THE CHARACTERIZATION OF CLAYS AND CETYLPYRIDINIUM-EXCHANGED CLAYS FOR THEIR ABILITY TO ADSORB ZEARALENONE

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Abstract

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The adsorption of zearalenone (ZEN) onto Ca-montmorillonite (STx-1), Na-montmorillonite (SWy-2), acid-activated montmorillonite (AAM), clinoptilolite and cetylpyridinium (CP) exchanged clays was studied. The results indicated that CP-exchanged clays were more effective than unmodified base clays in adsorbing ZEN, i. e. STx-1 (7.4 ± 2.3 mg/g) < clinoptilolite (7.6 ± 1.1 mg/g) < AAM (10.0 ± 0.0 mg/g) < SWy-2 (11.9 ± 0.4 mg/g), whereas the extent of ZEN adsorption into modified clay products did not follow the order seen in the based clays. The adsorption efficiency of CP-exchanged clays is in the order of CP*STx-1 (56.9 ± 1.1 mg/g) = CP*AAM (56.9 ± 1.8 mg/g) > CP*SWy-2 (47.9 ± 2.6 mg/g) > CP*clinoptilolite (15.5 ± 1.1 mg/g). Three CP-exchanged clays were further characterized by isotherm studies. The results indicated that the isotherm shapes of CP*exchanged clays exhibited a S-shape. The isotherm plot is concave up to the inflection point and then plateaus, which suggests multiple, specific binding sites and saturation at each site. It is important to rigorously test and thoroughly characterize potential mycotoxin binding agents both *in vitro* and *in vivo* to provide an understanding of the mechanism of adsorption, in order to modify or develop adsorbents for enhanced products and multi-mycotoxin binding agents.

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บทคัดย่อ

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การอธิบายคุณลักษณะดินเหนียวและดินเหนียวที่ถูกแลกเปลี่ยนด้วยสารเซทิลไพริดิเนียมคลอไรด์ในการดูดซับซีราลีโนน

การศึกษาดินเหนียวชนิด แคลเซียมมอนโมลินโลไนต์ (STx-1) โซเดียมมอนโมลินโลไนต์ (SWy-2) มอนโมลินโลไนต์ที่ถูกกระตุ้นด้วยกรด (AAM) ไคลนอพติโลไลต์ และดินเหนียวที่ถูกแลกเปลี่ยนด้วยสารเซทิลไพริดิเนียมคลอไรด์ ในการดูดซับสารพิษซีราลีโนน พบว่าดินเหนียวที่ถูกแลกเปลี่ยนด้วยสารเซทิลไพริดิเนียมคลอไรด์ มีประสิทธิภาพสูงกว่าดินเหนียวพื้นฐาน ในการดูดซับซีราลีโนน ดังนี้ STx-1 (7.4±2.3 มก./ก.) < ไคลนอพติโลไลต์ (7.6±1.1 มก./ก.) < AAM (10.0±0.0 มก./ก.) < SWy-2 (11.9±0.4 มก./ก.) ในขณะที่การดูดซับซีราลีโนนของดินเหนียวที่ถูกดัดแปลงโครงสร้างไม่เป็นไปตามลำดับเหมือนกับดินเหนียวพื้นฐาน ประสิทธิภาพในการดูดซับซีราลีโนนของดินเหนียวที่ถูกแลกเปลี่ยนด้วยสารเซทิลไพริดิเนียมคลอไรด์ มีลำดับดังนี้ CP*STx-1 (56.9±1.1 มก./ก.) = CP*AAM (56.9±1.8 มก./ก.) > CP*SWy-2 (47.9±2.6 มก./ก.) > CP*clinoptilolite (15.5±1.1 มก./ก.) นอกจากนี้ดินเหนียวที่ถูกแลกเปลี่ยนด้วยสารเซทิลไพริดิเนียมคลอไรด์ 3 ชนิดถูกนำมาการอธิบายลักษณะการดูดซับโดยวิธีไอโซเทอม ผลการทดลองบ่งชี้ว่า รูปทรงไอโซเทอมมีลักษณะเป็นตัว S ซึ่งการดูดซับเป็นเส้นเว้าเข้าข้างในก่อนโค้งขึ้น จนกระทั่งถึงจุดเปลี่ยนลักษณะราบซึ่งชี้แนะว่าจุดดูดซับเฉพาะมีหลายที่ และแต่ละที่ถึงจุดอิ่มตัว เป็นสิ่งสำคัญที่ต้องมีการทดสอบและอธิบายลักษณะสารที่สามารถจับสารพิษจากเชื้อรา ทั้งนอกกายและในกาย เพื่อที่จะได้ทราบกลไกระบวนการดูดซับ เพื่อเปลี่ยนแปลงหรือพัฒนาสารดูดซับ ให้มีประสิทธิภาพสูงขึ้นและสามารถจับกับสารพิษจากเชื้อราหลายชนิด

คำสำคัญ : ซีราลีโนน การดูดซับ ดินเหนียว ดินเหนียวที่ถูกแลกเปลี่ยนด้วยสารเซทิลไพริดิเนียมคลอไรด์

Introduction

Zealarenone (ZEN) or F-2 toxin is an important mycotoxin in temperate and warm regions of the world. The toxin is an estrogenic substance produced by Fusarium fungi. ZEN occurs in maize and small grains like barley, wheat, sorghum, millet and rice (Miller, 1995). ZEN causes alterations in the reproductive systems of several animals. The toxin produces a potent hyperestrogenic response in susceptible animal species. Swine are the most affected, other animals, such as cattle, poultry and laboratory animals, are also affected, but to a lesser extent. Symptoms of ZEN poisoning include precocious sexual development in gilts, uterine enlargement, swollen vulva and mammae, anestrous, pseudopregnancy, reduced litter size and reduced viability of newborn pigs

(Pier, 1981). In pregnant gilts, ZEN causes embryo loss and the retention of corpora lutea in affected pregnant gilts (Long and Diekman, 1986). In boars, ZEN exposure resulted in inflammation of the prepuce, smaller testes, epididymitis, reduced vesicular gland weights, cessation of spermatogenesis and reduced libido. Also, ZEN causes a reduction in the conception rate of dairy heifers (Diekman and Green, 1992).

Methods for the inactivation of mycotoxins in contaminated feedstuffs are critically needed. Recently, a reduction in the bioavailability of ZEN by adding adsorbents to contaminated feedstuffs has been considered as a cost-effective and practical strategy for the animal production industry to persue. Several adsorbents, including clays and modified clays are able to detoxify

mycotoxins (Alegakis et al., 1999; Huwig et al., 2001). The modified clays that by altering morphological properties and binding capacities by exchanging with organic cations, have been recently studied extensively for their ability to remove toxins in the environment including mycotoxins (Ake et al., 2001; Pal and Vanjara., 2001; Tomasevic-Canovic et al., 2003). Probably, the most favorable approach for the study of mechanisms of adsorption of toxins onto the surfaces of sorbents, is through the use of isotherm analysis (where the amount of toxin adsorbed is plotted against the concentration of toxin in the external phase, at a constant temperature and usually under equilibrium concentration). Previous studies have utilized and developed multi-tiered methods to the *in vitro* prescreening of clay-based adsorbents for the adsorption of mycotoxins, including isothermal analysis (Lemke et al., 2001). These methods have allowed an insight into the molecular mechanisms of sorbents and enabled a comparison of critical similarities and differences. Various sorbents and clay minerals possess a distinct structure which can bind mycotoxins in different ways. Important structural differences provide unique functional and chemical characteristics. The objective of this study was to compare the ability of clays (Ca-montmorillonite (STx-1), Na-montmorillonite (SWy-2), acid-activated montmorillonite (AAM), clinoptilolite) and these clays exchanged with cetylpyridinium chloride (CP) to adsorb ZEN *in vitro* and include isotherm studies to characterize the toxin adsorption of the modified clay products.

Materials and Methods

Chemicals and reagents: Zearalenone was purchased from Sigma Chemical Co. (St. Louis, MO). Cetylpyridinium chloride (CP) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Ca-montmorillonite (STx-1) and Na-montmorillonite (SWy-2) were obtained from a source of clay minerals (Columbia, MO). Acid-activated montmorillonite (AAM) was obtained from Kaiser Aluminum (Pleasanton, CA). Clinoptilolite was obtained from Engelhard Co. (Cleveland, OH). All chemi-cals and reagents were of the highest purity commercially available. The high-purity water used in this study was prepared by processing deionized water through a Milli-Q^{uf+} system.

Clay preparation: Exchanged clays were prepared by washing clays in the highly purified water (100 ml/1 g clay) for 24 hrs, while shaking at 500 rpm. The clays were then spun down by centrifugation for 20 min at 2000 rpm. The supernatant was poured off and the clays were resuspended in water. All clays were rehydrated with 100 ml water to which cetylpyridinium chloride was added at an amount of approximately 1.5 times the cation exchange capacity of the clays (based on an estimated of 90 cmol$_{charge}$/kg of clay). The resulting mixture was then agitated and allowed to exchange for a 24 hrs period. The CP-exchanged clays were then separated by centrifugation and the supernatant was removed and subsequent washing took place with deionized water, under agitation, for another 24 hrs. The washed clays were then dried at 50ºC overnight, ground and sieved through a 325-mesh sieve to obtain particles that were less than 45 μm (Herrera et al., 2000).

Adsorption capacity: A stock solution of ZEN was prepared by dissolving the pure crystals in acetonitrile. A volume of the stock solution was injected into purified water (pH 6.5 ± 0.2) to yield a 4 μg/ml solution of ZEN. The concentration was then checked with a recording UV-Visible spectrophotometer (UV-1601 PC, Shimadzu, Shimadzu Scientific Instrument). Approximately 10 mg of clay was weighed into a 16x125 ml, disposable, borosilicate test tube. Purified water was then added to the clay to make a 2 mg/ml suspension. The suspension of clay was vortexed to achieve homogeneity. From this suspension, 50 μl (containing 100 μg of clay) was added to each 5 ml solution of ZEN (4 ppm). There were three controls for each clay tested: a purified water control, a toxin control containing 4 μg/ml of ZEN without clay, and a clay control containing 5 ml purified water and 100 μg of clay. The samples and controls were capped and placed on an electric shaker (IKA-VIBRAX-VXR, Bacter, McGraw, IL) running at 1000 rpm for 24 hrs and held in

an incubator, at 25°C. After shaking, the samples were centrifuged (International Centrifuge, Model UV, International Equipment Co., Needham, MA) at 2000 rpm for 30 min, to separate the clay from the supernatant. The supernatant from the samples was analyzed for absorbance at 236 nm, to determine the concentrations of ZEN remaining in solution.

Isotherm studies: The isotherm method used in these studies was modified from one described previously published study (Lemke et al., 1998). A stock solution of ZEN was prepared as described earlier. Each isotherm study consisted of triplicate 5 ml solutions of ZEN made from dilutions of the stock. These yielded approximate concentrations of 0.2, 0.4, 0.8, 1.2, 1.6, 2.0, 2.4, 3.0, 3.2, 3.6, and 4.0 µg/ml. These concentrations were achieved by adding an appropriate amount of working solution to 16x125 ml, disposable, borosilicate test tubes and then adding the complimentary amount of purified water, to make a total volume of 5 ml. Approximately 10 mg of clay was weighed in a 16x125 ml, disposable, borosilicate test tube. Purified water was then added to the clay to make 2 mg/ml suspension. The suspension of clay was vortexed to achieve homogeneity. From this suspension, 50 µl (containing 100 µg of clay) was pipetted out with an autopipetter to each 5 ml test sample. Four controls were also prepared: a purified water control containing no toxin, a toxin control containing 0.2, 4 µg/ml ZEN, without clay and a control containing 5 ml purified water and 100 µg of clay. The samples and controls were capped and placed on an electric shaker running at 1,000 rpm (for 24 hr) and held in an incubator at 25°C. After shaking, the samples were centrifuged at 2000 rpm, for 30 min, to separate the clay from the supernatant. The supernatant was analyzed for absorbance at 236 nm using a UV-Visible spectrophotometer to determine the concentrations remaining in solution.

Data calculations and curve fitting: The UV/Visible absorption data was entered into an Excel spreadsheet (Microsoft, Redmond, WA) to calculate the amount of ZEN left in solution (CW), and the amount

adsorbed (q) for each data point. The data was then transferred to a Table Curve 2D v 3 (Jandel Scientific, San Rafael, CA) to fit the modified Freundlich isotherm equation so as to determine the distribution constant (Kd) (Grant et al., 1998; Pimpukdee et al., 2002).

Statistical analysis: ZEN adsorption data were represented as the arithmetic mean±SEM of the three replicates per sample. Means showing significant differences in ANOVA were compared using Duncan's multiple range test (Ott, 1993). All statements of differences were based on a significance of $p < 0.05$.

Results

Figure 1 shows the binding capacities of clays and cetylpyridinium-exchanged clays, for ZEN, under equilibrium adsorption conditions. Binding capacities of these clays ranged from 7.38±2.30 mg/g to 56.90±1.09 mg/g. The lowest average adsorption of ZEN from solution was STx-1 (7.38±2.30 mg/g) whereas the highest average adsorption of ZEN from solution were CP*STx-1 (56.90±1.09 mg/g) and CP*AAM (56.90±1.80 mg/g). SWy-2 and AAM demonstrated similar binding with an average adsorption of 11.90±0.41 mg/g and 10.00±0.00 mg/g, respectively. CP*clinoptilolite (15.48±1.09 mg/g) showed about two times increased adsorption capacity as compared to clinoptilolite (7.62±1.09 mg/g).
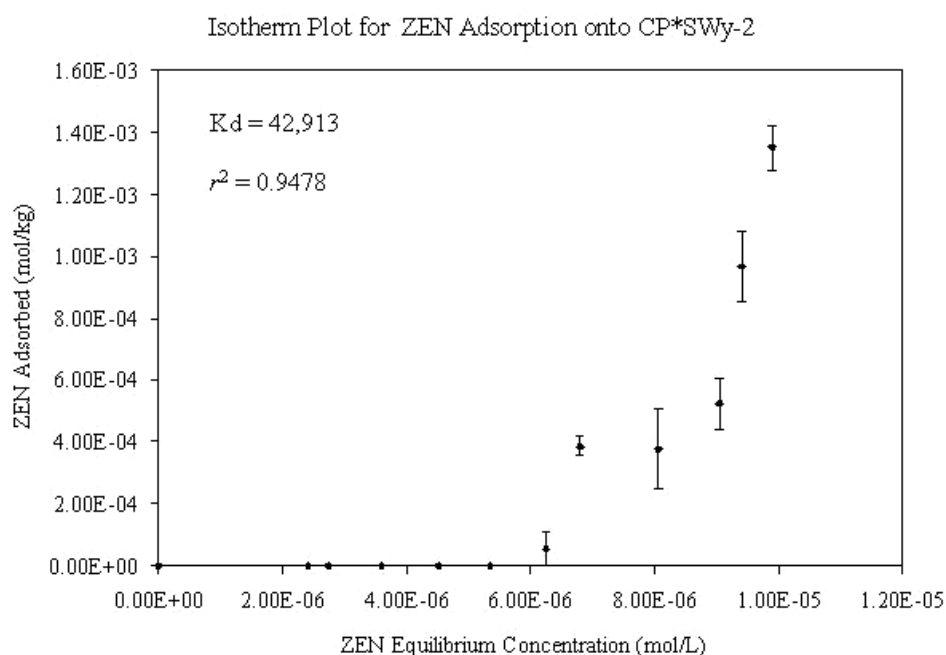
The data were fitted to a modified Freundlich isotherm to determine the distribution constant (Kd). The coefficient of determination (r^2) and the distribution constant (Kd) are displayed in the graph (Figure 2-4). CP*SWy-2 had highest Kd value (42,913) as compared to Kd values of CP*STx-1 (23,263) and CP*clinoplilote (29,519), which suggests that the CP*SWy-2 is more effective for removal ZEN in solution. Also, the modified Freuendlich isotherm gave a good fit for CP*SWy-2 ($r^2 = 0.9478$) but not for CP*STx-1 ($r^2 = 0.7104$) and CP*clinoplilote ($r^2 = 0.8827$). This study showed that experimental adsorption isotherms obtained for ZEN on CP*exchanged clays, may be classified, according to the Giles classification (Giles et al., 1974), as a S-shape
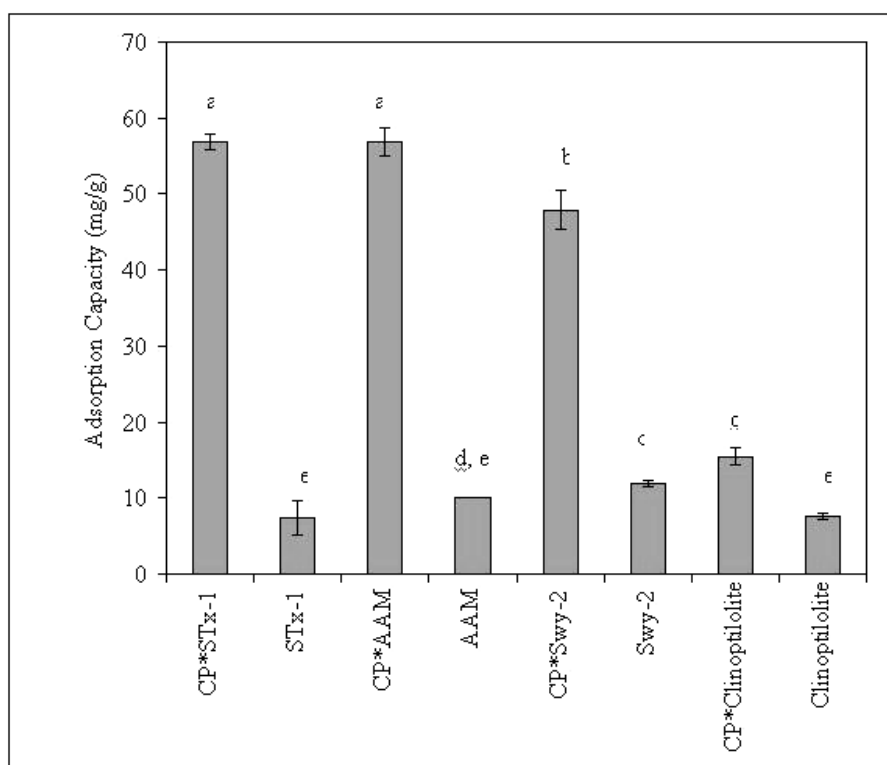
Figure 1 Average zearalenone adsorption onto clays and cetylpyridinium-exchanged clays. Means with the same letter are not significantly different by Duncan's multiple range test at p < 0.05. The data represent mean ± standard error for triplicates in each sample.
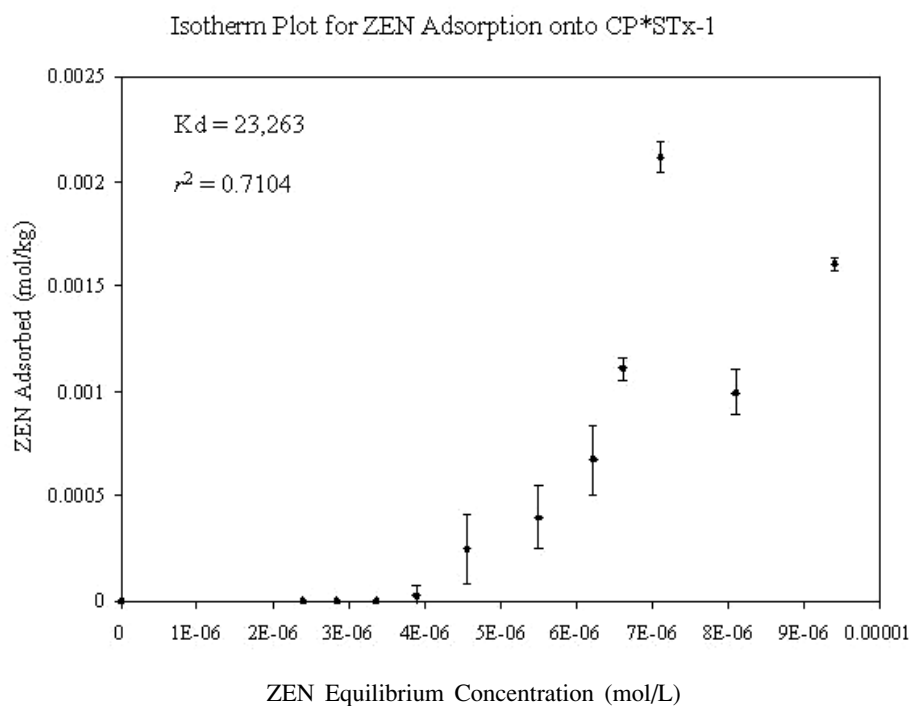
Figure 2 Adsorption isotherm for ZEN on CP*STx-1. The data represents the mean adsorption to clay ± SD from three replicate experiments. Data were fitted using the modified Freundlich isotherm equation; values of Kd and r^2 are displayed in the graph.

Figure 3 Adsorption isotherm for ZEN on CP*SWy-2. The data represents the mean adsorption to clay ± SD from three replicate experiments. Data were fitted using the modified Freundlich isotherm equation; values of Kd and r^2 are displayed in the graph.
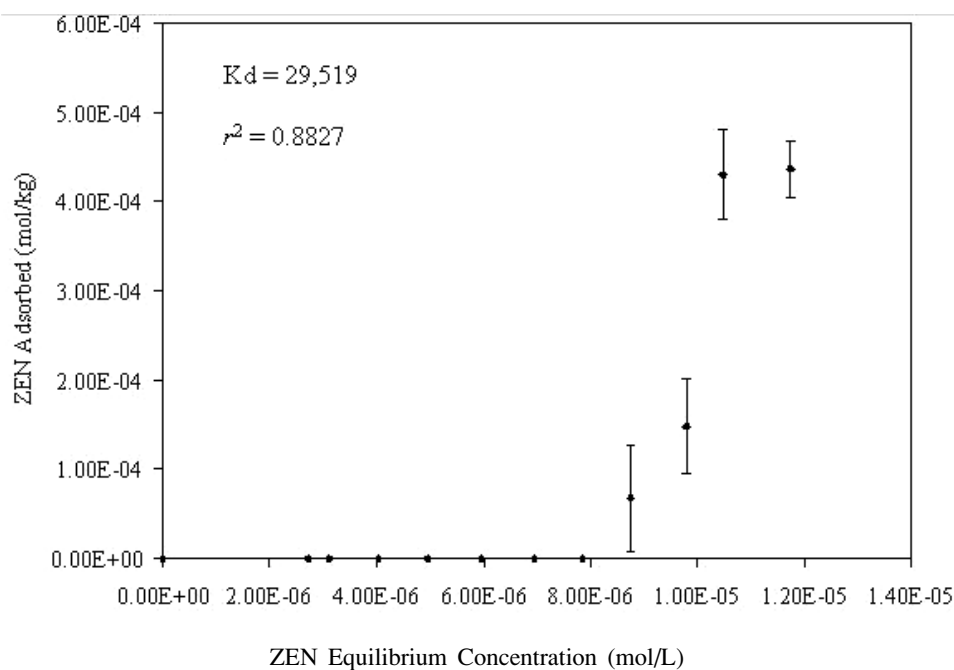
Figure 4 Adsorption isotherm for ZEN on CP*clinoptilolite. The data represents the mean adsorption to clay ± SD from three replicate experiments. Data were fitted using the modified Freundlich isotherm equation; values of Kd and r^2 are displayed in the graph.

subgroup max (Figure 2), a S-shape subgroup 3 (Figure 3), and a S-shape subgroup 2 (Figure 4), for CP*STx-1, CP*SWy-2 and CP*clinoptilolite, respectively.

Discussion

The montmorillonite is a dioctahedral smectite and the isomorphous substitution in montmorillonite occurs in the octahedral sheet rather than in the tetrahedral sheet. The large cation exchange capacity (CEC) of smectites arises from the substitution of Al^{3+} for Si^{4+} in the tetrahedral sheets and the substitution of divalent cations, such as Mg^{2+}, Ca^{2+}, and Fe^{2+}, for trivalent cations such as Al^{3+} and Fe^{3+} in the octahedral sheets. Montmorillonites swell in water, and the extent of the swelling is influenced by the hydration of the interlayer cations. Ca-montmoril-lonites, for example, can swell when immersed in water to give a basal spacing corresponding to the inclusion of three to four layers of water. Na-montmorillonites swell more than Ca- montmorillonites when immersed in water and in many instances the mean layer separations of these Na-exchanged clays can amount to hundreds of Å units. Molecular sorption on montmorillonites may involve chemical as well as physical effects. The large internal surface area of montmorillonite (up to $8 \times 105 \ m^2 \ kg^{-1}$) provides most of the adsorption surface. In addition, the external surface area of montmorillonite is generally greater than that of other clay minerals, due to its small particle size. The most important chemical properties of montmorillonites include high CEC, ion selectivity and molecular sorption. The most important physical properties of montmorillonites are expansion and collapse, retention of large quantities of water, high cohesion and adhesion, small particle size and an extremely large specific surface area (Barrer, 1978). The chemical structures of montmorillonite and AAM are different in that montmorillonites, activated in acids, yield products with a highly amorphous SiO_2 content and a new microstructure with pores that have greater adsorption properties. However, our results, indicated that montmo-rillonite and AAM were little different in their adsorption of ZEN.

Clinoptilolite is one of the zeolite group minerals, it is non expandable clay. Zeolite is a type of hydrous aluminosilicate belonging to the tectosilicates in which the SiO_4 tetrahedra form a 3-dimensional cagelike frame work. In the zeolite structure, some Si^{4+} ions are replaced by Al^{3+}, which results in a net negative charge, that needs to be balanced by exchangeable cations. The approximate chemical formula of clinoptilolite is $(Ca, Na, K)_6A_{16}Si_{30}O_{72}.24H_2O$ (Li, 1999). Clinoptilolite's framework consists of four channels. Three channels are formed with 8-membered rings of oxygen and one channel with 10-membered rings. The dimensions of the open channels in clinoptilolite are $0.89 \times 0.35 \ nm^2$ for the 10-member ring allowing exchangeable cation to enter and exit freely, but too small for ZEN sorption. Thus, the sorption of ZEN on clinoptilolite is limited to the external surface.

Our results indicated that cetylpyridinium-exchanged clays were more effective than unmodified base clays in adsorbing ZEN. Natural clays contain exchangeable metal ions (Ca^{2+}, Na^+, K^+), which make their surfaces hydrophilic; consequently they are quite ineffective in sorbing nonionic compounds from aqueous solutions. However, it has been found that if the metal ions are exchanged with large surfactant cations, such as long-chain cetylpyridinium (CP) or hexadecyltrimethy-lammonium (HDTMA), the clay surfaces become hydrophobic or organophilic. These clays are commonly referred to as organoclays and are very effective in sorbing organic pollutants (Mortland, 1970). This study suggests that natural clays exchanged with large surfactant cation (CP) greatly improved their adsorption abilities. It might be explained that CP interacts with clays and replaces the exchangeable cations on their surfaces, forming a stationary phase in the clay particles. The modification produces a change of surface property from hydrophilic to hydrophobic. In addition, as a result of the larger size of the CP cations, compared with that of the replaced cations, the interlamellar distance (basal spacing)

of the clay increases and additional space is produced in the clay particles, thus facilitating the entry of ZEN into interlayer region. Preliminary studies suggests that CP-exchanged clays can also be effective for the adsorption of bacteria (Herrera et al., 2000). Recently attempts have been made to modify natural clinoptilolite, with different amounts of octadecyldimethyl benzyl ammonium chloride and dioctadecyldimethyl ammonium chloride, for the effective adsorption of aflatoxin B_1, zearalenone and ochratoxin A, with varying degrees of success (Tomasevic-Canovic et al, 2003).

Isotherm shapes are categorized into four types of curve, designated as H, L, C, and S, which represent different adsorption mechanisms (Giles et al., 1960). Generally, S isotherms have a concave shape at low concentrations and the amount adsorbed increases as a semilinear function of the concentration. The isotherm plot is concave, up until an inflection point and then plateaus. Subgroups are defined by sorption behavior at high concentrations. Subgroup 2 is characterized by one plateau, whereas, subgroup 3 has an inflection point due to a change from the plateau to a concave shape. Subgroup max is defined by the existence of a maximum. Isotherms of the S-shape have two mechanisms. First, solute-solute attractive forces on the surface may cause cooperative adsorption which lead to the S-shape. Secondly, the sorption of a solute may be inhibited by a competing reaction within the solution, such as a complexation reaction with a ligand (Hinz, 2001).

Clays with different structures are not all equal in their ability to adsorb ZEN. This article details the characterization of clays and modified clay products for the adsorption of ZEN. Further research could be made to modify natural based clays with chemical or physical treatments for producing mycotoxin binding agents with a higher adsorption capacity or multi-mycotoxin sequestering agents. It is important to rigorously test and thoroughly characterize potential mycotoxin binding agents *in vitro* and *in vivo* to provide a comprehensive understanding of the mechanism of adsorption. It will be useful for both the investigation and the modification of safe and effective clay products in order to prevent ZEN-induced diseases in animals and ZEN residues in food of animal origin.

Acknowledgements

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