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In vitro and *in vivo* characterization of mycotoxin-binding additives used for animal feeds in Mexico

A. Marroquín-Cardona[a], Y. Deng[b], J.F. Taylor[a], C.T. Hallmark[b], N.M. Johnson[a] and T.D. Phillips[a]*

[a]*College of Veterinary Medicine and* [b]*Soil & Crop Sciences, Texas A&M University, College Station, TX, USA*

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The study was conducted to characterize and compare twelve different additives distributed in Mexico as mycotoxin binders utilizing: (1) equilibrium isothermal analysis for aflatoxin B_1 (AFB_1) adsorption, (2) a variety of mineralogical probes, and (3) *Hydra* toxicity bioassay. The test additives Milbond-TX® (MLB), Mycoad® (MCA), Volclay FD181® (VOL), Fixat® (FXT), Toxinor® (TOX), Mexsil® (MEX), Mycosil® (MYC), Klinsil® (KLS), Zeotek® (ZEO), Duotek® (DUO), Mycosorb™ (MSB), and Mycofix® Plus 3.0 (MIX) were compared with NovaSil™ Plus (NSP). Isotherms for AFB_1 adsorption were conducted at pH 2 and pH 6.5, mimicking pH conditions in the stomach and small intestine. Mineralogical analysis included determination of swelling volume, X-ray diffraction analysis, and fractionation procedures. A *Hydra vulgaris* toxicity study was performed to evaluate the potential safety of the additives. Computer-generated isotherm data were fit using the Langmuir model, and parameters of Q_{max} and K_d were estimated. The most effective additives for AFB_1 at both pH conditions were NSP, MLB, MCA and VOL, while the least effective was MSB. The amounts of sand, silt and clay fractions varied among the additives. Nine of the additives showed the presence of smectite. Most of the additives were found to be non-toxic to *Hydra* except for the organoclays (ZEO, DUO) and MSB. In general, NSP demonstrated the highest sorption capacity in the bulk material and the different fractions. Studies to characterize these binding additives further and to evaluate their multiple mycotoxin sorption claims are ongoing.

Keywords: aflatoxin; mycotoxins; binding additives; binders; isothermal analysis; adsorption; smectite

Introduction

Aflatoxin B_1 (AFB_1) is the most toxic of four naturally occurring aflatoxins (i.e., B_1, B_2, G_1 and G_2). It is classified as a Class A carcinogen by the US Environmental Protection Agency (USEPA) and Group 1 human carcinogen by the International Agency for Research on Cancer (IARC 1993, 2002). Because of its common occurrence in animal feed and its potent hepatotoxicity and carcinogenicity, considerable research has been directed at protecting animals against this toxin (Grant and Phillips 1998). The use of clay-based products as enterosorbents for aflatoxins is a frequently used strategy to reduce aflatoxin exposure in animals. Natural bentonites (e.g. montmorillonite) and zeolites (e.g. clinoptilolite) are the common sorbents used for this purpose. In pioneering studies in Texas, inclusion of a calcium montmorillonite clay (NovaSil, NS) in animal feed has been shown notably to reduce the adverse effects associated with aflatoxin exposure in different animal species (Phillips et al. 1988; Phillips 1999) and to decrease the level of an AFB_1 metabolite (aflatoxin M_1) in milk from lactating dairy cows and goats (Harvey et al. 1991; Smith et al. 1994). Equilibrium adsorption isotherms, molecular modelling, and *in vivo* studies have been used to demonstrate that NS preferentially binds AFB_1 in the gastrointestinal tract, thereby reducing its bioavailability to blood, liver, and other organs (Phillips 1999; Phillips et al. 2002). Sodium bentonites have also been shown to be effective against aflatoxins *in vitro* (Diaz et al. 2002) and *in vivo* (Pasha et al. 2007), although earlier investigations revealed that a 1% inclusion rate in the diet was not enough to protect rats against liver lesions due to aflatoxin (Voss et al. 1993). Zeolites (Mayura et al. 1998; Lemke et al. 2001b) and organozeolites (Dakovic et al. 2005) have been used as aflatoxin adsorbents as well, but some studies have reported that these minerals do not bind AFB_1 as effectively as the bentonites (Harvey et al. 1993). Importantly, there is evidence that clinoptilolite (the most commonly used zeolite) was ineffective in preventing maternal and developmental toxicity of AFB_1 in rats (Mayura et al. 1998). This could be due to the

*Corresponding author. Email: tphillips@cvm.tamu.edu

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natural conformational properties of zeolites (i.e. clinoptilolite) that prevent large molecules like aflatoxins from entering the zeolitic pores. The size of AFB_1 is estimated to be 12.78 Å, (from the hydrogen at C_2 to the hydrogen at C_8), and 10.38 Å, from oxygen at C_{11} to the exocyclic carbon in the *O*-methyl group (Phillips et al. 1995). The size of the pores on natural clinoptilolite can range from 4 to 7 Å (Li et al. 2005); hence, the adsorption of AFB_1 in zeolites (e.g. clinoptilolite) can be limited to the external surface only. Decreased aflatoxin-binding capacities for organozeolites could also be due to the exchange of these materials with synthetic surfactants like octadecyldimethylbenzylammonium (ODMBA) (Dakovic et al. 2005) and this may interfere with toxin/surface interactions.

Due to low inclusion rates and easy management of mycotoxin-binding additives, the widespread acceptance of these products by the farm animal industry has led to the introduction of a variety of diverse materials labelled as mycotoxin binders for use in feed. Thus, it is important to determine the effectiveness and safety of these products. The objective of this research was to characterize and compare twelve different products that are routinely distributed in Mexico as mycotoxin-binding additives, by means of: (1) equilibrium isothermal analyses to delineate sorbent/toxin surface interactions; (2) a variety of fundamental mineralogical probes; and (3) a hydra toxicity bioassay.

Materials and methods

Chemicals and reagents

AFB_1 from *Aspergillus flavus* was purchased from Sigma Chemical Co. (St Louis, MO, USA); CAS No. 1162-65-8. Acetonitrile, HPLC-grade, was purchased from Fisher Scientific; CAS No. 75-05-8. Na_2CO_3 (ACS grade) was obtained from Fisher Scientific. $MgCl_2$ (ACS grade) was purchased from Mallinckrodt Chemicals. All of the adsorption experiments were performed using high-purity deionized water (18.2 MΩcm).

Tested additives included Milbond-TX® (MLB) (Milwhite, Inc.), Mycoad® (MCA) (Avimex SA), Volclay FD181® (VOL) (Volclay de Mexico), Fixat® (FXT) (Süd Chemie), Toxinor® (TOX) (Norel Nature), Klinsil® (KLS) (HELM de Mexico), Mycofix® Plus 3.0 (MIX) (Biomin), Mexsil® (MEX) (Mexsil), Mycosil® (MYS) (Dresen), Zeotek® (ZEO), Duotek® (DUO) (Nutek SA) and Mycosorb™ (MSB) (Alltech). All these additives for use in Mexico claim to bind aflatoxins to some extent, and some also claim multiple mycotoxin-binding and/or inactivation potential. These additives were compared with NovaSil™ Plus (NSP) (Engelhard, Inc.), an effective aflatoxin binder that has been extensively studied (Mayura et al. 1998; Phillips et al. 1998, 2006). The composition of matter for each additive based on information provided by individual websites and/or other publications was reported as follows: NSP (HSCAS, Ca-montmorillonite), VOL (Na-montmorillonite), FXT and KLS (aluminosilicates), MEX, TOX, MLB, MYC, MCA (HSCAS), ZEO, DUO (organo-aluminosilicates), MSB (glucomannan/β-D-glucan containing yeast product) and MIX (a synergistic mixture of minerals, biological constituents, including enzymes and BBSH microbe, plant-derived extracts such as flavonolignans, saponins and terpenoids, and some algae materials). Since kaolinite, mica, silica, and clinoptilolite can be present in some of these products, adsorption isotherms of AFB_1 were also conducted on these reference minerals at pH 6.5. The above-mentioned reference minerals were obtained from the Clay Mineral Repository (Purdue University, IN, USA) and were used as negative controls (i.e., those materials that do not effectively sorb AFB_1) (Masimango et al. 1978; Phillips et al. 1988, Mayura et al. 1998; Huwig et al. 2001).

Sample characterization

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

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

and the mixed nature of MIX (i.e. algae, plants, and bacteria).

Random powder mounts were prepared for the whole product of each additive. A random sample (approximately 300 mg) for each additive was placed in a standard rectangular aluminium plate holder (about 2 mm thick) with a rectangular cavity in which the sample was gently packed. All the additives were passed through a 140 mesh (105 μm) sieve to prevent interference due to differences in particle size. The presence of organic matter in the additives can interfere with the detection of mineral clays in the powder mounts due to aggregation effects or dilution. For that reason, the definite evidence of smectite presence was determined in the clay fraction following Mg^{2+} saturation in most of the additives, except MSB. Carbonate and organic matter in MEX and MIX were destroyed in order to minimize the aggregation problems. The X-ray diffractograms were recorded with CuKα-radiation on a Phillips X-ray diffractometer equipped with a graphite monochromator and theta-compensation slit. The pattern was measured in 0.05°2θ intervals from 2 to 32°2θ.

The particle size distribution in the additives was determined using an automatic fractionator. The percentage of particles with diameter >0.05 mm (sand), 0.05–0.002 mm (silt), and ≤ 0.002 mm (clay), as reported by the US Department of Agriculture, Natural Resources Conservation Service (2007), was calculated based upon the weight of each fraction from an initial amount of 30 g of air dried sample. The samples were then dried in an oven at 105°C to adjust for moisture content. Briefly, the procedure consisted of placing the sample into one 400 ml glass bottle filled with 30 ml of 5% $NaCO_3$ and 250 ml of deionized water. The bottles were placed in a reciprocating shaker and shaken at 120 cycles min^{-1} overnight at room temperature. Once the shaking process was complete, the samples were passed through a 300 mesh (53 μm) sieve to separate the sand particles. The sieved suspension containing the silt and clay fraction was collected into a polypropylene cylinder and placed on the fractionator to run the following program consisting of seven cycles: distilled water addition; 5 min for mixing and stirring; and 8 h for settling. After each settling step, the suspension containing the clay fraction was siphoned automatically and saved in plastic containers. The sediment in the cylinders (silt fraction) was collected at the end of the fractionation process. In order to remove possible remaining clay and silt particles, the sand and silt fractions were rinsed with distilled water several times until the water was clear and then dried in a 105°C oven overnight. The clay fraction collected in 20 liter containers was flocculated by additions of 1 N $MgCl_2$. Following flocculation, the supernatant was siphoned off and clays were concentrated by centrifugation. The amount of clay was calculated by subtraction of the sand and silt weights after drying from the total sample weight.

The pH of each additive was measured by adding 4 g of each into a beaker with 200 ml of deionized water. The suspension was stirred for 10 min, and the pH was measured with a pH meter according to the procedure described in the US Pharmacopeia for pH measurement of bentonite samples (US Pharmacopoeia 2005).

Isothermal and single-concentration AFB_1 adsorption analyses

Isotherms were performed as previously described (Grant and Phillips 1998). A stock solution of AFB_1 was prepared by dissolving pure crystals (5 mg) in acetonitrile (1 ml) and then injecting a volume from the previous dissolved AFB_1 (approximately 0.16 ml) into 100 ml of purified water to yield a solution concentration of 8 μg ml^{-1}. The concentration was further verified by measuring the absorbance at 362 nm on a Shimadzu scanning UV-visible spectrophotometer, Model 1601PC.

Isothermal analyses for each additive were conducted at pH values of 2 and 6.5 to simulate stomach and small intestine pH conditions, respectively. The pH of the solutions used was adjusted with concentrated hydrochloric acid (HCl) and 1 M sodium hydroxide (NaOH). Briefly, an isotherm consisted of eleven concentrations of AFB_1 (0.4, 0.8, 1.2, 2.4, 3.6, 4, 4.8, 5.6, 6.4, 7.2 and 8 μg ml^{-1}) mixed with 0.1 mg of each additive in a total volume of 5 ml in 16 × 125 mm borosilicate glass tubes. Three replicates were used for each solute concentration. A suspension of each additive was prepared by weighing 100 mg into a 50 ml Erlenmeyer flask and adding 50 ml of water to yield a concentration of 2 mg ml^{-1}. The suspension was mixed to keep the slurry homogeneous. From this suspension, 50 μl (0.1 mg of additive) were added to each of the dilution tubes and the suspensions were vigorously stirred during the addition of the additive. Along with the dilution tubes, there were three controls consisting of 5 ml of water, 5 ml of AFB_1 stock solution and 5 ml of water with 0.1 mg of additive. The samples and controls were capped and placed on a shaker at 1000 rpm for 2 h in an incubator at 25°C. After shaking, the samples were centrifuged at 2000 rpm for 20 min. The supernatant was measured for absorbance at 362 nm. Isotherms for negative controls (kaolinite, mica, colloidal silica and clinoptilolite) were run at pH 6.5 following the same procedure as for the additives.

In order to compare the binding properties of the clay fraction with sand and silt fractions, an additional single-concentration adsorption analysis of AFB_1 at

pH 6.5 was performed with the sand, silt and clay fractions from the additives that exhibited the highest AFB_1 sorption capacities based on the isotherms. The analysis consisted of triplicate aliquots of AFB_1 ($8\,\mu g\,ml^{-1}$) in 5 ml reaction vials plus controls. The addition of sorbent, incubation time and the UV absorbance measurements were the same as the isotherm procedures. The presence of smectite in the sand and silt fractions was also evaluated by XRD.

Data calculation, curve fitting and statistics

The UV adsorption data were used to calculate the amount of AFB_1 left in solution at equilibrium (C_w) and the amount adsorbed to the additive (q) using Table Curve 2D v.2 software and an Excel program developed in our laboratory to fit the data to the standard Langmuir-derived isotherm equation:

$$q = Q_{max}(K_d C_w/1 + K_d C_w),$$

where the binding capacity (Q_{max}) and the distribution constant (K_d) were determined for each additive as previously described (Grant and Phillips 1998).

SPSS 14.0 software was used to calculate the Pearson's correlation coefficient for the data obtained from the parameters measured in the pre-screening analysis and from the isotherms. Data were first verified for normal distribution and the absence of outliers as required for this model.

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

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

Hydra *toxicity bioassay*

Adult *Hydra vulgaris* are maintained in culture in our laboratory and have been reported to be a sensitive *in vivo* indicator of toxicity for environmental and food-borne chemicals (Mayura et al. 1991; McKenzie et al. 1997; Ottinger et al. 1999; Huebner et al. 2000). Two main reasons justify the use of the hydra bioassay in this study: (1) previous work in our laboratory has reported the use of hydra as very sensitive method to evaluate toxicity of clay minerals and other sorbents (i.e. organoclays) (Afriyie-Gyawu et al. 2005); and (2) since most of the additives in our study contain clay minerals, and these clays are usually contaminated with various priority toxic metals (other than the framework metals) that have been shown to be toxic to adult hydra (Karntanut and Pascoe 2002), we have utilized the hydra bioassay as an initial indicator of safety for these materials.

Maintenance and feeding of hydra were carried out according to methods previously described (Mayura et al. 1991; Huebner et al. 2000; Ake et al. 2001). Hydra were not fed for 24 h before initiating the experiments and were maintained clean and free from bacteria and fungi contamination by treating with iodine tablets.

The assay was performed by exposing the hydra to three different concentrations of each additive. The concentrations (0.1%, 0.3% and 0.5%) were selected according to the inclusion rates for animal feed recommended by the additive's manufacturers. Each Pyrex® 60×15 mm test dish contained three normal healthy adult hydra in 4.0 ml of medium containing 1 mM $CaCl_2$ dihydrate, and 0.458 mM TES [*N-tris*(hydroxymethyl)-methyl-2-aminoethane-sulfonic acid, sodium salt] buffer (adjusted to pH 7). The dishes were maintained at 18°C. Hydra were examined for signs of toxicity at 0, 4, 20, 28, 44, 68 and 92 h. The toxic endpoint was determined by the 'tulip' or 'disintegration' stage of the hydra. In each test, experimental treatments were compared with untreated and solvent controls.

Results and discussion

Sample characterization

Selected physical and chemical properties of the additives are summarized in Table 1. The twelve additives showed a large range of SV from zero to greater than 4. MEX showed a value of zero. In contrast, VOL had a value slightly greater than 4 due to its strong swelling nature upon hydration. The remaining additives had SV values between 0.28 and 2.92. According to traditional estimation of COLE values, samples with a value greater than 0.03 are considered to contain significant amounts of smectite/montmorillonite clays (Buol et al. 1980). In our study, we used the same parameter from the COLE value (0.03) to determine qualitatively if the binding additives had significant smectite content. The results indicated that all the additives analyzed, except MEX, possessed a detectable amount of smectite. It is important to mention that traditional COLE values

Table 1. Physical, mineralogical, and chemical characterization of mycotoxin binding additives. Smectite presence was determined by both X-ray diffractograms with Mg^{2+} (air dry, heated at 300°C and 550°C) and ethylene glycol treatments. Q_{max}, K_d and r^2 values were from pH 6.5 isotherms. Color classification from Munsell® soil color charts.

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

SV: Swelling Volume; NA: not applicable; +: Positive for smectite; −: Negative for smectite: G: Gray; Y: Yellow.

were used to describe bulk soils, not pure clays, raw materials and mixtures as seen in this study. For this reason, the presence of smectite was confirmed by means of XRD powder mounts and clay fraction analysis. According to the X-ray diffractograms of the powder mounts, most of the additives except for MSB, DUO, MEX and MIX showed evidence of possible smectite presence (Figure 1). After carbonate and organic matter removal, powder mounts of MIX showed evidence of poorly crystallized aluminosilicates and quartz, while MEX confirmed previous results (no smectite evidence). Most of the additives except MSB showed quartz peaks, and interestingly, the additive DUO showed a peak compatible for clinoptilolite.

The pH values of the additives ranged from 5.4 to 9.6. MIX and MSB registered the lowest pH values of 5.4 and 5.6, respectively, which could be due to the presence of biological materials other than clay minerals as reported by other authors in similar products (Kannewischer et al. 2006). The rest of the additives had pH values higher than 6.7. It is not uncommon to find alkaline pH values with various aluminosilicates (García-Morales et al. 2004), as well as in confirmed smectite-containing products (Kannewischer et al. 2006). The US Pharmacopeia has reported a pH of 9.5–10.5 for natural bentonites and 9.0–10.0 for pure bentonites (montmorillonites) (US Pharmacopoeia 2005). In our study, we found the presence of smectite in nine of the additives and (on average) they had pH values of 8.55; however, there were two additives that did not correlate with these pH values. MLB had a pH of 6.7 with confirmed smectite presence, and MEX had a pH of 7.5 without evidence of smectite. For this reason, pH values cannot be definitively used as a characterization tool for smectite presence or for effectiveness of sorbents for AFB_1 binding.

In the fractionation analysis, ZEO and DUO (known as organoclays) registered the highest silt and the lowest clay-sized particle content, even though they had SV values of 0.80 and 0.68, respectively. The processing of these clays with long chain surfactants (Lemke et al. 1998) can aggregate clay minerals and prevent the action of dispersant agents used during the fractionation process. This effect may explain the low clay content obtained for these samples. MEX registered the highest percentage of sand-sized particles. Most likely, the sand fraction of complete soils is composed of quartz (SiO_2) or other primary silicates (Brady and Weil 2002). However, sometimes clay aggregates can be found in the sand and silt fractions. According to fractionation analysis, 46% of MEX was composed of sand-sized particles and this additive did not effectively bind AFB_1. The lowest percentage of sand-sized particles was obtained for MCA (6%), and this additive showed favourable sorption characteristics based on isothermal analysis (see below). The same trend was observed for VOL which had the highest clay percentage (51%) and a favourable sorption pattern. The rest of the additives ranged from 4 to 47% clay content. It is important to mention that, according to the XRD on powder mounts, the high percentage of clay-sized particles observed for MSB (60%) was mainly composed of organic matter (Figure 1). However, some XRD peaks observed for this additive were compatible with calcite mineral. A similar phenomenon (organic matter in the clay fraction) was also observed for MIX and it is been reported that, significant dilution of minerals with other materials (e.g. organic carbon)

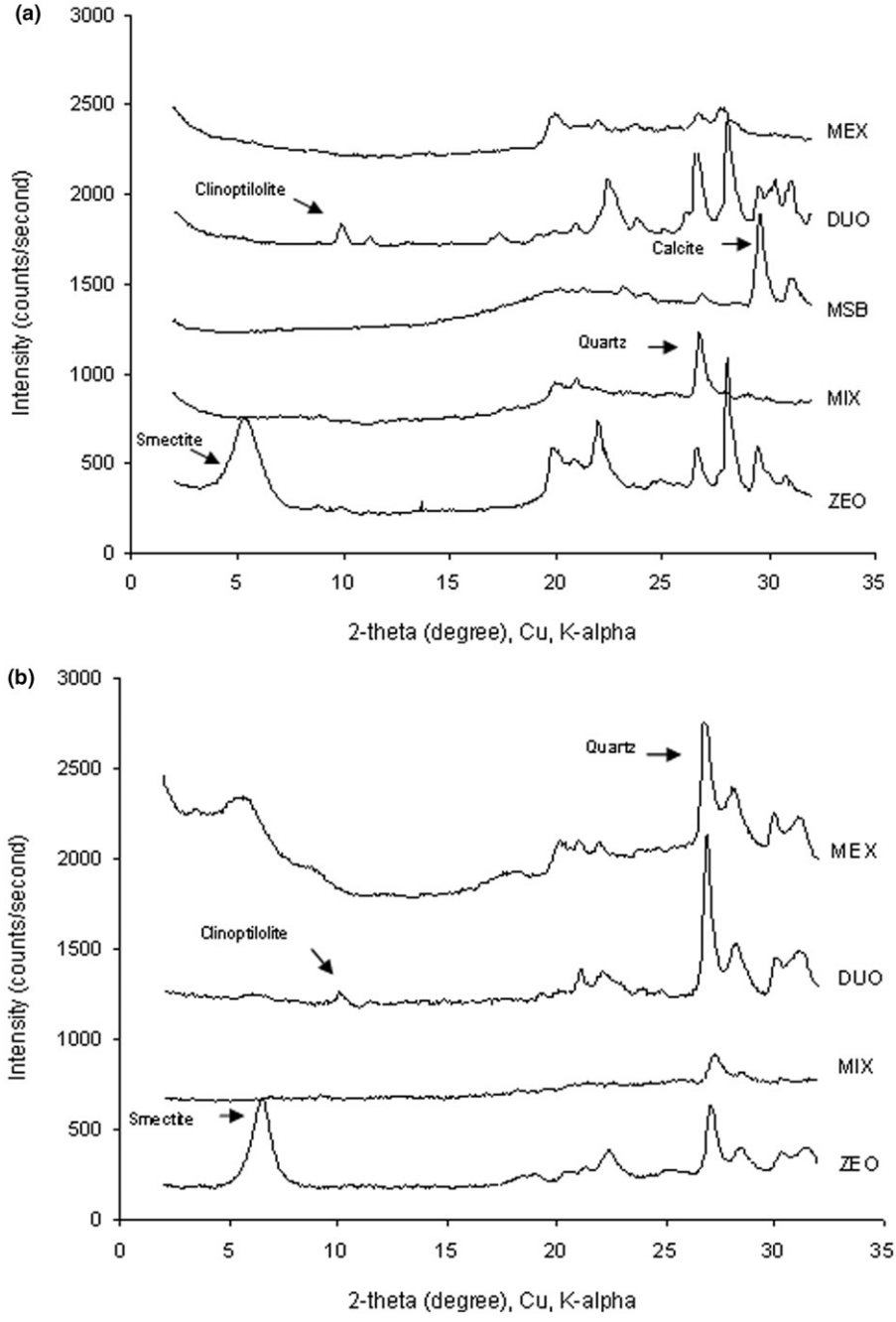

Figure 1. (a) Powder mounts X-ray diffractograms of a smectite containing additive (ZEO) and 4 potential additives without smectite (DUO, MIX, MEX and MSB). (b) Mg^{2+} saturated clay samples confirmed the smectite containing additive (ZEO) and the 3 potential samples without smectite (DUO, MIX, MEX). MSB was not included in Figure (b) due to difficulties preparing an oriented sample because of its mainly organic composition. Arrows display the mineral identification of the peaks.

observed for similar additives, can have a negative influence on the quality of XRD patterns (Kannewischer et al. 2006).

Isothermal and single-concentration AFB_1 adsorption analyses

Computer-generated equilibrium isotherms were extrapolated from additives data and fit to the Langmuir model (based on r^2-values and randomness of the residuals). The parameters of Q_{max} (mol AFB_1 kg^{-1} additive) and K_d were estimated to delineate the maximum sorption to the surface and the affinity of the sorption interaction. Major differences in AFB_1 binding were observed among the additives at pH 2, with just five additives fitting the Langmuir model, i.e., NSP, MCA, VOL, MLB and MIX. The remaining products did not fit the Langmuir model; instead, they showed evidence of a constant (non-saturable) partition trend (Figure 2). In contrast, at pH

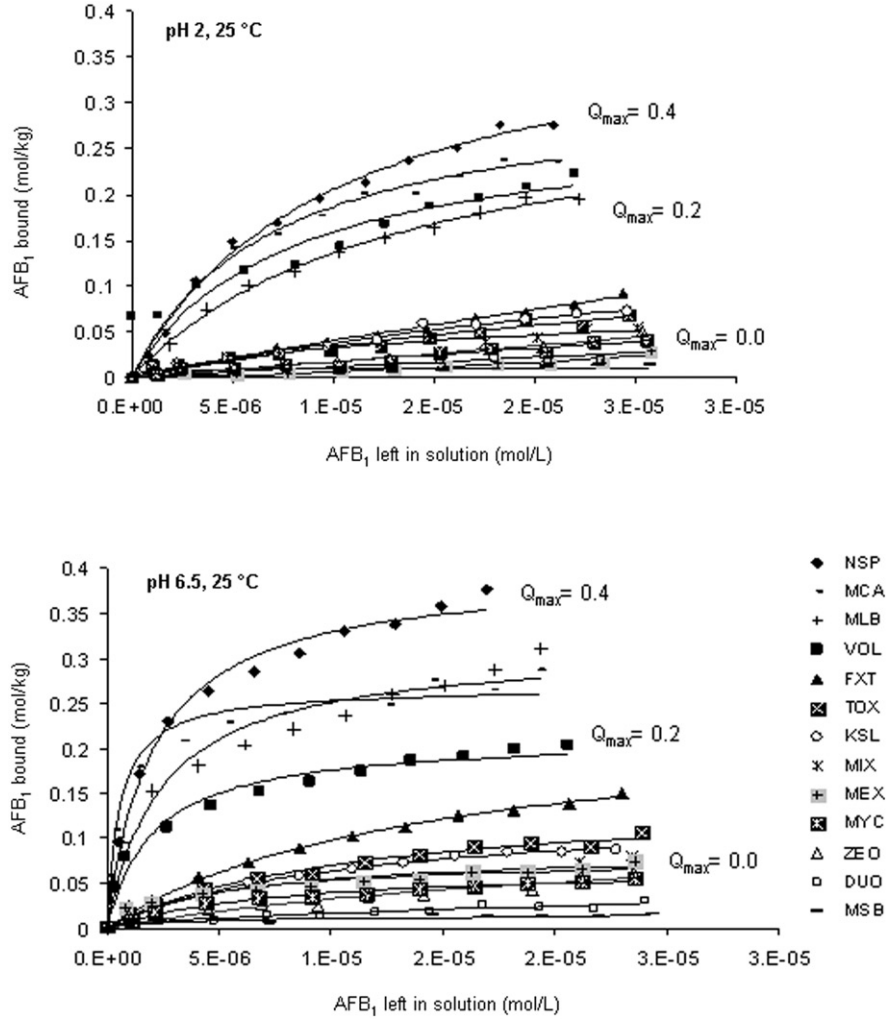

Figure 2. AFB_1 sorption isotherms on binding additives at pH 2 and pH 6.5. Each point represents the values calculated for AFB_1 bound and left in solution for the corresponding 11 dilutions. Isotherms were performed at 25°C. Q_{max} is expressed as mol AFB_1/kg of additive.

6.5, L-shaped curve characteristics of the isotherms were observed for many of the additives (Figure 2) with some variation in the r^2-values. MSB and ZEO did not fit the Langmuir model at pH values of 2.0 or 6.5. The most effective binding additives for aflatoxin at both pH values were NSP, MLB, MCA and VOL. The highest Q_{max} value was obtained for NSP ($Q_{max} = 0.4$) at pH values of 2.0 and 6.5.

In order to compare directly the additives, all adsorption isotherms were force-fitted to the Langmuir equation obtaining Q_{max} and K_d values regardless of the shape or fit of the curve (Table 1). By doing so, the lowest value was obtained for MSB ($Q_{max} = 0.009$ and 0.015) at pH values of 2.0 and 6.5, respectively. The results obtained for glucomannan-based products like MSB in our study, differ from the *in vitro* study of Diaz et al. (2002). In his study, MTB-100 at an approximate level of 1% in solution was reported to sorb 96.6% of 5 μg of aflatoxin, although the sorption trend was not determined by isothermal analysis. In recent reports, the molecular mechanism of binding of AFB_1 to *β*-D-glucans (ingredient of glucomannan products) was shown to be Van der Waals attractions and hydrogen bonds (Yiannikouris et al. 2006). It is well established that these bonding forces are reversible and depend largely on the orientation of the molecules. In contrast, the binding of AFB_1 on interlayer surfaces of smectites (e.g. NSP, Ca-montmorillonite) involves chemisorption bonding mechanisms (Grant and Phillips 1998) that are stronger than Van der Waals forces and hydrogen bonding interactions. Importantly, this interlayer interaction is thermodynamically favoured (Grant and Phillips 1998) and has been confirmed by XRD in another laboratory (Kannewischer et al. 2006).

Both organoclays (ZEO and DUO) showed low aflatoxin-binding capacities. This observation could be attributed to the small amount of clay-sized particles

contained in these two products. Another possibility is that exchanged organic cations used to produce these additives are also bound in the interlayer of the parent clays and compete for the active binding sites available for AFB_1. In studies comparing NSP (Ca-montmorillonite) with the negative controls, it was confirmed that the active ingredient for AFB_1 binding is smectite clay (Figure 3), and this conclusion has been supported by other studies (Phillips et al. 2002; Kannewischer et al. 2006).

In the single-concentration adsorption analysis, NSP showed the highest percentage of AFB_1 binding in the bulk material, the sand, silt and the clay fractions followed by MLB and MCA. Other additives showed variable sorption percentages in the different fractions (Figure 4). All of the additives were processed under the same standard protocols that are used in mineralogy studies to separate the fractions of soil samples. XRD methodology is capable of detecting very small amounts of clay minerals, and while the sand fraction is composed mainly of quartz, in NSP, the sand fraction contained considerable amounts of smectite clay (XRD data not shown). The silt fraction of NSP was also found to contain smectite. This suggests that smectite aggregates with the size of sand or silt are prevalent in this material and in some of the other additives (e.g. VOL and MLB). Smectite presence in the sand and silt fractions of additives may be attributed to a variety of causes including, lack of complete dispersion of the sample, or inadequate fractionation.

Pearson's correlation coefficient

The data from these studies were shown to be normally distributed and without the presence of outliers before calculating Pearson's correlation coefficient.

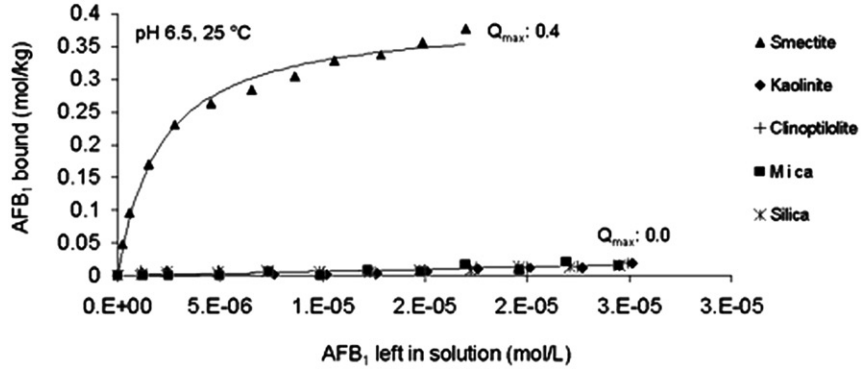

Figure 3. AFB_1 sorption at pH 6.5 on smectite clay (NSP) versus non-binding materials such as kaolinite, clinoptilolite, mica and silica. The highest capacity (Q_{max}) for AFB1 sorption was obtained for smectite (0.4 mol AFB_1/kg sorbent). Q_{max} values of 0.0 obtained for the other minerals demonstrate their negligible sorption capacity for AFB_1. Sorption assays were performed at 25°C.

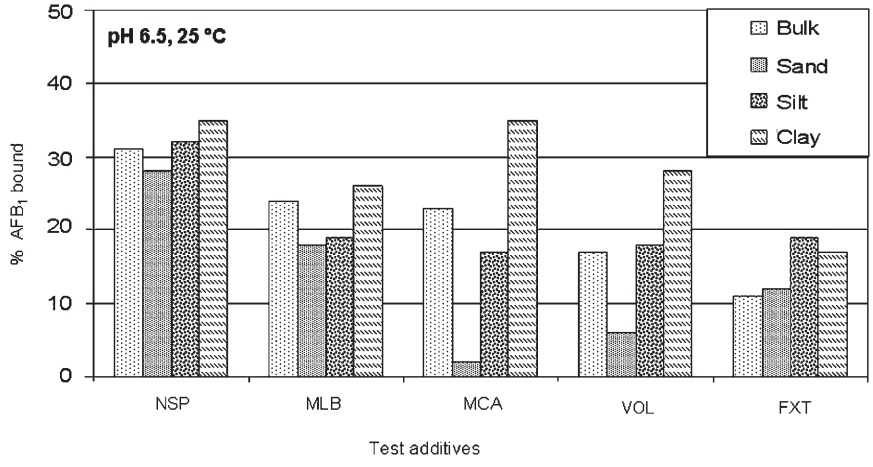

Figure 4. Percentage of AFB_1 binding at pH 6.5 with bulk versus fractionated additives in the single sorption assay at 25°C. Percentage was calculated based on the amount adsorbed from a total concentration of 8 µg ml-1 AFB_1. NSP had the highest percentage of AFB_1 binding in the bulk, sand and silt fractions. MCA and NSP registered the highest AFB_1 sorption in the clay fraction.

Significant correlation values at the 0.01 level were found for SV with sand [−0.859 correlation coefficient (CC) ($p = 0.001$)] and clay [0.747 CC ($p = 0.008$)]. Significant correlation values at the 0.05 level were found for Q_{max} with sand [−0.660 CC ($p = 0.027$)] and clay [0.606 CC ($p = 0.048$)]. The pH values were not included in the correlation analysis because the data for this parameter was not normally distributed. Q_{max} values from isotherms at pH 6.5 were used in the correlation analyses.

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

Based on the XRD-pattern from Mg^{2+}-treated samples, most of the additives except MSB, DUO, MEX and MIX showed evidence of smectite according to XRD analyses (Figure 1); however, the amount of smectite could not be accurately quantified without additional chemical analysis. Mg^{2+}-saturated MSB ceramic tile could not be prepared due to difficulties forming an oriented sample with organic clay-size materials. The only XRD pattern for this sample was the powder mount. Previous degradation of carbonates and organic matter in the clay fraction (air dry), MEX showed a pattern suggestive of smectite (Figure 1), however the Mg^{2+} EG treatment did not confirm the smectite presence and MEX was classified as an additive without smectite. Interestingly, MIX additive is known to contain silicate minerals (bentonite according to a personal communication) but evidence of smectite was not found in this material according to our procedures; nevertheless quartz and apparently poorly crystallized aluminosilicates were present in this sample. A possible reason for non-detection of smectite in MIX could be a low inclusion/dilution of bentonite. The aflatoxin-binding capacities of the additives that contained smectite were considerably different, even though they were all shown to contain smectite. This disparity may be due to a variety of factors. For example, the smectite inclusion level in the sample may be too low for effective binding. In addition, a lack of purity of the materials and contamination in mixtures (organic materials) could be a risk for masking the effects of smectite due to clay aggregation problems or dilution. Finally, processing procedures to make the materials more lipophilic (Lemke et al. 1998) may interfere with AFB_1 adsorption onto smectite.

Hydra *toxicity bioassay*

As a well-established and sensitive *in vivo* indicator of toxicity, hydra were used to estimate the potential toxicity of these additives. In the hydra toxicity bioassay, most of the additives were non-toxic to hydra except for ZEO, DUO and MSB at levels of 0.3 and 0.5% (equivalent to the common levels of inclusion in animal feed) (Figure 5). Importantly, organoclays were toxic to hydra (even at the 0.1% inclusion rate). These data reinforce previous studies in mice that demonstrated the toxicity of organoclays (Lemke et al. 2001a; Afriyie-Gyawu et al. 2005). Concurrently, MSB was also toxic to hydra. This effect may be due to the growth of intact yeast and microorganisms that have been observed in these types of products (Kannewischer et al. 2006).

Conclusions

In accordance with isothermal and single-sorption analyses from these studies, NSP showed the best sorption characteristics for AFB_1, followed by MCA, MLB and VOL. Since considerable amounts of clay minerals were found in the sand and silt fractions of some of the additives, further work is warranted to delineate this phenomenon. In order to determine the relationship of particle size to binding capacity, aflatoxin-binding additives need to be studied on a case-by-case basis, determining potential

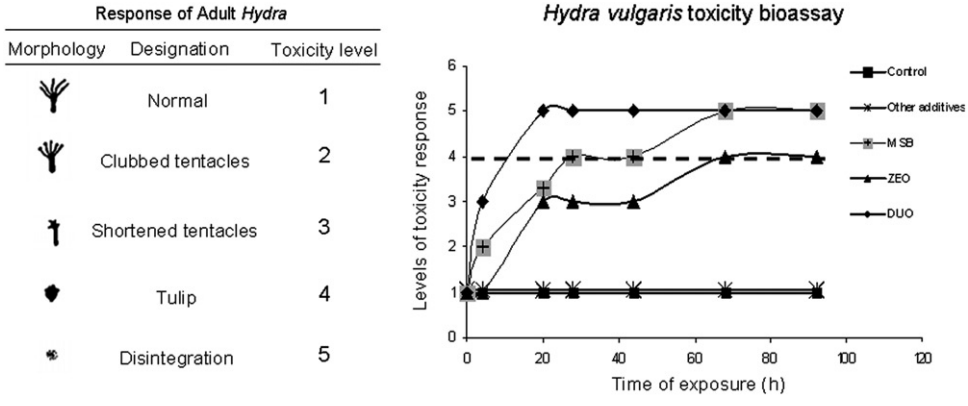

Figure 5. Hydra toxicity bioassay for binding additives. Dashed line represents the toxic endpoint (tulip stage). Each point represents the toxicity levels at different times of exposure.

contaminants versus the primary clay mineral content. Even though most of the additives tested showed evidence of smectite presence, the binding capacities were significantly different, and only four of them showed better adsorption characteristics for AFB_1. The best binding additives showed an L-shape pattern of sorption at both pH conditions, and a $Q_{max} \geq 0.283$ or 0.212 at pH values of 2.0 and 6.5, respectively. The particle size distribution in the additives was found to be significantly correlated with the binding capacity for AFB_1. Again, the presence of smectite clays in the additives was responsible for the capacity to bind AFB_1 when compared with other major clay minerals. Major differences in the ability of these additives to bind aflatoxins verify the critical need for further research and screening of these types of products. Based on our work, aflatoxin-binding additives intended for animal feed must contain smectite (i.e., montmorillonite) clay mineral as a primary component and should be rigorously evaluated *in vitro* and *in vivo*. Apart from favourable thermodynamic characteristics of sorption and other *in vitro* characterization analysis, these additives should be challenged *in vivo* to evaluate their safety and efficacy under realistic contamination levels of aflatoxins and other mycotoxins. Before using these additives in animal feed, clear evidence of their purity (free from hazardous contaminants) and their negligible nutrient interactions must be considered. Further investigations in the area are clearly warranted. Studies should focus on claims of multiple mycotoxin binding, which would suggest non-selectivity and potential interference with nutrients and other critical feed additives.

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