2001. Baseline studies of the Clay Minerals Society source clays: powder x-ray diffraction analyses. Clays and Clay Minerals 49:398-409.

Daigle, D.J., and P.J. Cotty. 1995. Formulating atoxigenic *Aspergillus flavus* for field release. Biocontrol Science and Technology 5:175--184.

Dakovic, A., M. Tomasevic-Canovic, V. Dondur, G.E. Rottinghaus, V. Medakovic, and S. Zaric. 2005. Adsorption of mycotoxins by organozeolites. Colloids and Surfaces B-Biointerfaces 46:20--25.

Dakovic, A., M. Tomasevic-Canovic, V. Dondur, A. Vujakovic, and P. Radosevic. 2000. Kinetics of aflatoxin B1 and G2 adsorption on Ca-clinoptilolite. Journal of the Serbian Chemical Society 65:715--723.

Dalvi, R. 1986. An overview of aflatoxicosis of poultry: Its characteristics, prevention and reduction. Veterinary Research Communications 10:429-443.

Davidson, J.N., J.D. Babish, K.A. Delaney, and D.R. Taylor. 1987. Hydrated sodium calcium aluminosilicate decreases the bioavailability of aflatoxin in the chicken. Poultry Science 66:89.

Deng, Y., A.L.B. Velázquez, F. Billes, and J.B. Dixon. 2010. Bonding mechanisms between aflatoxin B1 and smectite. Applied Clay Science 50:92-98.

Deng, Y., G.N. White, and J.B. Dixon. 2009. Soil mineralogy laboratory manual. Texas A&M University, College Station.

Devegowda, G., and T.N.K. Murthy. 2005. Mycotoxins: their effect in poultry and some practical solutions, p. 25-50, *In* D. E. Diaz, ed. The mycotoxin blue book. Nottingham University Press, Nottingham, England.

Diaz, D., and T. Smith. 2005. Mycotoxin sequestring agents: practical tools for the neutralization of mycotoxins, p. 323-334, *In* D. E. Diaz, ed. The mycotoxin blue book Nottingham University Press, Nottingham, England.

Dixon, J.B., I. Kannewischer, M.G. Tenorio Arvide, and A.L. Barrientos Velazquez. 2008. Aflatoxin sequestration in animal feeds by quality-labeled smectite clays: an introductory plan. Applied Clay Science 40:201--208.

Dorner, J.W. 2004. Biological control of aflatoxin contamination of crops. Toxin Reviews 23:425-450.

Dorner, J.W., and R.J. Cole. 1997. A method for determining kernel moisture content and aflatoxin concentrations in peanuts. Journal of the American Oil Chemists Society 74:285--288.

Doyle, M.P., R.S. Applebaum, R.E. Brackett, and E.H. Marth. 1982. Physical, chemical and biological degradation of mycotoxins in foods and agricultural comodities. Journal of Food Protection 45:946-971.

Dvorackova, I. 1990. Aflatoxins and human health CRC Press, Inc, Boca Raton, FL.

Eaton, D.L., and J.D. Groopman. 1994. The toxicology of aflatoxins: human health, veterinary and agricultural significance. Academic Press, Inc, New York.

Edrington, T.S., A.B. Sarr, L.F. Kubena, R.B. Harvey, and T.D. Phillips. 1996. Hydrated sodium calcium aluminosilicate (HSCAS), acidic HSCAS, and activated charcoal reduce urinary excretion of aflatoxin M1 in turkey poults. Lack of effect by activated charcoal on aflatoxicosis. Toxicology Letters 89:115-121.

Eisenhour, D.D., and R.K. Brown. 2009. Bentonites and its impact on modern life. Elements 5(2):83-88.

Eshel, G., G.J. Levy, U. Mingelgrin, and M.J. Singer. 2004. Critical evaluation of the use of laser diffraction for particle-size distribution analysis. Soil Sci Soc Am J 68:736-743.

Gates, W.P. 2005. Infrared spectroscopy and the chemistry of dioctahedral smectites, p. 125-68, *In* I. Kloprogge, ed. Vibrational Spectroscopy to Clay Minerals and Layered Double Hydroxides, Vol. 13. The Clay Minerals Society, Aurora, Co.

Grant, P.G., and T.D. Phillips. 1998. Isothermal adsorption of aflatoxin B1 on HSCAS clay. Journal of Agricultural and Food Chemistry 46:599--605.

Harvey, R.B., L.F. Kubena, M.H. Elissalde, and T.D. Phillips. 1993. Efficacy of zeolitic ore compounds on the toxicity of aflatoxin to growing broiler-chickens. Avian Diseases 37:67--73.

Heathcote, J.G., and J.R. Hibbert. 1978. Aflatoxins: chemical and biological aspects. Elsevier, Amsterdam.

Heinrich, M. 2003. IARC Monographs on the evaluation of carcinogenic risks to humans: some traditional herbal medicines, some mycotoxins, naphthalene and styrene: IARC working group on the evaluation of carcinogenic risks to humans 0378-8741. International Agency for Research on Cancer (IARC), Lyon, France.

Huff, W.E., L.F. Kubena, R.B. Harvey, and T.D. Phillips. 1992. Efficacy of hydrated sodium calcium aluminosilicate to reduce the individual and combined toxicity of aflatoxin and ochratoxin-A. Poultry Science 71:64-69.

Jaraprakash, M., R.N.S. Gowda, S.K. Vijasarathi, and S.J. Seshadri. 1992. Adsorbent efficacy of hydrated sodium calcium aluminosilicate in induced aflatoxicosis in briolers. Indian Journal of Veterinary Pathology 16:102-105.

Jouany, J.P., and D.E. Diaz. 2005. Effects of mycotoxins in ruminants, p. 295-299, *In* D. E. Diaz, ed. The mycotoxin blue book. Nottingham University Press, Nottingham.

Kabak, B., A.D.W. Dobson, and I. Var. 2006. Strategies to prevent mycotoxin contamination of food and animal feed: A review. Critical Reviews in Food Science and Nutrition 46:593--619.

Kannewischer, I., M.G.T. Arvide, G.N. White, and J.B. Dixon. 2006. Smectite clays as adsorbents of aflatoxin B1: initial steps. Clay Science 12:199--204.

Kermanshashi, H., A.R. Hazegh, and N. Afzali. 2009. Effect of sodium bentonite in broiler chickens feed diet contamiated with aflatoxin B1. Journal of Animal and Veterinary Advances 8:1631-1636.

Kubena, L.F., R.B. Harvey, R.H. Bailey, S.A. Buckley, and G.E. Rottinghaus. 1998. Effects of a hydrated sodium calcium aluminosilicate (T-Bind^{TM}) on mycotoxicosis in young broiler chickens. Poultry Science 77:1502-1509.

Kubena, L.F., R.B. Harvey, W.E. Huff, and D.E. Corrier. 1990. Efficacy of a hydrated sodium calcium aluminosilicate to reduce the toxicity of aflatoxin and T-2 toxin. Poultry Science 69:1078-1086.

Kubena, L.F., R.B. Harvey, W.E. Huff, M.H. Elissalde, A.G. Yersin, T.D. Phillips, and G.E. Rottinghaus. 1993a. Efficacy of hydrated sodium calcium aluminosilicate to

reduce the toxicity of aflatoxin and daicetoxyscirpenol. Poultry Science 72:51-59.

Kubena, L.F., R.B. Harvey, T.D. Phillips, and B.A. Clement. 1992. The use of sorbent compound to modify the toxic expression of mycotoxins in poultry. Proceedings of 19th World's Poultry Congress XIX:357-361.

Kubena, L.F., R.B. Harvey, T.D. Phillips, and B.A. Clement. 1993b. Effect of hydrated sodium calcium aluminosilicates on aflatoxicosis on broilder chicks. Poultry Science 72:651-657.

Leeson, S., G.J. Diaz G, and J.D. Summers. 1995. Poultry metabolic disorders and mycotoxins University Books, Ontario, Canada.

Lindemann, M.D., D.J. Blodgett, E.T. Kornegay, and G.G. Schurig. 1993. Potential ameliorators of aflatoxicosis in weanling growing swine. Journal of Animal Science 71:171--178.

Madejova, J., and P. Komadel. 2001. Baseline studies of the Clay Minerals Society source clays: infrared methods. Clays and Clay Minerals 49:410-432.

Madejova, J., P. Komadel, and B. Cicel. 1994. Infrared study of octahedral site populations in smectites. Clay Minerals 29:319-326.

Madejová, J. 2003. FTIR techniques in clay mineral studies. Vibrational Spectroscopy 31:1-10.

Magnoli, A.P., L.R. Cavaglieri, C.E. Magnoli, J.C. Monge, R.D. Miazzo, M.F. Peralta, M.A. Salvano, C.A. Da Rocha Rosa, A.M. Dalcero, and S.M. Chiacchiera. 2008a. Bentonite performance on broiler chickens fed with diets containing natural levels of aflatoxin B1. Revista Brasileira de Medicna Veterinaria 30:55-60.

Magnoli, A.P., L. Tallone, C.A.R. Rosa, A.M. Dalcero, S.M. Chiacchiera, and R.M. Torres Sanchez. 2008b. Commercial bentonites as detoxifier of broiler feed contaminated with aflatoxin. Applied Clay Science 40:63-71.

Mann, R., and H.J. Rehm. 1975. Degradation of aflatoxin B_1 by microorganisms Naturwissenschaften 62:537-538.

Marquez Marquez, R. 1995. Aflatoxin adsorbent capacity of two Mexican aluminosilicates in experimentally contaminated chick diets. Food Additives and Contaminants 1995.

Miazzo, R., M. Peralta, C. Magnoli, M. Salvano, S. Ferrero, S. Chiacchiera, E. Carvalho, C. Rosa, and A. Dalcero. 2005a. Efficacy of sodium bentonite as a detoxifier of broiler feed contaminated with aflatoxin and fumonisin. Poult Sci 84:1-8.

Miazzo, R., M. Peralta, C. Magnoli, M. Salvano, S. Ferrero, S. Chiacchiera, E. Carvalho, C. Rosa, and A. Dalcero. 2005b. Efficacy of sodium bentonite as a detoxifier of broiler feed contaminated with aflatoxin and fumonisin. Poultry Science 84:1-8.

Mulder, I., A.L. Barrientos Velazquez, M.G. Tenorio Arvide, G.N. White, and J.B. Dixon. 2008. Smectite clay sequestration of aflatoxin B1: mineral dispersivity and morphology. Clays and Clay Minerals 56:559--571.

Murray, H.H. 2006. Structure and composition of the clay minerals and their physical and chemical properties., p. 7-31, *In* H. H. Murray, ed. Developments in Clay Science, Vol. Volume 2. Elsevier, Amsterdam.

Ostrowski-Meissner, H.T. 1984. Biochemical and physiological responses of growing chickens and ducklings to dietary aflatoxins. Comparative Biochemistry and Physiology Part C: Comparative Pharmacology 79:193-204.

Pasha, T.N., M.U. Farooq, F.M. Khattak, M.A. Jabbar, and A.D. Khan. 2007. Effectiveness of sodium bentonite and two commercial products as aflatoxin absorbents in diets for broiler chickens. Animal Feed Science and Technology 132:103-110.

Pawlowski, N.E., G.L. Schoenhard, D.J. Lee, L.M. Libbey, P.M. Loveland, and R.O. Sinnhuber. 1976. Reduction of aflatoxin B_1 to aflatoxicol. Agricultural and Food Chemistry 25:437-438.

Peers, F.G., and C.A. Linsell. 1975. Aflatoxin contamination and its heat stability in Indian cooking oils. Tropical Science 17:229-232.

Phillips, T.D. 1999. Dietary clay in the chemoprevention of aflatoxin-induced disease. Toxicological Sciences 52:118--126.

Phillips, T.D., E. Afriyie-Gyawu, J. Williams, H. Huebner, N.A. Ankrah, D. Ofori-Adjei, P. Jolly, N. Johnson, J. Taylor, A. Marroquin-Cardona, L. Xu, L. Tang, and J.S. Wang. 2008. Reducing human exposure to aflatoxin through the use of clay: a review. Food Additives and Contaminants, Part A: Chemistry, Analysis, Control, Exposure and Risk Assessment 25:134-145.

Phillips, T.D., B.A. Clement, and D.L. Park. 1994. Approaches to reduction of aflatoxins in foods and feeds, p. 383-399, *In* D. L. Eaton, ed. The toxicology of aflatoxins. Academic Press, Inc, San Diego, CA.

Phillips, T.D., L.F. Kubena, R.B. Harvey, D.R. Taylor, and N.D. Heidelbaugh. 1987. Mycotoxin hazards in agriculture: new approach to control. Journal of Animal Veterinary Medicine:1617-1618.

Phillips, T.D., L.F. Kubena, R.B. Harvey, D.R. Taylor, and N.D. Heidelbaugh. 1988. Hydrated sodium calcium aluminosilicate: a high affinity sorbent for aflatoxin. Poultry Science 67:243-247.

Phillips, T.D., S.L. Lemke, and P.G. Grant. 2002. Characterization of clay-based enterosorbents for the prevention of aflatoxicosis. Advances in Experimental Medicine and Biology 504:157--171.

Phillips, T.D., A.B. Sarr, and P.G. Grant. 1995. Selective chemisorption and detoxification of aflatoxins by phyllosilicate clay. Natural Toxins 3:204-213.

Pimpukdee, K., L.F. Kubena, C.A. Bailey, H.J. Huebner, E. Afriyie-Gyawu, and T.D. Phillips. 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. Poultry Science 83:737--744.

Piva, G., F. Galvano, A. Pietri, and A. Piva. 1995. Detoxification methods of aflatoxins. A review. Nutrition Research 15:767-776.

Quezada, T., H. Cuéllar, F. Jaramillo-Juárez, A.G. Valdivia, and J.L. Reyes. 2000. Effects of aflatoxin B1 on the liver and kidney of broiler chickens during development. Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology 125:265-272.

Ramos, A.J., J. FinkGremmels, and E. Hernandez. 1996. Prevention of toxic effects of mycotoxins by means of nonnutritive adsorbent compounds. Journal of Food Protection 59:631--641.

Reid-Soukup, D.A., and A.L. Ulery. 2002. Chapter 15 Smectites, p. 467- 495, *In* J. B. Dixon and D. G. Schulze, eds. Soil mineralogy with environmental applications. Soil Science Society of America, Madison, WI.

Rosa, C., R. Miazzo, C. Magnoli, M. Salvano, S. Chiacchiera, S. Ferrero, M. Saenz, E. Carvalho, and A. Dalcero. 2001. Evaluation of the efficacy of bentonite from the south of Argentina to ameliorate the toxic effects of aflatoxin in broilers. Poultry Science 80:139-144.

Rustom, I.Y.S. 1997. Aflatoxin in food and feed: occurrence, legislation and inactivation by physical methods. Food Chemistry 59:57--67.

Santurio, J.M. 1999. Effect of sodium bentonite on the performance and blood variables of broiler chickens intoxicated with aflatoxins. British Poultry Science 40:115 - 119.

Scheideler, S.E. 1993. Effects of various types of aluminosilicates and aflatoxin-B1 on aflatoxin toxicity, chick performance, and mineral status. Poultry Science 72:282--288.

Southern, L.L., T.L. Ward, T.D. Bidner, and L.G. Hebert. 1994. Effect of sodium bentonite or hydrated sodium-calcium aluminosilicate on growth-performance and tibia mineral concentrations in broiler chicks fed nutrient-deficient diets. Poultry Science 73:848--854.

Staff, S.S.L. 2004. Soil Survey laboratory methods manual. Soil Survey Investigation Report No. 42. USDA, NRCS, Lincoln, NE.

Stroud, J., E. English, S. Davidson, B. Hopkins, and G. Latimer. 2006. Effect of feed additives on aflatoxin in milk of dairy cows fed aflatoxin-contaminated diets. Journal of Animal Science 84:129--129.

Tejada-Castaneda, Z.I., E. Avila-Gonzalez, M.T. Casaubon-Huguenin, R.A. Cervantes-Olivares, C. Vasquez-Pelaez, E.M. Hernandez-Baumgarten, and E. Moreno-Martinez. 2008. Biodetoxification of aflatoxin-contaminated chick feed. Poultry Science 87:1569-1576.

Tenorio Arvide, M.G., I. Mulder, A.L. Barrientos Velazquez, and J.B. Dixon. 2008. Smectite sorption of aflatoxin B1 and molecular changes suggested by FTIR. Clays and Clay Minerals 56:572--579.

Tessari, E.N.C., C.A.F. Oliveira, A.L.S.P. Cardoso, D.R. Ledoux, and G.E. Rottinghaus. 2006. Effects of aflatoxin B1 and fumonisin B1 on body weight, antibody titres and histology of broiler chicks. British Poultry Science 47:357 - 364.

Teunisson, D.J., J.A. Robertson, and G.J. Boudreaux. 1970. Isolation and structure of a biologically reduced aflatoxin B1. Journal of Agricultural and Food Chemistry 18:1090-1091.

Thieu, N., B. Ogle, and H. Pettersson. 2008. Efficacy of bentonite clay in ameliorating aflatoxicosis in piglets fed aflatoxin contaminated diets. Tropical Animal Health and Production 40:649-656.

van der Marel, H.W., and H. Beutelspacher. 1976. Atlas of infrared spectroscopy of clay minerals and their admixtures. Elsevier, Amsterdam.

Vekiru, E., S. Fruhauf, M. Sahin, F. Ottner, G. Schatzmayr, and R. Krska. 2007. Investigation of various adsorbents for their ability to bind aflatoxin B1. Mycotoxin Research 23:27-33.

Zain, M.E. 2011. Impact of mycotoxins on humans and animals. Journal of Saudi Chemical Society 15:129-144.

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Experience

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Selected awards

* Best graduate student poster presentation. Joe B. and Martha J. Dixon Award 2008. SSSA S-9 soil mineralogy division. Joint Annual Meeting (GSA, ASA-CSSA-SSSA, and GCAGS), October 2008.

* Fourth place graduate student oral presentation. 46th Annual Meeting of Clay Mineral Society, June 2009