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TRANSFORMATION OF ADSORBED AFLATOXIN B_1 ON SMECTITE AT ELEVATED TEMPERATURES

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Abstract—Aflatoxins cause liver damage and suppress immunity. Through adsorption, smectites can be used to reduce the bioavailability of aflatoxins. To further reduce the toxicity of aflatoxins and to eliminate the treatments of aflatoxin-loaded smectites, the ability to degrade the aflatoxin adsorbed to non-toxic or less toxic compounds is desirable. The objective of the present study was to investigate the effects of temperature and the exchange cation on the transformation of adsorbed aflatoxin B_1 on smectite. An AfB_1-Ca-smectite (sm) complex was synthesized. To enhance the Lewis acidity of the complexes, the exchanged calcium in the complex was replaced with Mn and Cu to obtain AfB_1-Mn-sm and AfB_1-Cu-sm complexes, respectively. The aflatoxin-sm complexes and pure aflatoxin B_1 were dried at 60ºC in aluminum cups, and heated from 100 to 200ºC in 25ºC steps. Aflatoxin B_1 and its transformation products were extracted with methanol after the heat treatment. The extracts were analyzed using UV spectroscopy, high performance liquid chromatography (HPLC)-fluorescence/UV, ultra-performance liquid chromatography (UPLC)-photodiode array (PDA), and electrospray ionization-tandem quadrupole-mass spectrometry (ESI-TQD-MS). The solid residues were analyzed using Fourier-transform infrared spectroscopy (FTIR). The UV and FTIR spectra of the AfB_1-sm clay residue extracts obtained after heating had decreased AfB_1 peak intensities and shifted peak positions with increased heating temperature. Significant shifts in band positions and changes in the shape of the UV spectra were observed in the extracts from the AfB_1-Ca-sm complex heated at 175ºC, the AfB_1-Cu-sm complex heated at 150ºC, and the AfB_1-Mn-sm complex heated at 125ºC. The HPLC and UPLCMS analyses of AfB_1-sm complex extracts indicated a gradual decrease in AfB_1 concentration with increased heating temperature and the formation of aflatoxins B_2, B_{2a}, M_1, M_2, and other unidentified compounds. No new compound was observed in the extracts of pure aflatoxin B_1 after a comparable heating experiment. These results suggest that smectite can effectively convert aflatoxin to other less toxic forms at elevated temperatures. Smectite ion exchange with Cu or Mn transition-metal cations and heat treatment induced more efficient conversion of the adsorbed aflatoxin B_1 molecules to other compounds.

Key Words—Aflatoxin, HPLC, Smectite, Transformation, UPLC-MS.

INTRODUCTION

Aflatoxins are carcinogenic mycotoxins produced by *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxin B_1, B_2, G_1, G_2, and M_1 are classified as class 1 carcinogens by the International Agency for Research on Cancer (IARC, 2002). The B and G letters in aflatoxin names refer to the blue and green fluorescence of the toxins under ultraviolet (UV) light and the 1 and 2 subscripts indicate the positions of the toxins relative to the solvent front on thin-layer chromatographic plates. Aflatoxin M_1 was first isolated from the milk of lactating animals fed an aflatoxin-contaminated diet.

Aflatoxin B_1 is the most toxic and hepatocarcinogenic aflatoxin (Grant and Phillips, 1998) and is also the biosynthetic precursor of the other aflatoxins. To reduce the bioavailability of aflatoxins to animals and humans, researchers investigated different physical methods such as UV or gamma irradiation, and the removal of aflatoxins by adding adsorbents to contaminated diets (Piva *et al.*, 1995). One of the most useful amendment strategies is the addition of clay minerals to animal feed and into the human diet (Huwig *et al.*, 2001; Phillips *et al.*, 2008). Bentonites are smectite-rich clays and have been tested as aflatoxin binders for >30 y in many countries.

Several factors are involved in the adsorption process of aflatoxin by smectite. The carbonyl groups of an aflatoxin B_1 molecule may undergo intermolecular H-bonding with the methoxy group of another aflatoxin B_1 molecule, and also with hydrogen atoms of water molecules present in the smectite interlayer. Ion-dipole interaction may also exist between exchange cations and carbonyl oxygen atoms (Deng *et al.*, 2010). The dicarbonyl groups of aflatoxin B_1 are an electron-rich system which readily forms chelates with transition metals having unfilled *d* orbitals. The chelate rings formed are planar and resonance stabilized (Phillips *et al.*, 1995). Exchange transition cations may form coordination bonds with carbonyl oxygen. An electron donor acceptor (EDA) model for the aflatoxin–smectite

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interactions was proposed by Phillips *et al.* (2008). According to this hypothesis, negatively charged smectite can share charge with carbonyl carbons of aflatoxin which have partial positive charge (Phillips *et al.*, 2008).

Abiotic and microbial degradation of aflatoxins is another important amelioration strategy in dealing with the mycotoxin contamination. Several studies have shown that aflatoxin can be degraded by heating (Hussain *et al.*, 2011; Farag *et al.*, 1996). Although aflatoxin is stable to heating below its melting point, partial destruction of aflatoxin has been shown by several studies. Roasting peanuts at 150ºC for 2 h degraded >95% of aflatoxin B_1 (Hussain *et al*., 2011). Mann *et al.* (1967) showed that a 1-h heat treatment of cottonseed meal at 100ºC degraded ~74.8% of aflatoxins (B_1 and B_2). Another study by Doyle and Marth (1978) showed that aflatoxin was stable when heated to 250ºC. The destruction of aflatoxin by irradiation with microwave, sunlight, and gamma radiation has also been reported (Herzallah *et al.*, 2008). Degradation products were not reported in these studies.

Smectites can adsorb aflatoxins in amounts as much as 20% of the smectite mass (Deng *et al*., 2010). To further reduce the toxicity of the aflatoxins and to eliminate treatments of the aflatoxin-loaded smectite, *in situ* degradation of the adsorbed aflatoxin molecules to non-toxic or less toxic compounds on smectite is desirable. Natural and acid-activated smectite have shown capability in degrading many organic compounds due to the clay's great surface acidity and surface area. When aflatoxin B_1 interacted with phyllosilicates, alumina, and zeolite, several fluorescence compounds in the methanol and chloroform extracts were detected on thin-layer chromatography columns and the major degradation product was identified as aflatoxin B_2 (Phillips *et al*., 1990, 1994). Most of the fluorescent degradation products, however, were not identified in these studies. The effect of temperature on the transformation of aflatoxins adsorbed on smectite has not yet been addressed.

As Brønsted acidity and Lewis acidity are often involved in the catalytic degradation of aflatoxin, and both types are present at smectite surfaces, the main objectives of this study were: (1) to investigate the potential use of smectite in degrading or transforming adsorbed aflatoxin B_1 at elevated temperatures; (2) to identify the degradation products; and (3) to investigate the effect of transition metal cations on the transformations.

MATERIALS AND METHODS

Synthesis of the aflatoxin B_1-smectite complex

The clay fraction (<2 μm) of a Ca-bentonite (referred to as 8TX) from Gonzales, Texas, USA, was collected and saturated with Ca and was then used to prepare an aflatoxin B_1-smectite complex as described below: 150 mL of 20 ppm AfB_1 solution was added to 50 mL of a smectite dispersion containing 30 mg of Ca-smectite in a 250 mL centrifuge bottle. The bottle was shaken overnight at 200 rpm on an orbital shaker (Cole-Parmer, model 51501-20, Vernon Hills, Illinois, USA). After centrifuging at $880 \times g$ for 10 min and decanting the supernatant, settled smectite was re-dispersed in 50 mL of deionized water and mixed again with 150 mL of 20 ppm aflatoxin B_1 solution. After centrifugation, the aflatoxin-smectite complex was transferred to a 15 mL centrifuge tube and washed twice with deionized water (10 mL each time). The resulting complex was dispersed in 10 mL of water. This Ca-saturated AfB_1-smectite complex was divided into three equal parts. One part was saved for later characterization, and the other two were used to prepare Cu- and Mn-saturated AfB_1-smectite complexes as described in the next section.

Cu and Mn saturation of aflatoxin B_1-smectite complexes

The Lewis acidity of smectite plays a critical role in the degradation of many organic compounds. To test whether Lewis acid sites can catalyze the degradation of adsorbed aflatoxin, transition metal cations, Cu and Mn, were introduced onto the exchange sites of the AfB_1-smectite complex to enhance its Lewis acidity. The Ca-saturated AfB_1-smectite complex containing 10 mg of smectite was saturated with Cu or Mn in separate 35 mL centrifuge tubes. To prevent precipitation of the Cu and Mn as hydroxide compounds, the AfB_1-smectite complex was acidified twice by mixing it with 10 mL of 1 mM HCl, shaking for 30 min at 200 rpm, centrifuging for 10 min at $880 \times g$, and decanting the supernatant. After the acidification, 10 mL of 0.1 M $CuCl_2$ or 0.1 M $MnCl_2$ prepared in 1 mM HCl was added to each respective tube. The tubes were shaken for 1 h at 200 rpm, and centrifuged at $880 \times g$ for 10 min. The supernatant was removed. These complexes in the tubes were treated with $CuCl_2$ or $MnCl_2$ one more time. The complexes were washed twice with water, each washing involved mixing with 10 mL of deionized water, shaking at 200 rpm for 20 min, centrifuging at $880 \times g$, and decanting the supernatant. The final complexes were re-dispersed in 3 mL of deionized water.

To address the contributions of smectite alone to the spectroscopic signals (UV, fluorescence, and FTIR) during the characterization of AfB_1-smectite complexes, controls of 100 mg of Cu-smectite and 100 mg of Mn-smectite were also prepared. The Ca-smectite was washed twice with 1 mM HCl and then twice with $CuCl_2$ and $MnCl_2$ solutions, as mentioned above. The Cu-smectite and Mn-smectite were re-dispersed in deionized water after removing the excess electrolytes by washing with deionized water.

Heating AfB_1-sm complexes at different temperatures

Dispersions containing 1 mg each of Ca-, Cu-, or Mn-saturated AfB_1-smectite complexes were dried in

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aluminum dishes firstly in an oven at 60ºC, and then heated for 1 h in the aluminum dishes at temperatures ranging from 100 to 200ºC using 25ºC steps. 30 µL of the 1000 ppm AfB_1 stock solution was dried in an aluminum dish at 60ºC to obtain 30 µg of pure aflatoxin B_1. The Ca-, Cu-, or Mn-saturated smectites and the 30 µg of pure aflatoxin B_1 were also dried and heated at the same temperatures as the controls.

Extraction of aflatoxin and transformation products with methanol

After the heat treatments, the AfB_1-smectite complexes were cooled to room temperature and dispersed in 5 mL of methanol (Fisher Optima, USA, UPLCMS grade) in 15 mL centrifuge tubes. These tubes were shaken at 200 rpm for 2 h and centrifuged at $4470 \times g$ for 30 min. Supernatants were saved in 20 mL glass vials while the clay residues were air dried in a fume hood.

The UV analysis of the methanol extracts

The methanol extracts obtained from heated AfB_1-smecite complexes were analyzed using a UV/visible spectrophotometer in wavelength scan mode to observe shifts in absorption bands. 1 mL of each extract solution was mixed with deionized water (1 mL). The UV/visible absorption spectrum was scanned over the range 200 to 800 nm.

High-performance liquid chromatography (HPLC) analysis of extracts

Extracts (1 mL) prepared in methanol were transferred into HPLC vials after filtration through 0.2 µm nylon membrane filters. The HPLC analysis was performed using an Agilent 1200 series HPLC system (Agilent, Santa Clara, California, USA) equipped with a diode array detector (DAD) and a fluorescence detector. The separation was carried out on a C18 column (Kinetex 2−6 µm) with dimensions 50 mm × 4.6 mm, pore diameter of 82−102 Å, and particle size of 2.4 to 2.6 µm. The mobile phase was a mixture of 65 parts of 0.05% formic acid and 35 parts of 1:1 methanol:acetonitrile. The injection volume was 5 µL with a flow rate of 1 mL/min. The column temperature was maintained at 30ºC. For fluorescence detection, the excitation and emission wavelengths were set at 360 nm and 440 nm, respectively. The UV-visible absorbance of each separated compound in the column was observed over the wavelength range of 210 to 700 nm using the DAD.

An aflatoxin standard solution mixture prepared in methanol containing AfB_1 (1 µg/mL), AfB_2 (0.3 µg/mL), AfG_1 (1 µg/mL), and AfG_2 (0.3 µg/mL) was purchased from Sigma Aldrich (St Louis, Missouri, USA). A series of solutions containing 3, 4, 5, 6, and 7 µg/L AfB_1 were prepared from the above standard solution mixture. A calibration curve drawn between the aflatoxin concentration and the chromatography peak area was linear with an R^2 value of 0.999.

Ultra-performance liquid chromatography-mass spectrometric (UPLC-MS) analysis of extracts

Ultra-performance liquid chromatography (UPLC) is the preferred technique for detection and quantification of aflatoxins due to its high sensitivity and speed (Pico and Barcelo, 2008). Degradation products of aflatoxin resulting from UV irradiation of aflatoxins had been identified with UPLC-MS (Liu *et al*, 2010, 2011). In the current study, aflatoxin B_1 transformation products in the extracts were analyzed on a Waters Acquity UPLC-MS, which can offer both photodiode array (PDA) ultra-performance liquid chromatography (UPLC/PDA) and electrospray ionization-tandem quadrupole-mass spectrometry (ESI-TQD-MS). The tandem-mass spectrometry is also referred to as 'MS-MS.' The MS-MS spectra were obtained using a collision energy of 30 or 35 eV. The compounds in the extracts were separated in a C18 column. The mobile phase was a gradient prepared from acetonitrile (component A) and 0.1% formic acid aqueous solution (component B). Full scan spectra were recorded from 150−100 m/z in positive ionization mode. The injection volume was 5 µL.

Infrared spectroscopic analysis of aflatoxin-smectite residues after methanol extraction

AfB_1-smectite residues left after extraction with methanol were re-dispersed in deionized water and air dried as films on ZnS windows (ClearTran, International Crystal Labs, Garfield, New Jersey, USA) for FTIR study. Infrared spectra were obtained in transmission mode using a Perkin-Elmer Spectrum 100 instrument over the wavenumber range 4000 to 700 cm^{-1} with a resolution of 2 cm^{-1}.

RESULTS

Characterization of aflatoxin degradation products

UV analysis of extracts. Aflatoxin B_1 showed absorption peaks, λ_{max}, at 361 nm, 264 nm, and 222 nm (Figure 1). No peak shift was observed in the UV spectra of extracts of the pure aflatoxin B_1 after heating at 100, 125, 150, 175, or 200ºC. The methanol extracts from the Ca-, Cu-, and Mn-smectite alone showed no absorption peaks in the UV region tested (data not shown). The methanol extracts of aflatoxin B_1-smectite complexes heated at 100ºC had similar spectra to that of pure aflatoxin B_1. Shifts in the absorption peak of the AfB_1-Ca-smectite extract were observed after heating at 150ºC or above. The absorption peaks became much broader. The intensities of the peaks at 361 and 264 nm decreased substantially. New peaks at 340 and 252 nm appeared after heating at 175ºC. The 340 and 252 peaks became prominent in the extract after heating at 200ºC.

Similar shifts were observed in the AfB_1-Cu-smectite extracts but in this case the peak at 361 nm became

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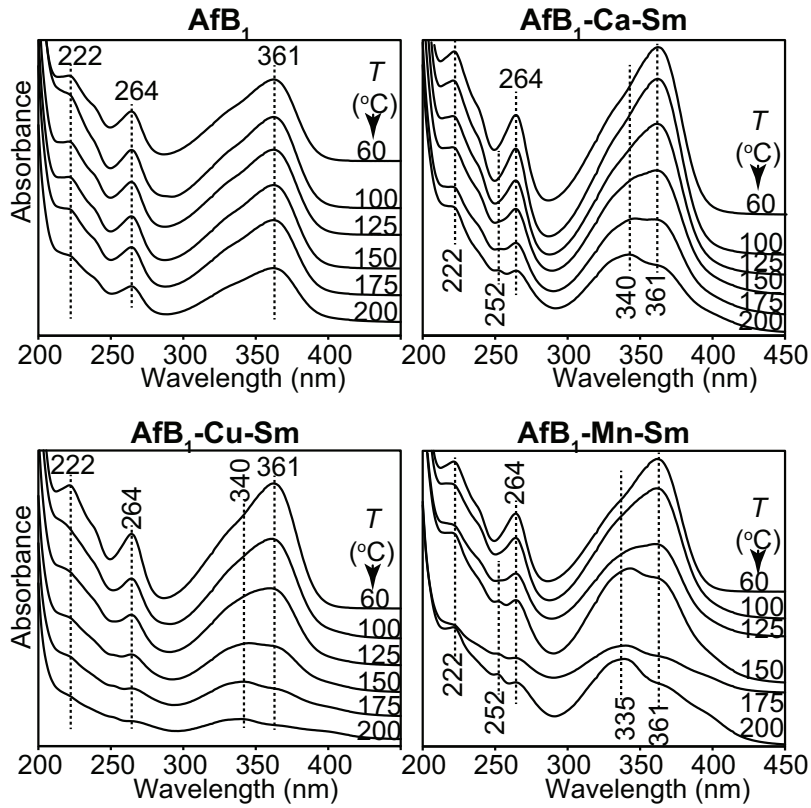

Figure 1. UV spectra of methanol extracts of pure aflatoxin B_1 and aflatoxin B_1-smectite complexes after heating.

broader in the extracts after heating at 125ºC. A new peak at 252 nm appeared after heating at 175ºC. After heating at 200ºC, the UV peak positions and shape changed completely. The peaks at 223 nm and 265 nm disappeared almost completely after heating at 175 and 200ºC. Similar results were observed for AfB_1-Mn-smectite complexes: the peak at 361 nm became much broader after heating at 125ºC and the peak shape changed completely after heating at 175 and 200ºC.

High-performance liquid chromatography (HPLC) analysis of extracts

HPLC-fluorescence chromatograms. On the HPLC chromatograms with the fluorescence detector, the retention time for standard AfB_1 was 6.2 min (Figure 2, left). Pure aflatoxin extracts showed no new peaks on the chromatograms. Aflatoxin B_1 was present in all of the extracts obtained from Ca-, Cu-, and Mn-saturated AfB_1-smectite complexes but its intensity decreased with increasing temperature. Additional peaks were also observed at retention times of 1.8 min, 2.5 min, 4.6 min, and 5.4 min (Figure 2, left). After heating at 200ºC, the intensity of the peak at 1.8 min became nearly equal to that obtained from AfB_1-smectite complexes heated at 60ºC. The peak at 5.4 min disappeared in all extracts when the AfB_1-smectite complexes were heated at 175ºC. The extracts of blank Ca-, Cu-, and Mn-smectite showed no new peaks on their chromatograms (data not shown).

HPLC-DAD UV chromatograms. Aflatoxin B_1 remained in the extracts but the concentration decreased with increasing temperature. The extracts from heated pure aflatoxin B_1 solutions showed only one peak for a retention time of 6.05 min (Figure2, right). The UV spectra and retention time of this compound were identical to those of the aflatoxin B_1 standard. A gradual decrease in concentration of aflatoxin B_1 was observed after heating at 125ºC, but no new peak was observed on the chromatograms.

The aflatoxin B_1 peak (retention time = 6.05 min) appeared on all of the aflatoxin-smectite complexes. Aflatoxin was the dominant compound shown in the extract of complexes after drying at 60ºC. Very weak or negligible peaks from other compounds were observed in the 60ºC chromatograms. The intensity of the AfB_1 peak (retention time of 6.05 min) decreased with increase in heating temperature. Some additional changes in retention time or intensity are described below. Five peaks can be seen in the chromatogram of extracts of the AfB_1-Ca-smectite complex after heating and the intensities of these peaks also increased with increasing temperature up to 150ºC. After heating at 100ºC, two more peaks at retention times of 3.17 and 2.32 min appeared in the

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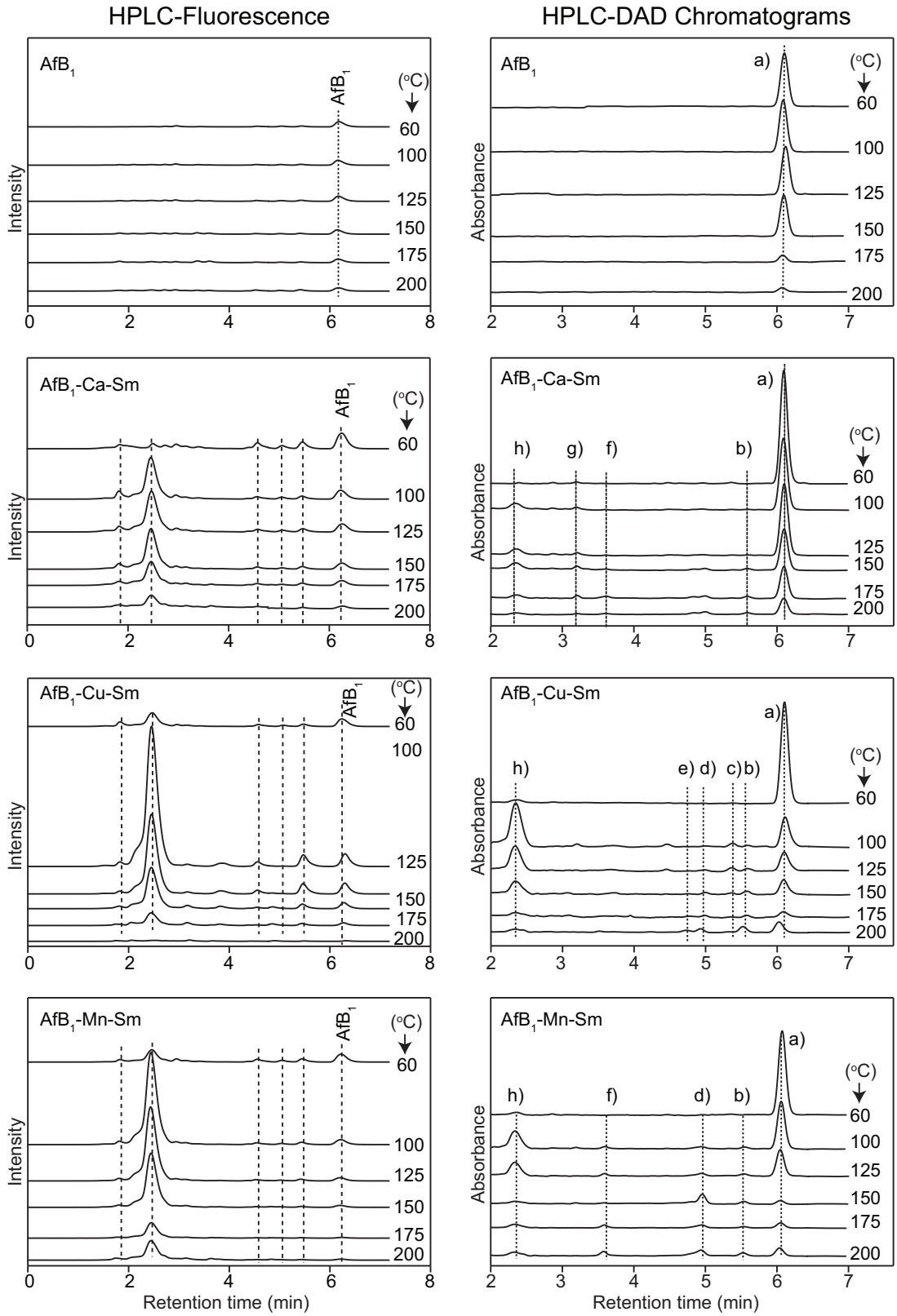

Figure 2. HPLC fluorescence (left) and HPLC-diode array detector (DAD, right) chromatograms of methanol extracts from aflatoxin B_1-smectite complexes and pure aflatoxin B_1 after heating at different temperatures.

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chromatogram. These peaks were of low intensity. The results of the HPLC analysis after heating at 125ºC and 150ºC were similar to that obtained at 100ºC. The chromatogram for the extract from the 175ºC-heated AfB_1-smectite complex showed two more peaks at retention times of 5.55 min and 5.0 min. After heating at 200ºC, a significant decrease in the intensity of aflatoxin B_1 was observed. The peak due to an unknown compound at a retention time of 2.32 min disappeared and the peaks at 5.35, 5.0, and 4.7 min were intense.

The extracts obtained from the AfB_1-Cu-smectite complexes also contained a mixture of compounds. After heating at 100ºC, the most intense peak due to a highly polar compound appeared at a retention time of 2.3 min. Other peaks observed were at retention times of 5.55 and 5.35 min. Less intense peaks at retention times of 4.43 and 3.17 min were also observed on the chromatogram. The chromatograms for the extracts of 125ºC- and 150ºC-heated complexes were similar to that of the 100ºC-heated sample except for the intensity reduction of the 2.3 min peak and the appearance of a new peak at a retention time of 5.0 min. After heating at 175ºC, a significant decrease in the peak intensities of all other compounds including AfB_1 was noted.

Similar responses to heating were observed for the AfB_1-Mn-smectite complex. When the complex was heated at 100ºC, three additional peaks other than AfB_1 appeared at retention times of 5.5, 5.0, 3.57, and 2.3 min. The chromatogram for the extract of complexes heated at 125ºC was similar to that obtained after heating at 100ºC. On further heating (150ºC), most of the peaks observed in previous chromatograms became much weaker but the intensity of the peak at a retention time of 5.0 min increased substantially. The intensity of this peak was comparatively less in extracts heated at 175 and 200ºC.

Ultra-performance liquid chromatography-mass spectrometry

Degradation/transformation products were identified by comparing the UV spectra, the retention time, and the molecular ion peak of the compounds with corresponding standards. Aflatoxin B_1 was detected in all of the extracts. The UV and MS-MS spectra of the compound at a retention time of 4.37 min were similar to that of the standard aflatoxin B_1. The base peak for aflatoxin B_1 appeared at m/z 240.9. Other prominent peaks were at m/z 285, 269, 242, and 214. Their corresponding fragment structures are proposed in Figure 3.

Aflatoxin B_2 ($C_{17}H_{14}O_6$) was detected in the extracts and identified based on UV spectra and the m/z value of the molecular ion in the mass spectrum. The retention time, UV, and MS-MS spectra of the compound were verified against the standard aflatoxin B_2. The main fragments were observed at m/z of 287, 271, and 243 with a base peak at m/z 259. Two fragment structures are proposed in Figure 3.

Other compounds in the extracts were aflatoxin B_1 metabolites: aflatoxins M_1 ($C_{17}H_{12}O_7$), M_2 ($C_{17}H_{14}O_7$), and B_{2a} ($C_{17}H_{14}O_7$), which were identified by comparing their MS-MS spectra with the reported spectra of these compounds in the literature and the m/z values of their fragments. The [M+1] ion of aflatoxin M_2 at m/z 331 was observed using a (+) ESI-MS technique in some extracts at retention time 3.13 min (Figure 3). Aflatoxin M_1 was detected in the extracts of AfB_1-smectite complexes after heating at 100 and 125ºC. The retention time for this compound was 2.60 min with λ_{max} values of 214, 265, and 358.9 nm. Another compound (AfB_{2a}) was detected in the PDA chromatogram at a retention time of 3.09 min (Figure 3). The molecular weight for aflatoxin B_{2a} ($C_{17}H_{14}O_7$) is 330 and λ_{max} values were 228, 256, and 363 nm. The main fragments observed for this compound were at m/z 312.9, 269, 257, and a base peak at m/z 285 (Figure 3).

Degradation evolution as indicated by the peak areas of the UPLC chromatograms

Analysis by UPLC-MS indicated chemical conversion of aflatoxin B_1 to other products and heating could accelerate these reactions because a gradual decrease in the amount of aflatoxin B_1 was observed in smectite extracts (Figure 4). The exchange cation in the interlayer of smectite affected the conversion rates of the aflatoxin B_1 to other compounds: much larger amounts of aflatoxin B_2 were extracted from the Mn-saturated AfB_1-smectite complex than from the Ca- or Cu-saturated complexes (Figure 4). A negligible amount of AfB_2 was observed in Ca- or Cu-saturated AfB_1-smectite complexes after heating at 150, 175, and 200ºC. Aflatoxin B_{2a} appeared in extracts after heating at 100ºC with a gradual decrease upon further heating. Only small amounts of AfM_1 and AfM_2 were detected in a small number of extracts (Figure 4).

FTIR spectra of clay residues after heating and extraction

The amounts of aflatoxin and degradation products extracted were small compared to the amount of aflatoxin adsorbed by the clay complexes, suggesting that most of the adsorbed aflatoxin was still present in the smectite interlayer with or without conversion to other compounds. Shifts and decreases in the intensity of some IR bands were observed in the FTIR spectra of residues. The width of the IR at 1722 cm^{-1}, which is attributed to carbonyl stretching vibrations, of AfB_1-Ca-smectite complexes increased after heating at 175 and 200ºC (Figure 5). In AfB_1-Cu-smectite, the absorption band at 1731 cm^{-1} was very weak in spectra of the clay residue after drying at 60ºC. This band was also absent from FTIR spectra of residues heated at 100 and 125ºC. A broad band at 1731 cm^{-1} appeared after heating at 150ºC. The intensity of this band increased gradually after heating at 175 and 200ºC (Figure 5). In the case of

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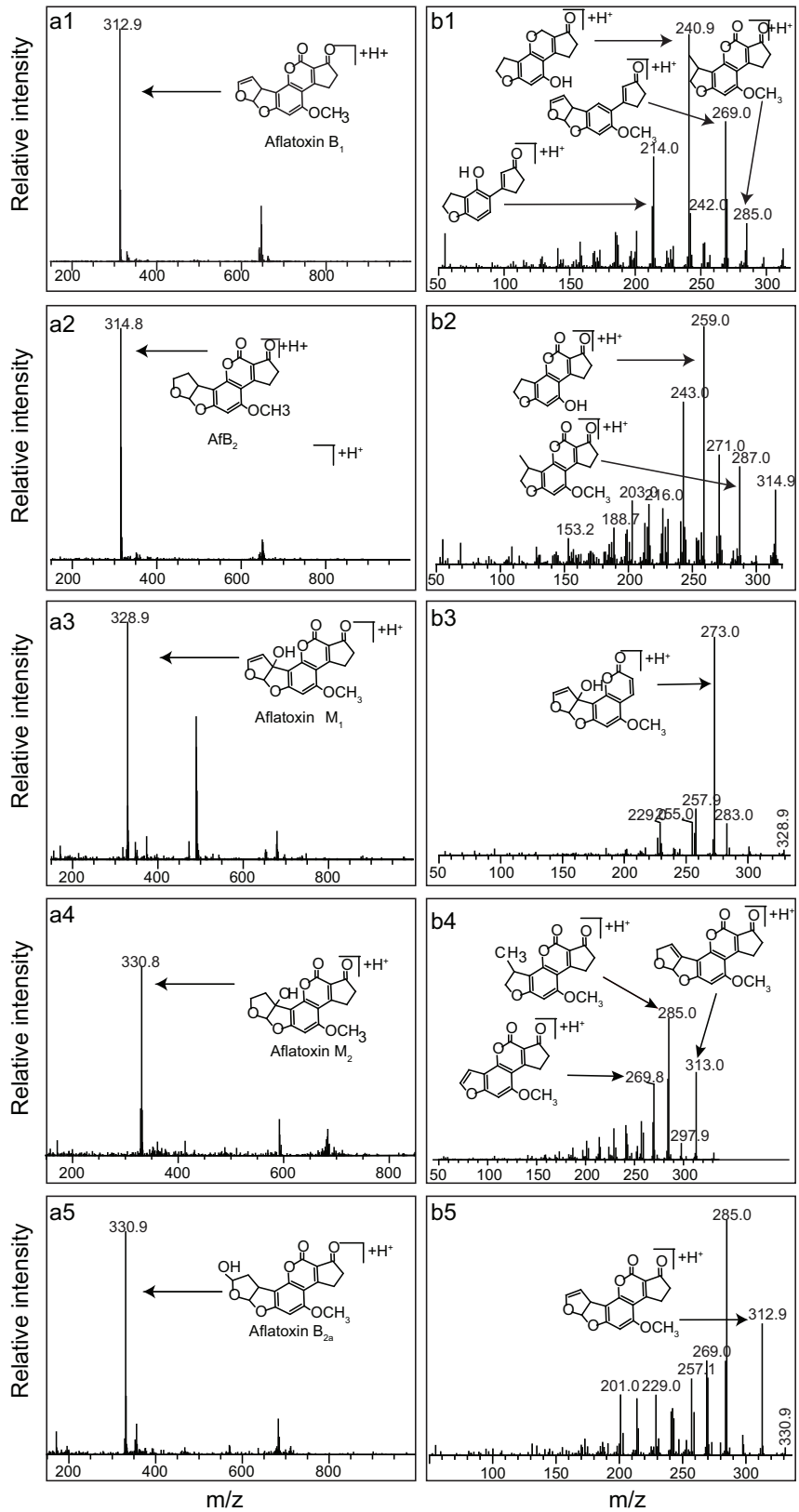

Figure 3. (a) The UPLC-MS and (b) the MS-MS spectra of pure aflatoxin B_1 and a few identified transformation products of adsorbed aflatoxin B_1 on smectite at elevated temperatures. Some fragments of aflatoxin molecules based on MS-MS spectra are proposed.

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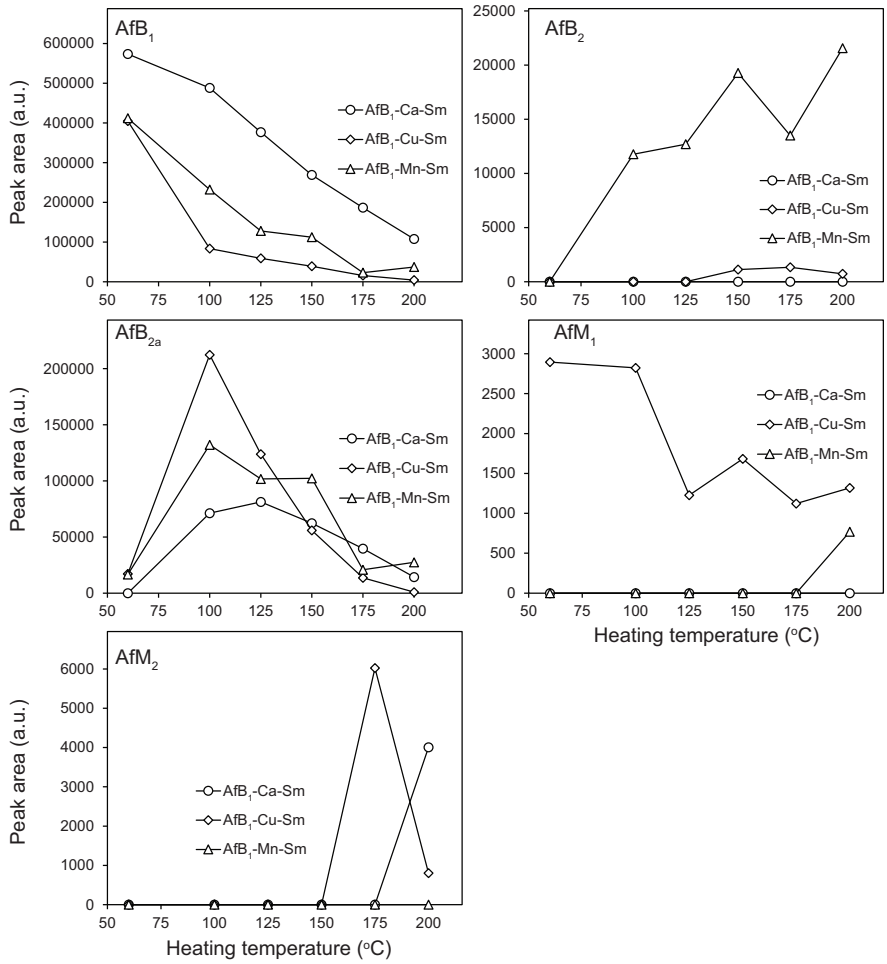

Figure 4. The UPLC-PDA peak areas of AfB_1 and four aflatoxin transformation products in the methanol extracts of the aflatoxin B_1-smectite complexes heated at elevated temperatures.

the residue from the AfB_1-Mn-smectite complex, the band at 1705 cm^{-1} was absent from the residue after drying at 60ºC but appeared after heating at 100ºC (Figure 5). Another prominent change in the IR spectra was the disappearance of the absorption band at 1627 cm^{-1} after heating at 175ºC.

DISCUSSION

The peak shifts in the UV spectra of the extracts of AfB_1-smectite complexes (Figure 1) indicated that chemical changes in the aflatoxin adsorbed occurred on smectite at elevated temperatures, but these changes were absent from pure aflatoxin heated at the same temperatures. The peaks at shorter retention times in the HPLC-fluorescence and HPLC-UV chromatograms (Figure 2) of the aflatoxin-smectite extracts indicated chemical transformations of the adsorbed aflatoxin B_1 on smectite. The shorter retention time on the chromatograph column also suggested that the new compounds were more polar than aflatoxin B_1. The new compounds were less stable at temperatures of >100ºC because, after heating at 125ºC, their intensity decreased.

The extracts obtained from the aflatoxin-smectite complexes (Figure 2) had six or seven peaks on the chromatograms dried at 60ºC, suggesting that the transformation of the aflatoxin adsorbed on smectite occurred even at that low temperature. Heating at elevated temperatures served only to change the intensity of these peaks.

The most prominent peak observed in HPLC-UV chromatograms of all complexes after heating was at the retention time of 2.3 min. The λ_{max} of this compound was 364 nm. Extracts obtained from complexes heated at 60ºC had unknown compounds which can be seen in fluorescence chromatograms (Figure 2).

The HPLC analysis indicated transformation of aflatoxin B_1 to other compounds when AfB_1-smectite complexes were heated. The type of exchange cation also caused variations in the transformations. Aflatoxin B_1 concentration decreased in all of the samples with increase in temperature. Interaction of aflatoxin B_1 or its

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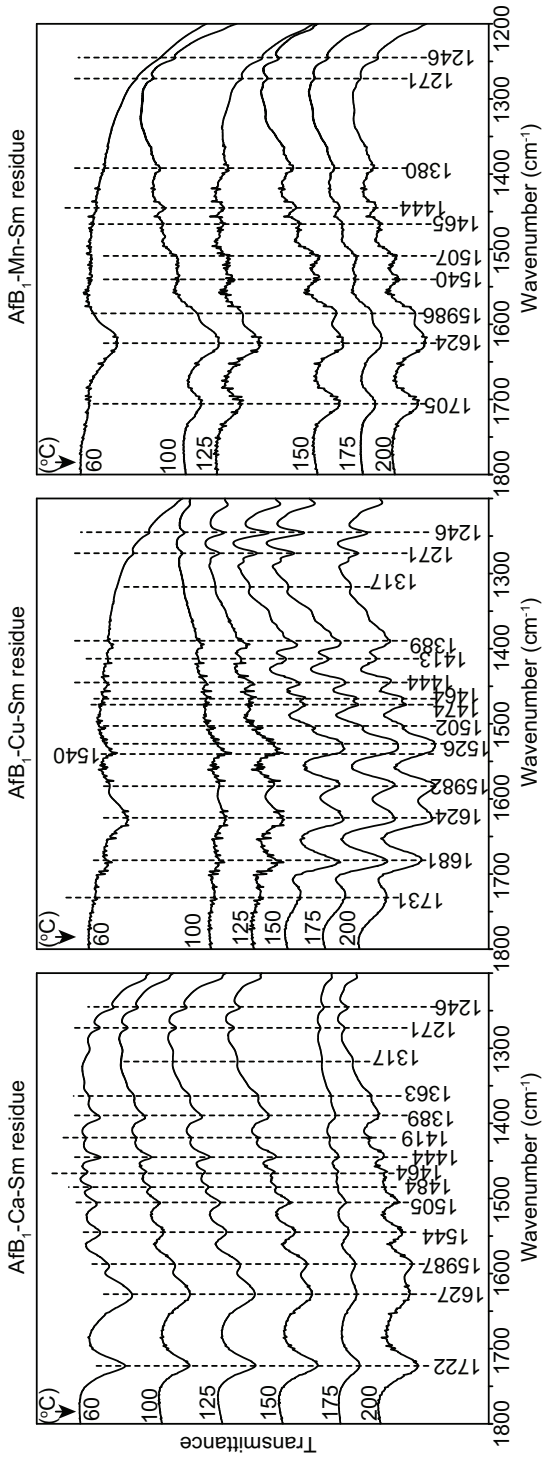

Figure 5. FTIR spectra for aflatoxin-smectite complex residues after heating and extraction with methanol.

degradation products with smectite increased on heating, resulting in less aflatoxin in the extracts.

The aflatoxin B_1 transformation products identified based on the UPLC-MS (Figure 3) spectra were aflatoxin B_2, B_{2a}, M_1, and M_2. Aflatoxin B_{2a} is considered a detoxification product of aflatoxin B_1 because it is 200 times less toxic than AfB_1 (Cole, 1981). Aflatoxin M_1 is a metabolic product of AfB_1 with a molecular weight of 328 and is less toxic than aflatoxin B_1. The median lethal dose, LD_{50} value, for aflatoxin M_1 is 16.6 μg/day in day-old ducklings (Dhanasekaran *et al.*, 2011). Aflatoxin M_2 is produced by *in vivo* hydroxylation of aflatoxin B_2 when animals are fed on aflatoxin-contaminated feed. Aflatoxin M_2 is less toxic than AfB_1 and has greater polarity due to the OH group at position 7 in the aflatoxin B_2 structure (Cole and Cox, 1981).

Three degradation products, $C_{17}H_{15}O_6$, $C_{16}H_{13}O_5$, and $C_{14}H_{11}O_6$ were identified by Liu *et al.* (2011) after UV irradiation of an aflatoxin B_1 solution in acetonitrile. Three new photo-degradation products $C_{17}H_{14}O_7$, $C_{16}H_{14}O_6$, and $C_{16}H_{12}O_7$ were identified when an aqueous solution of aflatoxin B_1 was irradiated with UV light. The degradation products AfB_{2a} and AfM_2 identified in the present study have a formula of $C_{17}H_{14}O_7$, which might be one of the compounds identified in the study of Liu *et al*. (2011), but other compounds reported in their study were not observed in extracts in the present work, suggesting that the transformation of adsorbed aflatoxin on smectites may have different pathways. The toxicities of these degradation products need be evaluated further. Different researchers have shown that heat treatments decreased aflatoxin concentrations (Méndez-Albores *et al*., 2013; Perez-Flores *et al*, 2005) but the degradation products were not identified.

The infrared band shifts of the methanol-extracted residues suggest that the compounds remaining in the interlayer space of smectite also changed to structures which are different from aflatoxin B_1 after heating at high temperatures and became more resistant to methanol extraction. Other more efficient solvents will be explored for extraction and identification of these compounds.

CONCLUSIONS

The new results here have confirmed the strong interactions between aflatoxin and smectite. A significant proportion of the AfB_1 or the transformation products were resistant to methanol extraction and remained in smectite after heating at elevated temperatures. The smectite reduced the thermal stability of the aflatoxin B_1 adsorbed: pure AfB_1 did not convert to other compounds after heating up to 200ºC, but some of the aflatoxin adsorbed on smectite converted to other compounds. Some of the degradation/transformation products were extractable by methanol and were

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identified as aflatoxin B_2, B_{2a}, M_1, and M_2. These are known metabolites of AfB_1 and are less toxic than Aflatoxin B_1. Copper or Mn saturation of smectite accelerated the transformation of AfB_1 at elevated temperatures. Manganese saturation degraded aflatoxin the most at the lowest temperature.

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