

## CHARACTERIZATION OF CLAY-BASED ENTEROSORBENTS FOR THE PREVENTION OF AFLATOXICOSIS

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### ABSTRACT

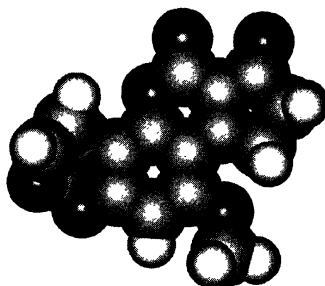
Appropriate chemical interventions that can block, retard, or significantly diminish foodborne exposure to aflatoxins are high priorities. A practical and effective approach to the aflatoxin problem has been the dietary inclusion of a processed calcium montmorillonite clay (HSCAS). HSCAS acts as an enterosorbent that rapidly and preferentially binds aflatoxins in the gastrointestinal tract resulting in decreased aflatoxin uptake and bioavailability. In mechanistic studies, we have shown that the intact dicarbonyl system in aflatoxin is essential for optimal sorption by HSCAS. Evidence also suggests that aflatoxins react at multiple sites on HSCAS clay surfaces (especially those within the interlayer region). Due to conceivable risks associated with the dietary inclusion of nonspecific binding agents, all aflatoxin enterosorbents should be tested in sensitive animal models for efficacy, safety, and the potential for nutrient interactions.

### INTRODUCTION: AFLATOXIN PROBLEM IN PERSPECTIVE

In historical context, the aflatoxin problem in foods is longstanding, unavoidable and seemingly inextricable. Aflatoxins are harmful by-products of mold growth and (though invisible to the naked eye), are potentially fatal to humans and animals (CAST, 1989; Phillips et al., 1995; 1997; 1998; Phillips, 1999). The aflatoxins are primarily produced by *Aspergillus flavus* and *A. parasiticus* fungi (Diener et al., 1983) and to a lesser extent by *Aspergillus nomius* (Kurtzman et al., 1987). These fungi are widespread and especially a problem during extended periods of drought. Thus, drought stress can be a frequent harbinger of intensified production of aflatoxins in the field. Also, the aflatoxins are heat stable and can survive a variety of food processing procedures and can occur as "unavoidable" contaminants of most foods (particularly those derived from maize and

peanuts). Animals can also secrete carcinogenic metabolites of aflatoxin in their milk. Consequently, a variety of dairy products (including cheese and ice cream) can be contaminated with these hazardous chemicals (CAST, 1989; Phillips et al., 1994).

Aflatoxin B<sub>1</sub> (AfB<sub>1</sub>), the most toxic of four naturally occurring aflatoxins, is a very potent carcinogen (IARC, 1976, 1987), and has been strongly implicated in the etiology of disease and death in man and animals (Figure 1). Aflatoxin B<sub>1</sub> is a direct acting mutagen and has been shown to disrupt genes involved in carcinogenesis (McMahon et al., 1986; 1987) and tumor suppression (Aguilar et al., 1993). In addition to DNA damage, AfB<sub>1</sub> interacts with RNA and intercellular proteins. The latter occurs through a reaction of the phenolate ion resonance form to produce a Schiff base with protein amino groups (e.g., lysine). These interactions may explain some of the non-genotoxic effects of aflatoxin (Eaton et al., 1994). There is substantial evidence that low-level exposure to aflatoxin may cause suppression of the immune system and increased susceptibility to disease (Richard et al., 1978; Peska and Bondy, 1994). Aflatoxin is also excreted in mother's milk and increases the morbidity of children with kwashiorkor (Adhikari, 1994). Clearly, the young of all species are more sensitive than adults to aflatoxin. What is not clear is how these numerous actions of aflatoxins may contribute to commonly perceived health problems in young humans and animals. Consequently, safe and sustainable dietary interventions that can prevent or reduce exposure of humans and animals to the aflatoxins are highly desirable.



**Figure 1.** Molecular model of aflatoxin B<sub>1</sub> showing the spatial orientation of functional groups, including the dicarbonyl system (Key: light gray, hydrogen; medium gray, carbon; dark gray, oxygen).

Since earliest recorded history, the unique properties and beneficial uses of clay minerals have been well-documented, and in many instances animals and humans have been reported to eat clay for the prevention of various illnesses (Munn, 1984; Phillips et al., 1995; Ramu et al., 1997; Clark et al., 1998; Phillips, 1999). Many of these clays vary considerably in their structures and ability to sequester toxic chemicals. This chapter will serve to review a simple, clay-based strategy for the prevention of aflatoxicosis in animals and further delineate the surface chemistry associated with aflatoxin sorption to HSCAS clay.

## **CLAY AND ZEOLITIC MINERALS**

### **Clay Mineral Chemistry**

Soil, the loose outer layer of the surface of the earth, is a surprisingly complex mixture. By volume, slightly less than 50% of soil is made up of mineral matter while

another 50% is the pore space between particles, filled with water or air. Soil can also contain a variable amount of organic matter (0.5 – 10%) and supports a diversity of living organisms such as bacteria, actinomycetes, fungi, algae and protozoa, which together make up much less than 1% of the soil. The solid particles of soil are classified into three categories based on size: sand (0.05 – 2 mm), silt (0.002-0.05 mm) and clay (less than 2 µm). The relative contribution of each type of particle to a particular soil determines its texture and other physical attributes and is used to name soil classes (Sylvia et al., 1997).

### **Soil Components**

Soil minerals are composed of relatively few elements. Oxygen and silicon are by far the most abundant components; silicates constitute 70-90% of the total mineral mass. Aluminum and iron are the next most abundant elements as they also participate in a variety of complexes with oxygen that form crystalline minerals. Smaller quantities of magnesium, calcium, sodium, potassium, titanium, manganese, nitrogen, phosphorus and sulfur are also present (Sylvia et al., 1997). Mineral classes are divided based on the identity of the dominant anionic group. Major mineral groups include: sulfides, oxides and hydroxides, halides, carbonates, nitrates, borates, phosphates, sulfates, tungstates and silicates. Within these classes, minerals are further subdivided based on structural similarities. The silicates are by far the largest class based on both number of mineral types they form and their overwhelming contribution to total mass of the earth's crust (Schulze, 1989). The basic structural unit for the silicates is a  $\text{SiO}_4$  tetrahedron in which  $\text{Si}^{4+}$  is located at the center and four  $\text{O}^{2-}$  are positioned at the apices. Tetrahedra can be linked together by sharing  $\text{O}^{2-}$ , and together can form a variety of more complex structures including rings (cyclosilicates), chains (inosilicates), sheets (phyllosilicates) and three-dimensional arrangements (tectosilicates) (Schulze, 1989).

### **Phyllosilicate Clay Minerals**

The phyllosilicates are a unique class of minerals that have been incorporated into a variety of production processes. Kaolinite is widely used in the ceramic industry and serves as a filler and coating for paper. Vermiculite, which becomes very light and porous when heated, is used as filler for concrete and also serves as a thermal and sound insulator. Montmorillonites have been employed in industry as decolorizing agents and for filtration of water and food products (Millot, 1979). The functionality of this class of minerals is a result of the distinctive structural and chemical properties of silicate layers. Phyllosilicates contain both tetrahedral and octahedral sheets. The tetrahedral sheets are composed of  $\text{SiO}_4$  tetrahedra linked together. Each tetrahedron shares three  $\text{O}^{2-}$  ions with three adjacent tetrahedra. This extends in all directions, forming a plane of basal oxygens. The fourth  $\text{O}^{2-}$  of each tetrahedron is referred to as the apical oxygen and is free to bond to other elements. The octahedral sheet is comprised of two planes of  $\text{OH}^-$  groups that form a hexagonal closest packing arrangement. To counter the negative charge of this structure, cations are located in the octahedral spaces between the layers. There are two possible ways to do this: a divalent cation (e.g.,  $\text{Mg}^{2+}$ ) must fill every available octahedral space to produce a trioctahedral arrangement, or a trivalent cation (e.g.,  $\text{Al}^{3+}$ ) fills two out of every three spaces to produce a dioctahedral arrangement. In a phyllosilicate, apical oxygens from the tetrahedral layer that replace  $\text{OH}^-$  groups from the octahedral layer and coordinate with the metal cation of the octahedral layer link the octahedral and tetrahedral layers. This commonly occurs in one of two ways: the 1:1 layer structure, in which one tetrahedral layer is bonded to one octahedral layer, and the 2:1 layer structure, in which an octahedral layer is bound on either side by a tetrahedral layer (Schulze, 1989). Frequently, cations in either the tetrahedral or octahedral layers are missing or have been replaced through an isomorphous substitution with another cation of lesser charge. The most common

substitutions are a replacement of  $\text{Si}^{4+}$  in the tetrahedral layer with  $\text{Al}^{3+}$  and a replacement of  $\text{Al}^{3+}$  or  $\text{Fe}^{3+}$  in the octahedral layer with  $\text{Mg}^{2+}$ . These substitutions result in a permanent negative charge on the phyllosilicate mineral (McBride, 1989). The amount of charge is dependent on the amount of isomorphous substitution. Variations in this rate and the identity of the substituted cations create the individual phyllosilicate types. To counteract the negative charge, these minerals attract cations such as  $\text{Na}^+$  and  $\text{Ca}^{2+}$  into the region between the layers (i.e., the interlayer). The amount of charge necessary to balance the negative charge of the clay is referred to as the cation exchange capacity (CEC). The interlayer cations are subject to the formation of layers of hydration, so when wetted, water enters the interlayer regions of the clay and causes it to swell (Bohn et al., 1979).

Phyllosilicates are grouped by the amount of substitution, and therefore, charge per layer. Of the 2:1 phyllosilicates, talc and pyrophyllite have almost no substitution and therefore very little charge; smectites have a layer charge per formula unit of 0.25 to 0.6; vermiculites have 0.6 to 0.9; and micas have complete substitution, resulting in a charge of 1 per formula unit (Bohn et al., 1979) (Table 1). The intermediate charge of smectites gives them optimal swelling properties. These clays attract cations into the interlayer, which hydrate and swell, expanding the space between layers. However, the negative surface charge of the mineral layer is not so high as to limit swelling due to electrostatic attraction to the cations. The montmorillonites are a subclass of smectites that contain isomorphous substitution in the octahedral sheets, resulting in a general formula of  $\text{Na}_x[(\text{Al}_{2-x}\text{Mg}_x)\text{Si}_4\text{O}_{10}(\text{OH})_2]$ . These clays are naturally abundant and have surface areas as high as  $800 \text{ m}^2/\text{g}$ , making them ideal sorbent materials (Borchardt, 1989).

**Table 1.** Classification of 2:1 Phyllosilicate Clays Based on Layer Charge

Mineral Group	Layer Charge per Formula Unit		Predominant Octahedral Cation	
	Tetrahedral Sheet	Octahedral Sheet	Dioctahedral ( $\text{Al}^{3+}$ )	Trioctahedral ( $\text{Mg}^{2+}$ )
Pyrophyllite/Talc	0	0	Pyrophyllite	Talc
Smectites	0.25 to 0.6	0	Beidellite	Saponite
	0	0.25 to 0.6	Montmorillonite	Hectorite
Vermiculites	0.6 to 0.9	0	Vermiculite	Vermiculite
Micas	1	0	Muscovite	Biotite

Adapted from Bohn et al. (1979) and Lemke (2000).

## Zeolites

Zeolites fall under the class of tectosilicates, containing three-dimensional frameworks of  $\text{SiO}_4$  tetrahedra. These minerals also undergo substitution of  $\text{Si}^{4+}$  with  $\text{Al}^{3+}$ , resulting in a net negative charge that is balanced by exchangeable cations. Clinoptilolite is a member of the zeolite class and possesses the general structure of  $(\text{Na}_3\text{K}_3)[\text{Al}_6\text{Si}_{30}\text{O}_{72}]24\text{H}_2\text{O}$ , where  $\text{Na}^+$  and  $\text{K}^+$  are exchangeable cations. Like the phyllosilicates, zeolites are also adsorbent materials due to their large internal surface and void volume (Ming and Mumpton, 1989).

## **Sorption of Chemicals onto Clay Surfaces**

The mechanisms by which compounds bond to or are retained by soil particles, and in particular, clays, have been extensively researched. Sorption can be loosely classified as one of two types: physical/chemical bonding to soil mineral surfaces or hydrophobic partitioning onto soil organic matter (Koskinen and Harper, 1990). A number of physical/chemical bonding mechanisms have been characterized that vary based on the strength of interaction. Van der Waals forces are short-range bonds that result from the interaction of natural or induced dipoles. London forces are a subclass of the van der Waals forces resulting from vibrational dipoles. These two types of interactions are relatively weak (2-4 kJ/mol) and decay rapidly with increasing distance, but can be important for large compounds with a sizeable surface area. A variation on dipole interaction is the hydrogen bond, which results from electrostatic attraction between a hydrogen and the electron pairs of an electronegative atom such as oxygen, nitrogen or sulfur. These forces are slightly stronger than the van der Waals forces (2-60 kJ/mol). The aluminosilicate clay surfaces contain numerous exposed OH groups from octahedral surfaces and edge sites (protonation is pH dependent), making hydrogen bonding an important mechanism for adsorption on these surfaces. A final relatively weak interaction is cation and water bridging. Anionic or polar compounds may interact through electrostatic attraction to exchangeable cations. If the compound cannot displace the waters of hydration surrounding a cation, the interaction is referred to as water bridging (Koskinen and Harper, 1990).

Electrostatic and covalent interactions provide stronger bonds between solutes and surfaces. In anion exchange, an anion becomes electrostatically attracted to an exposed cation at the surface or edge. Ligand exchange involves covalent binding of a compound with a metal ion at the surface. This generally occurs with a carboxylate or hydroxyl functional group that can displace one of the natural surface hydroxyl groups. Cation exchange involves the exchange and retention of a positively charged compound onto the negative clay surface. Protonation involves the same mechanism of exchange, but relies on the mineral surface (assisted by low pH) to transfer a proton to a compound, producing a cation (Koskinen and Harper, 1990).

Alternatively, a variety of compounds become sorbed to soil through partitioning. The organic matter found in soil is a combination of decomposition products from plants, animals and microbes such as carbohydrates, proteins, lignin, fats and waxes. Over time, these materials undergo enzymatic and chemical reactions and become incorporated into humus, a colloidal polymer. This organic matter is important for the structure of the soil as well as buffering pH and increasing the water holding capacity. The identity of humus has not been fully characterized; rather, humus is observationally divided into constituents based on a pH fractionation and relative oxygen and carbon contents (Bohn et al., 1979). These non-humic and colloidal humic substances in the soil are able to interact with other non-polar compounds through hydrophobic sorption (Senesi, 1993; Fetter, 1993).

## **CLAY-BASED INTERVENTIONS FOR AFLATOXIN CONTROL**

The strategy of reducing foodborne exposure to aflatoxins through the inclusion of various binding agents or “detoxifying clays” in the diet has been given considerable attention in the scientific literature. Many of these binding agents have been reported to prevent the deleterious effects of diverse mycotoxins in a variety of animals, apparently by diminishing toxin uptake and distribution to the blood and target organs. As early as 1979, adsorbent clay minerals were reported to bind aflatoxin B<sub>1</sub> in liquids (Masimango et al.,

1979). Also, bleaching clays, that had been used to process canola oil, were found to lessen the effects of T-2 toxin (Carson and Smith, 1983; Smith, 1984).

### **Enterosorption of Aflatoxins (HSCAS clay)**

In the first enterosorbent study with aflatoxins, HSCAS (NovaSil™), a calcium montmorillonite clay that is used as an anticaking additive for animal feeds, was reported to significantly adsorb aflatoxin B<sub>1</sub> with high affinity and high capacity in aqueous solutions and to protect broiler and Leghorn chicks from the toxic effects of 7,500 ppb aflatoxins in the diet (Phillips et al., 1987, 1988). Since this initial study, HSCAS and other similar montmorillonite clays have been reported to diminish the toxic effects of aflatoxins in a variety of young animals including rodents, chicks, turkey poults, ducklings, lambs, pigs, and mink (Phillips et al., 1990, 1991, 1994, 1995; Colvin et al., 1989; Bonna et al., 1991; Harvey, 1994; Harvey et al., 1989, 1991a, 1991b, 1993; Voss et al., 1993; Kubena et al., 1990a, 1990b, 1991, 1993; Ledoux et al., 1999; Smith et al., 1994; Marquez and Hernandez, 1995; Cerdchai et al., 1990; Lindemann et al., 1993; Abdel-Wahhab et al., 1998; Nahm, 1995; Jayaprakash et al., 1992). Importantly, HSCAS clay has also been shown to decrease the bioavailability of radiolabeled aflatoxins and to reduce aflatoxin residues in poultry (Davidson et al., 1987; Jayaprakash et al., 1992), rats (Sarr et al., 1995) and pigs (Beaver et al., 1990). Levels of aflatoxin M<sub>1</sub> in milk from lactating dairy cattle and goats were also diminished in the presence of HSCAS in the diet (Ellis et al., 1990; Smith et al., 1994; Harvey et al., 1991b).

### **Specificity of HSCAS for Aflatoxins**

Recent studies (*in vivo*) have supported our earlier findings (*in vitro*) that HSCAS has a notable preference (and capacity) for the aflatoxins at levels in the diet at, or below, 0.5% w/w. For example, HSCAS at a level of 0.5% in the diet of poultry, did not impair phytate or inorganic phosphorous utilization (Chung and Baker, 1990). In other poultry nutrition studies, the addition of HSCAS at concentrations of 0.5 and 1.0% did not impair the utilization of riboflavin, vitamin A, or manganese; however, there was a statistically significant reduction in zinc utilization in the presence of 1.0% clay (Chung et al., 1990). It is worthy to note that 0.5% or lower (not 1.0%) of HSCAS is recommended for anticaking activity. Also, in earlier studies, HSCAS (at an inclusion rate of 0.5%) has been shown to protect young chickens from very high levels of aflatoxins (i.e., 7,500 ppb) which represents a “worst-case scenario” for enterosorption.

While clay-based interventions are clearly effective for aflatoxins, an analogous technology is not yet available for other important mycotoxins. For the most part, unmodified HSCAS clays do not “tightly” bind other structurally diverse mycotoxins, e.g., zearalenone, deoxynivalenol, T-2 toxin, ochratoxin A, cyclopiazonic acid, ergota-mine, and fumonisins, nor do they significantly prevent the adverse effects of these mycotoxins when included in the diet of animals. For example, in enterosorbent studies in poultry with mycotoxins (other than the aflatoxins), the inclusion of HSCAS clay in the diet did not significantly prevent the adverse effects of cyclopiazonic acid (Dwyer et al., 1997), T-2 toxin (Kubena et al., 1990a), diacetoxyscirpenol (Kubena et al., 1993), ochratoxin A (Huff et al., 1992), and fumonisins (Lemke, 2000).

The use of HSCAS in mink fed zearalenone helped to alleviate some fetotoxicity but did not reduce the hyperestrogenic effects (Bursian et al., 1992). Also, the addition of HSCAS at 0.5 and 1.0% w/w in the diet, did not influence the average daily gain of pigs exposed to deoxynivalenol. Dilution of the contaminated maize with uncontaminated maize was the only efficacious method for decreasing the toxicity of deoxynivalenol (Patterson and Young, 1993). The possibility of supplementing livestock diets with HSCAS clay to protect from fescue toxicity has also been investigated (Chestnut et al.,

1992). Although *in vitro* experiments predicted good binding of ergotamine to montmorillonite clays in aqueous solution (Chestnut et al., 1992; Huebner et al., 1999), HSCAS (at levels of 2.0% by weight) did not protect rats or sheep from fescue toxicosis and impaired the absorption of magnesium, manganese, and zinc at this high level. Even though lower levels of HSCAS may have less of an effect on these minerals, further work is warranted to determine the dosimetry of this effect and the potential for nutrient interactions in livestock.

## MECHANISMS OF AFLATOXIN SORPTION ONTO HSCAS

Earlier studies *in vitro* have assessed the sorption of aflatoxins onto the surface of HSCAS clay (Ellis, 1990, 1991; Sarr, 1992; Ramos and Hernandez, 1996; Grant, 1998; Grant and Phillips, 1998; Phillips et al., 1995; Phillips, 1999). HSCAS, in aqueous solution, was shown to tightly and preferentially bind AFB<sub>1</sub> and similar analogs of AFB<sub>1</sub> that contain an intact  $\beta$ -dicarbonyl system (Phillips et al., 1988; Sarr, 1992).

Prior to isothermal analysis, the physical characteristics of AFB<sub>1</sub>, HSCAS, and heat-collapsed HSCAS were confirmed (Grant and Phillips, 1998) (Table 2). The octanol water partition coefficient ( $K_{ow}$ ) for AFB<sub>1</sub> was estimated by two separate methods, including an energy-minimized molecular model and HPLC analysis. In the scientific literature, the values for the solubility of AFB<sub>1</sub> in water vary from 10-30  $\mu\text{g/ml}$  (Busby and Wogan, 1984). Using estimated  $\log K_{ow}$  values and the molecular weight and melting point of AFB<sub>1</sub>, a solubility range of 11-33  $\mu\text{g/ml}$  was derived from equations described by Meylan and coworkers (1996). The total organic carbon (TOC) in HSCAS was determined to be < 0.05% using a Leco model 523-300 induction furnace. Following the removal of carbonates, organic carbon was converted into CO<sub>2</sub> which was measured with a Horiba IR detector and HP 3369a integrator. The negligible amount of TOC and the specificity of aflatoxin sorption onto the surfaces of HSCAS further confirm that organic partitioning is not an important mechanism in the aflatoxin enterosorption process and protection of animals *in vivo*.

**Table 2.** Physical characteristics of AFB<sub>1</sub>, HSCAS and collapsed HSCAS

<b>Aflatoxin B<sub>1</sub></b>	
Log $K_{ow}$	1.46 and 1.98
Solubility	11 to 33 $\mu\text{g/mL}$
$\epsilon$	21,865 [1/(Mcm)]
Vertical cross-sectional area	52.8 $\text{\AA}^2$
Horizontal cross-sectional area	88.3 $\text{\AA}^2$
<b>HSCAS</b>	
Total surface area	848 $\text{m}^2/\text{g}$
External surface area	70 $\text{m}^2/\text{g}$
Total organic carbon	<0.05%
<b>Collapsed HSCAS</b>	
Total surface area	77 $\text{m}^2/\text{g}$

HSCAS was found to have a high total surface area of  $848 \pm 11 \text{ m}^2/\text{g}$  by the ethylene glycol adsorption method (Dyal and Hendricks, 1950). The surface area was calculated based on the relationship that  $3.1 \times 10^{-5} \text{ g}$  of ethylene glycol covers each square meter of surface. The external surface area of HSCAS was measured by N<sub>2</sub> adsorption and indirectly by measuring heat-collapsed HSCAS with ethylene glycol. Collapsed HSCAS was prepared by heating HSCAS clay to 200°C for 30 minutes and then heating the same

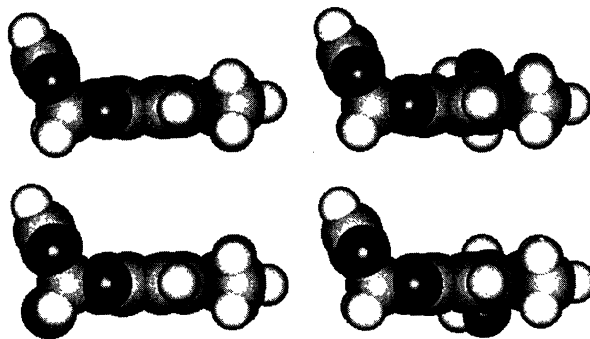
sample at 800°C for one hour. The collapsing of the interlamellar region leaves only the exterior surface available for ethylene glycol adsorption. The two methods resulted in values for the external surface area equal to 70 and  $77 \pm 2$  m<sup>2</sup>/g, respectively (Table 2). The near agreement between the two methods confirmed the collapsing of HSCAS and the loss of the interlamellar region in the collapsed clay.

The isotherm shape for the sorption of AfB<sub>1</sub> onto HSCAS can be categorized as an L1 or L2 plot that is reaching (or has reached) a plateau. The maximum amount of AfB<sub>1</sub> that was sorbed by HSCAS was 0.336 mol/kg which was 72.9% of the theoretical maximum capacity ( $Q_{\max}$ ) of 0.461 mol/kg derived from the fitting of the Langmuir model to the data (Grant and Phillips, 1998). The shape of the isotherm plot of AfB<sub>1</sub> binding to collapsed HSCAS was an L2 that had a capacity notably smaller (i.e., 0.0567 mol/kg) than untreated HSCAS (i.e., 0.461 mol/kg). These findings suggested that a significant portion of the binding of AfB<sub>1</sub> to HSCAS is within the interlayer region of the clay. The fitting of the data to the Toth isotherm model gave an exponent of 0.64, indicating more than one type of site for the sorption of AfB<sub>1</sub> and supports the data of Ramos and Hernandez (1996), which suggested that different sites and/or mechanisms of action were involved in aflatoxin binding at the surface of a montmorillonite clay. The Langmuir model was also used to estimate the  $Q_{\max}$  at different isotherm temperatures and to calculate individual distribution constants ( $K_{ds}$ ) to use in enthalpy calculations. The enthalpy of AfB<sub>1</sub> sorption (near or above -40 kJ/mol) showed some variation, suggesting multiple sites on HSCAS with dissimilar thermodynamic properties. Based on this value, it is conceivable that multiple sites on the surface of HSCAS clay act to chemisorb AfB<sub>1</sub>.

In previous work, the strength of adsorption to HSCAS had been quantified utilizing an adsorption index ( $C_{\alpha}$ ) (Sarr, 1992). The  $C_{\alpha}$  index expressed the ratio of the retained amount of ligand (difference between the amount bound and the amount desorbed) to the initial concentration of ligand. Based on data from these early studies, a proposed mechanism of action was the formation of a stable chelate with metals ions in HSCAS. However, a comparison of  $C_{\alpha}$ s indicated a significant difference in ligand adsorption to HSCAS, even when the ligands possessed a similar structure and the same number of carbonyl functional groups. It was also observed that two pairs of analogs had the same dicarbonyl system, but a slight difference in stereostructure which resulted in a dramatic change in the  $C_{\alpha}$  values. These included: AfB<sub>1</sub>/aflatoxin M<sub>1</sub> and aflatoxicol I (AfTI)/aflatoxicol II (AfTII) (Figure 2). These findings suggested a mechanism involving more than the dicarbonyl system and led to further investigation of AfB<sub>1</sub> and analogs to determine if there was a structural correlation between this set of compounds and the  $C_{\alpha}$  data (Grant, 1998). The partial charges of AfB<sub>1</sub> and the various analogs were estimated by drawing the chemical structures in ISIS Draw 2.0 (MDL Information Systems, San Leandro, CA) and then importing them into HyperChem 4.5 (HyperCube, Waterloo, Ontario, Canada). The structures were energy-minimized using the semi-empirical quantum mechanical AM1 method, which is an improvement of the MNDO method (Dewar, 1985; HyperChem, 1994). The structural information was then imported into the ChemPlus module for the determination of  $K_{ow}$ . ChemPlus utilizes previously derived atomic parameters to estimate each individual atom's contribution to the molecule's Log  $K_{ow}$  (ChemPlus, 1993; Ghose et al., 1988; Vellarkad et al., 1989). These analogs of aflatoxin included: aflatoxin B<sub>2</sub>, G<sub>1</sub>, G<sub>2</sub>, G<sub>2a</sub>, M<sub>1</sub>, P<sub>1</sub>, Q<sub>1</sub> and aflatoxicol I and II, coumarin, dimethoxycyclopentenone[2,3-*c*]coumarin, dimethoxycyclopenteno[*c*]coumarin, esculentin, 4-methylumbelliferone, tetrahydrodeoxy-aflatoxin B<sub>1</sub> and xanthotoxin. ChemPlus was used to measure the cross-sectional areas of AfB<sub>1</sub> and AfM<sub>1</sub>. The van der Waals radii of C, O, and H were set to 1.85, 1.40, and 1.20 Å, respectively (Emsley, 1991). The structures were oriented on edge with the dicarbonyl in view and planar with the dihydrofuran in view. The cross-sectional area method was modified by the use of a carbon atom as the reference for the area calculations (Gray et al., 1995). The relative surface coverages were constructed by orienting the test ligand in the proposed orientation with a *periodic box* scribing out the



relative surface area. Since the unit cell coordinates for HSCAS clay are not available in the literature, the model for HSCAS was constructed from unit cell coordinates of muscovite, a structurally similar 2:1 phyllosilicate clay (Radoslovich, 1960). The unit cell coordinates were converted to orthogonal coordinates and replicated to form an individual platelet. The platelet was duplicated and arranged to a  $d_{001}$  spacing (19 Å) of fully hydrated calcium substituted montmorillonite clay (MacEwan, 1980).



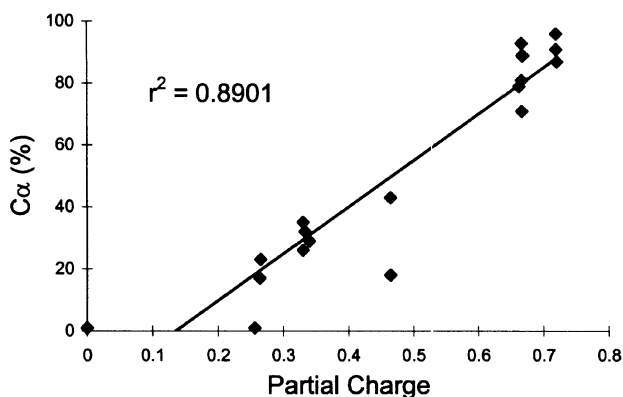
**Figure 2.** Molecular models of AfB<sub>1</sub> (upper left), AfM<sub>1</sub> (lower left), AfT<sub>1</sub> (upper right), and AfT<sub>2</sub> (lower right) illustrating important stereochemical differences.

In structure-activity studies, a log-linear plot of the index of adsorption and the calculated  $K_{ow}$  for individual aflatoxins and analogs indicated a lack of correlation. These results confirmed that hydrophobicity of AfB<sub>1</sub> and similar ligands was not critical to the mechanism of sorption onto HSCAS. Molecular models were constructed to estimate the relative surface coverage of the adsorbent based on the possible locations and orientations of the adsorbed ligand. The  $Q_{max}$  which was obtained from fitting the Langmuir equation in isotherms, was applied to the total, internal and external surface areas in order to calculate the amount of coverage of AfB<sub>1</sub> on HSCAS clay. These values were used to predict the significance of adsorption in the interlamellar region and to indicate whether a multi-layer formation was possible. The surface area of interest was divided by the number of molecules adsorbed by 1.0 kg of either HSCAS, collapsed-HSCAS or the difference between HSCAS and collapsed-HSCAS to yield the available surface area per adsorbed molecule. This value was then compared to the cross-sectional areas of the probable orientations of adsorbed molecules. The vertical orientation was defined as the dicarbonyl system bound to the clay, since this moiety was found to be important in previous research (Sarr, 1992). The horizontal orientation was defined as the molecule lying planar on the surface with the dihydrofuran away from the surface (resulting in minimal steric hindrance to docking). The total surface area of HSCAS relative to the capacity of the adsorbed AfB<sub>1</sub> molecules was calculated to be 305 Å<sup>2</sup>/molecule. This was the area that could bind the the AfB<sub>1</sub> molecule in either orientation without requiring a multi-layer coverage (Grant and Phillips, 1997). Even though the vertical orientation of AfB<sub>1</sub> would be less than a monolayer, its insertion in the interlamellar space would require a slight tilt from the basal surface. The outer area relative to the amount of AfB<sub>1</sub> bound is 25.2 Å<sup>2</sup>/molecule which is smaller than the area required by either orientation (Grant and Phillips, 1998). This confirms that an intact interlamellar region is important in the adsorption of AfB<sub>1</sub> which is also supported by the findings with collapsed-HSCAS. The relative surface coverage for the interlamellar region was calculated by difference between the surface areas and capacities of HSCAS and collapsed-HSCAS. The results of this calculation show that the

interlamellar region would have enough surface area to adsorb AfB<sub>1</sub> based on its estimated capacity.

Insight into potential mechanisms for the adsorption of AfB<sub>1</sub> onto the surface of HSCAS came from the observation that stereochemical differences between some of the aflatoxin analogs resulted in a significant effect on the C<sub>α</sub> (even though the carbonyl functional groups were identical). AfB<sub>1</sub> and analogs are relatively planar compounds except for the dihydrofuran groups. Interestingly, the analogs that possess a functional group extending out of the major plane of the molecule in the opposite direction of the furan group have a significantly lower C<sub>α</sub> and Q<sub>max</sub> versus similarly structured compounds. For example, AfB<sub>1</sub> (C<sub>α</sub> = 0.93, Q<sub>max</sub> = 0.461 mol/kg) and AfM<sub>1</sub> (C<sub>α</sub> = 0.71, Q<sub>max</sub> = 0.157 mol/kg) have the same dicarbonyl structure, and aflatoxicol I (C<sub>α</sub> = 0.43) and aflatoxicol II (C<sub>α</sub> = 0.18) differ only in the orientation of the hydroxyl group (Sarr, 1992; Grant and Phillips, 1997; Ellis, 1991; Ellis, 1994). Aflatoxicol (like AfM<sub>1</sub>) contains a hydroxyl group pointing out of the plane away from the dihydrofuran group. These results also suggest that the molecular mechanism for the adsorption of aflatoxins onto HSCAS may favor an optimal orientation where the furan is aligned away from the surface (Figure 2).

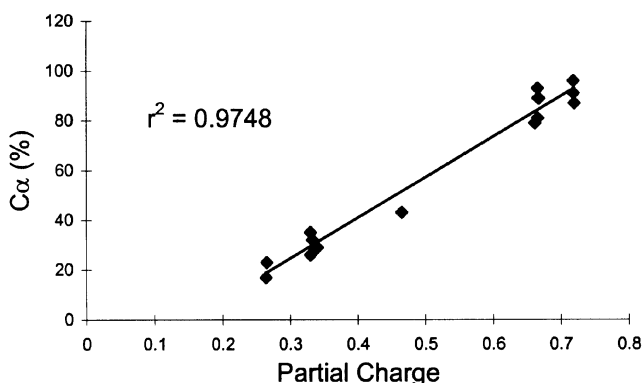
AfB<sub>1</sub> is strongly bound (chemisorbed) to HSCAS. A potential chemical reaction that may explain these results is an electron donor acceptor (EDA) mechanism. This mechanism involves sharing electrons from the negative surface of the clay with atoms in the adsorbed molecule that are partially positive (Haderlein, 1996). The carbons comprising the dicarbonyl system in aflatoxins are partially positive (electron poor) and have also been shown to be essential to the adsorption process. When the summation of partial charges of the carbons of the carbonyl functional groups for each ligand was plotted versus the C<sub>α</sub> there was a significant correlation (Figure 3). When the ligands that were not planar on the side of the molecule opposite the dihydrofuran functional group were removed from the set of test compounds, the correlation was significantly improved (Figure 4).



**Figure 3.** Plot of % chemisorption of ligand onto the surface of HSCAS clay versus the summation of partial charges of the carbons of the carbonyl functional groups of ligands, i.e., aflatoxin B<sub>1</sub> and various analogs. Partial charges: AfB<sub>1</sub> (.67); AfB<sub>2</sub> (.67); AfG<sub>1</sub> (.72); AfG<sub>2</sub> (.72); AfG<sub>2a</sub> (.72); AfM<sub>1</sub> (.67); AfP<sub>1</sub> (.67); AfQ<sub>1</sub> (.66); AfII (.47); AfTII (.47); Coumarin (.33); DMCI (.67); DMCI (.26); Esculetin (.33); 4MU (.34); THDAfB<sub>1</sub> (.27); Xan (.33). Adapted from Grant, 1998.

Interference from compounds with stereochemical restrictive groups could also play an important role in the adsorption process. For the analogs that contain functional groups that make them thicker than AfB<sub>1</sub>, their insertion, docking and adsorption at surfaces in the

interlamellar channel might be restricted. In summary, HSCAS clay has been previously shown to protect animals from aflatoxins when included in contaminated feed. With molecular modeling we have demonstrated that this protection may be attributed to the chemisorption of aflatoxins at surfaces within the interlamellar region of HSCAS; the exterior surfaces of the clay were responsible for only minor sorption of aflatoxins. The optimal orientation of the AFB<sub>1</sub> molecule is probably planar on the interlamellar surface. Our results also indicate a good correlation between the magnitude of partial positive charges on carbons C11 and C1 of the β-dicarbonyl system and the strength of adsorption of planar ligands, suggesting an EDA mechanism with the surface of the clay. Functional groups on the aflatoxin analogs may cause steric hindrance to the adsorption at the surface of HSCAS or may block adsorption by interacting across the interlamellar region. Other mechanisms of AFB<sub>1</sub> sorption to HSCAS surfaces involve the potential chelation of interlayer cations (especially Ca<sup>2+</sup>) and various edge-site metals (data not shown) (Grant, 1998; Phillips, 1999).



**Figure 4.** Plot of % chemisorption of ligand onto the surface of HSCAS clay versus the summation of partial charges of the carbons of the carbonyl functional groups of ligands, excluding compounds that are not planar. Adapted from Grant (1998).

## CONCLUSIONS

Many concerns about the aflatoxins have originated from the strong implications of their involvement in disease and death in man and animals. Even, at the beginning of a third millennium, scientists and clinicians are still seeking practical ways to control these elusive toxins. The prevention of toxicity and carcinogenicity of aflatoxins through clay-based dietary interventions (i.e., aflatoxin-selective enterosorbents) shows great promise and significant advantages over other methods of detoxification. Evidence suggests that aflatoxins react tightly at multiple sites on HSCAS clay surfaces (especially those within the interlayer region). However, clay and zeolitic minerals comprise a broad family of diverse aluminosilicates and are not created equal; all aflatoxin binding agents should be rigorously tested for efficacy, paying particular attention to their effectiveness and safety in aflatoxin-sensitive animals and their potential for interactions with nutrients and/or synergy with aflatoxins before inclusion in diets.

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