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Mass Spectrometry/Mass Spectrometry Study on the Degradation of B-Aflatoxins in Maize with Aqueous Citric Acid

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Abstract: Degradation of B-aflatoxins in maize by means of 1N aqueous citric acid was confirmed by the AFLATEST immunoaffinity column method, High Performance Liquid Chromatography (HPLC), tandem mass spectrometry (MS/MS) and computational information. The AFLATEST and HPLC assays showed that 96.7% degradation occurred in maize contaminated with 93 ng g^{-1} when treated with the aqueous citric acid. Two major products, produced during the acidification process, were identified by their corresponding mass spectral data: a nonfluorescent compound lacking the lactone group evidenced by the presence of a peak m/z 286 and a nonfluorescent compound retaining the difurane moiety but lacking the lactone carbonyl and the cyclopentenone ring of the AFB_1, also suggested by the peak m/z 206; the title fragments correspond to molecular ions in agreement with their respective molecular weights. According to the theoretical calculations, applying density functional theory, it was confirmed that the active site may be assigned to the carbonylic carbon of the lactonic moiety.

Key words: Detoxification, aflatoxin B_1, aflatoxin B_2, acidic treatment, tandem mass spectrometry, theoretical calculations

INTRODUCTION

Mycotoxins are fungal-generated secondary metabolites that are ubiquitous and "unavoidable" contaminants of grains and oil seeds. A variety of fungi, including numerous species of Aspergillus, Penicillium and Fusarium, are capable of producing mycotoxins[1]. These compounds have been frequently detected in food and feedstuffs and their ingestion by humans and animals can result in disease and death[2].

Aflatoxin B_1 (AFB_1) is the most potent of the four naturally-occurring aflatoxins. Because of its hepatotoxicity and carcinogenicity, this molecule has been the focus of considerable research since its discovery. Like aflatoxins, fumonisin B_1 is commonly produced as a contaminant of maize[3] and has been linked to the etiology of disease in pigs and horses, namely porcine pulmonary edema and equine leukoencephalomalacia[4,5]. Importantly, it has been also reported to cause and promote cancer[6]. Other common mycotoxins, including ochratoxin A[7], patulin[8] and zearalenone[9], have generated concern due to their frequent occurrence in food and feeds. Because of the adverse effects of these and other mycotoxins on human and animal health, practical and effective detoxification procedures are highly desirable[10]. Currently, aflatoxins are the only mycotoxins which are specifically and formally regulated. Thus, management of the mycotoxin problem has focused on aflatoxins, instead of other potentially hazardous contaminants as mentioned.

Ideally, detoxification procedures will not only reduce the concentration of toxins to safe levels, but will also prevent production of toxic degradation products as well as any reduction of the nutritional value of the treated commodity. A number of approaches have been taken to detoxify aflatoxins; however, only a few have practical applications. Among these, ammoniation, an effective and low cost-efficient means for reducing the aflatoxin content of a variety of foods[11].

Tandem mass spectrometry, also known as Mass Spectrometry-Mass Spectrometry (MS/MS), is an appropriate method for the detection of trace amounts of non-volatile compounds in complex biological mixtures[12,13], as well as for identification of the major reaction products during the chemical treatment of aflatoxin contaminated commodities[14,15]. MS/MS has been used to identify AFB_1 in maize extracts at nanograms per gram levels[16].

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Consequently, the aim of this research was to define the possible mechanism of the acidification procedure for B-aflatoxins degradation in maize by means of MS/MS, through the identification of the major reaction products during the chemical reaction and verify the adequate active site in the aflatoxin molecule using the information obtained by means of theoretical calculations.

MATERIALS AND METHODS

Chemicals: B-aflatoxins, anhydrous citric acid, as well as other chemicals were obtained from Sigma Chemical Co. Ltd (St Louis, MO, USA).

Safety: Procedures used for handling contaminated AFB materials were adopted from recommendations published by the International Agency for Research on Cancer[17].

Maize grain: Maize grain of the commercial hybrid AS-900 was utilized. At its arrival to the laboratory, the grain had a Moisture Content (MC) of 11.7%. The MC was determined by drying replicate portions of 5-10 g each of whole grain at 103°C for 72 h, the MC was expressed as percentage calculated on a wet-weight basis. The grain was aflatoxin-free, as tested with the AFLATEST method described below.

Toxigenic fungus: The A. flavus Link strain UNIGRAS-1231 (Culture Collection of the Grain and Seed Research Unit of the National Autonomous University of Mexico), originally isolated from aflatoxin-cultivated maize, was inoculated into Petri dishes containing MSA medium (malt extract, 2%; sodium chloride, 6% and agar, 2%) at 25°C for 7 days. This strain, UNIGRAS-1231 is capable of producing only B-aflatoxins.

Fungal inoculation technique: To inoculate the maize grain, the fungus spores were removed from the Petri dishes with a spatula, a sterile water spore suspension was prepared with approximately 100,000 conidia mL^{-1} and this suspension was used to elevate the MC of the grain. This amount of inoculum, was used in an attempt to eliminate competition with other storage fungi that potentially can grow under such moisture and temperature conditions. The MC of the maize was adjusted to 18%. The maize grain was stored in plastic bottles (5 kg of maize per replicate). Bottles were covered with thin polyethylene film to minimize the loss of moisture from the grain; however, ten perforations with a pin were made to such film to avoid the accumulation of carbon dioxide generated by the respiration of maize grain and fungi. Bottles were incubated at 27°C during 14 days to obtain the required aflatoxin concentration. After the incubation period, the grain was put under a 1000 mg L^{-1} ethylene oxide gas atmosphere for 5 h, to stop further development of the toxigenic fungus and to avoid the dispersal of viable spores. Finally, the aflatoxin-contaminated grain was dried to 12.5% MC.

Maize acidification procedure: An aflatoxin-contaminated maize sample (5 kg) was ground in a mill (Pulvex-200, sieve 0.8 mm. Pulvex S.A. de C.V. Plutarco Calles 290, Mexico, DF) and thoroughly mixed. Three sub-samples (1000 g each), were treated with 1N aqueous citric acid for 15 min (3 mL g^{-1} of contaminated maize). The treated-maize was filtered through a microfiber filter to remove excess water and then dried in a vacuum oven at 40°C for 48 h. Samples were transferred to clean plastic bags, labeled and stored at 4°C until further aflatoxin analysis.

Aflatoxin assay

Aflatoxin quantification: The aflatoxin content was determined according to the 991.31 AOAC (1995) method[18] using monoclonal antibody columns for aflatoxins B_1 and B_2 (VICAM Science Technology, 303 Pleasant St., Watertown, MA, USA). When the concentration of total aflatoxins was greater than 25 ng g^{-1}, dilutions from the extract were made for their quantification in the fluorometer after reaction with a bromine solution at 0.002%[19]. The detection limit for aflatoxins with the immunoaffinity column (IAC) via fluorescence measurement is approximately 0.5 ng g^{-1}[20].

Aflatoxin identification: Aflatoxin identification was carried out by means of a Waters HPLC equipment with two pumps (Mod 510. Waters Associates, Milford, MA.) and a Waters Nova-Pak C18, reverse phase column (5 μm, 3.9×150 mm). Standards, as well as samples collected from the IAC (20 μL) were injected into a HPLC and eluted isocratically with a mobile phase of 12.5 mN acetic acid: acetonitrile (1:1, v/v) at a flow rate of 1 mL min^{-1}. Aflatoxins were fluorometrically detected and identified using a fluorescence detector Waters 470 AC; the excitation and emission wavelengths were 338 and 425 nm, respectively. The aflatoxins were identified by their retention time, compared with those for a pure aflatoxin standard solution under identical conditions. The performance of the AOAC method was tested by the

percentage of aflatoxin recovery by means of the HPLC method.

MS and MS/MS analysis: A Finnigan GCQ-plus mass spectrometer was used for the electron impact fragmentation mode and the respective MS/MS experiments. Electron Impact Mass Spectra (EIMS) were recorded with a source temperature of 230°C, ionization energy of 70 eV and ionization trap current of 100 μA. During tandem mass spectral analysis, the precursor ion was isolated in the ion trap and fragmented via collision-induced dissociation (CID). The mass range for isolation of the peak of interest was centered at the precursor mass and ranged to one-half of the isolation width to either side of the precursor mass. The default value for isolation width was 1 Da and the isolation time was 8 ms. The radio-frequency voltage applied to the endcap electrodes of the ion trap mass analyzer was 1 V for 15 ms. The q value was 0.45. An AC voltage of constant frequency (1.03 MHz) and variable amplitude (0 to 8500 V zero-to-peak) was applied to the ring electrode of the ion trap mass analyzer. Argon was the target gas in collision-activated dissociation MS/MS daughter experiments. The pressure of the collision cell was maintained between 1×10^{-3} to 3×10^{-3} Torr and the equivalent collision energy from the helium gas was 20 V. Samples were introduced via direct insertion probe.

Computational procedure: The AFB_1 molecule was studied with the density functional theory (DFT) method, specifically B3LYP with the 6-31G (d,p) basis set implemented in the program Gaussian 03[21]. The charges of the respective atoms of the aflatoxin molecule were determined with natural population analysis (NPA) to determine a first approach to the reactive site.

Experimental design and statistical analysis: The experiment was conducted as a completely randomized design and the experimental conditions were carried out with three replicates. Data was assessed by analysis of variance (ANOVA) using the Statistical Analysis System[22].

RESULTS AND DISCUSSION

As stated above, the performance of the AOAC method was tested by measuring the percentage of aflatoxin recovery using the HPLC method, spiking four replicates of six different aflatoxin concentrations (from 0.78, 1.56, 3.13, 6.25, 12.50 to 25 ng g^{-1}), attaining an aflatoxin recovery of 92%, with a standard deviation of 3.5. These results indicate that the method used was applicable.

The technique used here (e.g., MC, incubation temperature/time, spore load and *A. flavus* strain) worked as expected to obtain the aflatoxin contamination level in the maize grain. The total aflatoxin contamination (93 ng g^{-1}), is a concentration that can be found in commercial maize grain used to produce food or feeds. Since the AFLATEST method gives the total aflatoxin concentration without discriminating between the kinds of B-aflatoxins; the HPLC identification showed that the toxins produced by the *A. flavus* isolate were only AFB_1 and AFB_2, with concentrations of 89.3 and 3.7 ng g^{-1}, respectively. It has been stated that *A. flavus* produces mainly the B-toxins[23]. In this case, AFB_1 was the more prevalent and abundant toxin in all samples evaluated.

The results showed that inoculated maize contained 93 ng g^{-1}. However, the acid post-reaction aflatoxin content was 3 ng g^{-1}. This reduction in aflatoxin content represents about 97%. As observed, the 1N acidic treatment in the contaminated maize, leads to a partial detoxification measured as loss of fluorescence. The chromatograms of the HPLC (not presented) showed that fluorescence of AFB_2 was not detected in acidified extracts, while AFB_1 fluorescence was much weaker than the untreated samples.

The HPLC results confirmed that acidified AFB_1 molecule is not a different substance from the parent compound and may provide support for detoxification activity. It also suggests that the molecular structure in post-treated AFB_1 samples changes, the lactone ring may be opened. We hypothesize that detoxification of AFB_1 initially involves the formation of the β-keto acid structure (catalyzed by the acidic medium), followed by hydrolysis of the lactone ring, yielding aflatoxin D_1, a nonfluorescent compound mol wt 286 g moL^{-1}, which exhibits phenolic properties and lacks the lactone group derivated from decarboxylation of the lactone ring-opened form of AFB_1 as shown in Fig. 1; the title molecule is less toxic and mutagenic than AFB_1[24-27]. Therefore, the fluorescence strength varies in the HPLC chromatograms at the same retention time value.

The acidification procedure can yield in less extent a second compound, a nonfluorescent phenol with mol wt 206 g moL^{-1}, commonly known as aflatoxin D_2 (AFD_2), which retains the difurane moiety but lacks both the lactone carbonyl and the cyclopentenone ring characteristic of the AFB_1 molecule (Fig. 1).

The mass spectral fragmentation pattern of AFB_1 standard (profile a) and AFB_1 purified from the contaminated maize (profile b), are presented in Fig. 2. Both mass spectrums exhibited the expected molecular

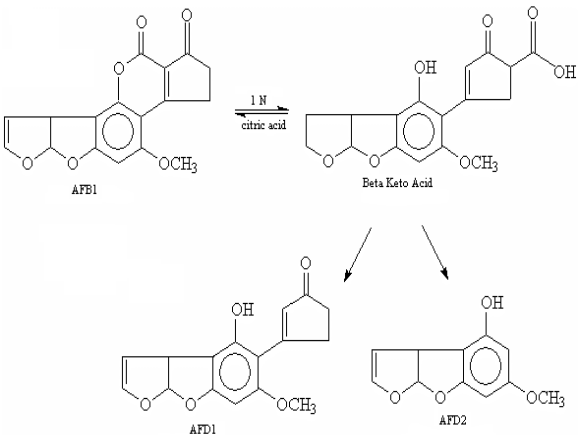

Fig. 1: Proposed mechanism for the acidification of AFB_1 to produce AFD_1 (mol wt 286) and AFD_2 (mol wt 206)

Table 1: Typical fragments of the AFB_1 molecule obtained by EIMS

		m/z (% relative abundance)	
Ion	Assignation	AFB_1 standard	AFB_1 purified
a	$M^{+\cdot}$	312 (100)	312 (100)
b	$\bar{M}CH_3$	297 (88)	297 (74)
c	$\bar{M}H_2O$	294 (12)	294 (08)
d	$\bar{M}CO$	284 (35)	284 (26)
e	$\bar{M}CH_3CO$	269 (73)	269 (36)
f	-	253 (25)	253 (20)
g	-	219 (46)	219 (46)
h	-	218 (45)	218 (55)
i	-	191 (43)	191 (58)
j	-	189 (86)	189 (64)

ion ($M^{+\cdot}$) at m/z 312 (100), in addition to other abundant fragments. The $M^{+\cdot}$ values are in agreement with the corresponding molecular weight of the AFB_1 molecule.

A great number of fragments in the spectrum of the AFB_1 purified from contaminated maize (summarized in Table 1) are consistent with those observed in the mass spectra of the AFB_1 standard, indicating that both samples correspond to the same molecule.

On the other hand, the mass spectrum of the crude reaction product (Fig. 3) showed intense peak values at m/z 286, 240, 206, 150. This mass spectrum also showed an m/z 312 value, attributable to residual AFB_1. Those ions were not present in the mass spectrum of pure AFB_1 (Fig. 2, profile a), consequently, we inferred them as by-products achieved during the aqueous citric treatment of the AFB_1 molecule.

Consequetly, the ions m/z 286 and 206 were independently treated by MS/MS (Fig. 4). The corresponding daughter fragments of m/z 286 were in strong agreement with the fragmentation pattern exhibited by the AFD_1 standard, reported previously[28]; thus, AFD_1 must be present in the acidified maize grain.

Figure 4 (profile a), showed an intense peak m/z 240, also present in the spectrum of the crude reaction product (Fig. 3). Consequently, it is not a product originated during the acidification of the AFB_1 molecule. Rather, it must correspond to the fragmentation of the mol wt 286 compound. However, the peak at 206 atomic mass unit (amu), found in the spectrum of the crude reaction product, undoubtedly belongs to a product formed during the detoxification procedure. All major m/z 206 daughter fragments (Fig. 4, profile b) are in good agreement with the relative intensities exhibited by the mol wt 206 compound obtained by Cucullu *et al.*[14] during the ammoniation of AFB_1. Moreover, Fig. 4 (profile b), shows an intense peak of 150 amu, also present in the spectrum of the crude reaction product (Fig. 3), suggesting that this mass does not correspond to a product formed during acidification. It must therefore be accounted as fragmentation of the moL wt 206 compound.

Knight *et al.*[29] identified the compound tetrahydro-4-hydroxy-6-methoxyfuro[2,3-b] benzofuran (moL wt 208), from degradation of sterigmatocystin (a mold metabolite containing the furobenzofuran moiety); this mol wt 208 compound is apparently the saturated analog of the mol wt 206 compound.

The experimental data indicated that the mol wt 206 compound produced during the acidification of the AFB_1 molecule in maize, is dihydro-4-hydroxy-6-methoxyfuro [2,3-b] benzofuran; the structure is also showed in Fig. 1. Thus, we propose that this compound arises with the opening of the lactone ring of AFB_1, in the presence of aqueous citric acid, followed by decarboxylation of the resultant β-keto acid structure, producing both AFD_1 and the mol wt 206 compound, commonly known as AFD_2

Recently, a computational study was also performed to contribute on the understanding of the activity of the AB_1 molecule, the results are summarized as follows: since the molecular geometry is one of the most important features in a fluorescent molecule; our results indicate that the ring A, B, C and D of the AFB_1 structure adopts a planar conformation, with all dihedral angles varying by less than one degree from planarity; whereas ring E is located slightly outside the plane, enabling the formation of an extended conjugated π electron system (Fig. 5).

Moreover, the charge values obtained with NPA, clearly demonstrated an electronic deficiency on the carbonylic carbon atom, favouring this site for a nucleophilic attack and providing in this sense the driving force to hydrolyse the lactonic moiety.

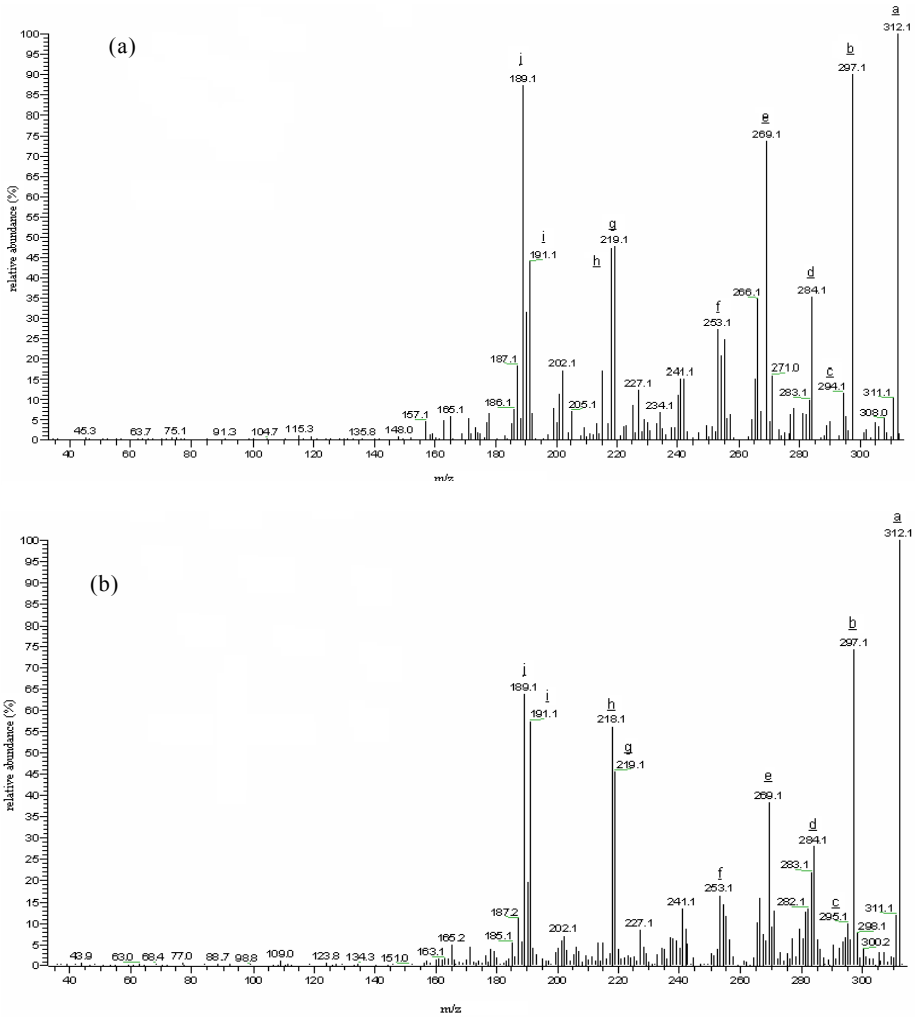

Fig. 2: Main spectrums of AFB_1: AFB_1 standard (profile a); AFB_1 purified from the contaminated maize (profile b)

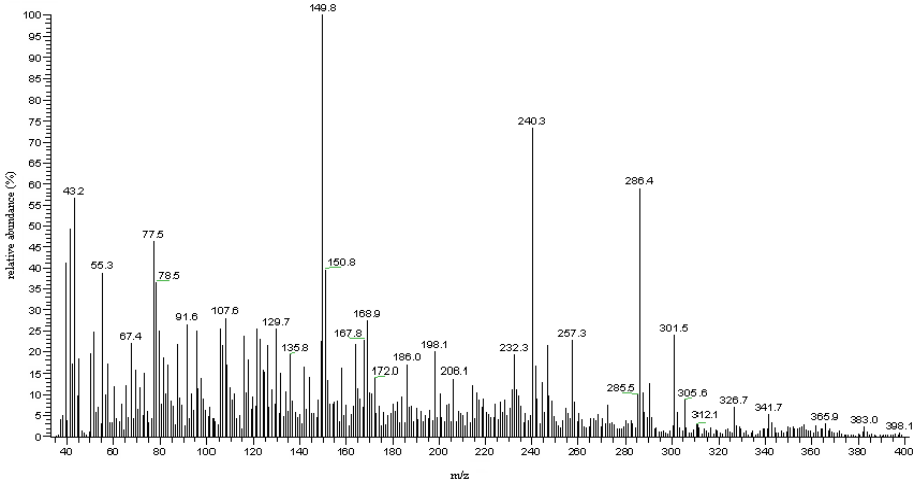

Fig. 3: Relative intensities of peaks obtained from the EIMS of the crude reaction product

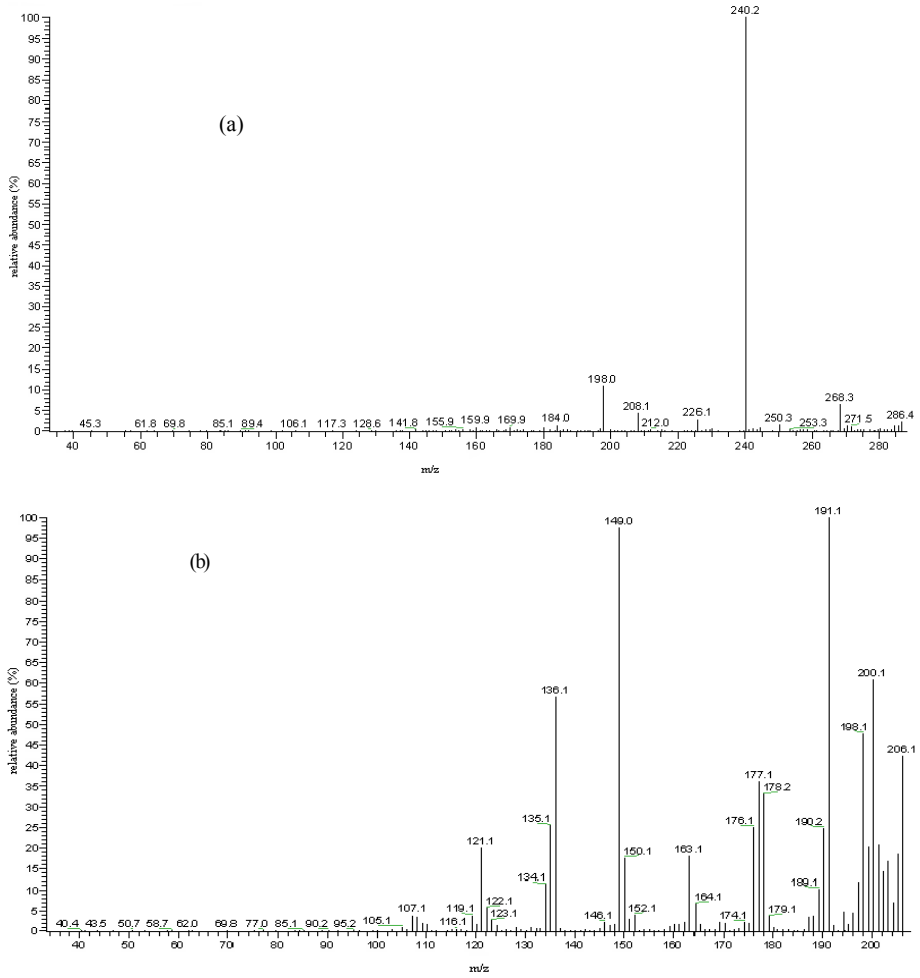

Fig. 4: Daughter ions of fragments obtained by MS/MS: m/z 286 (profile a), m/z 206 (profile b)

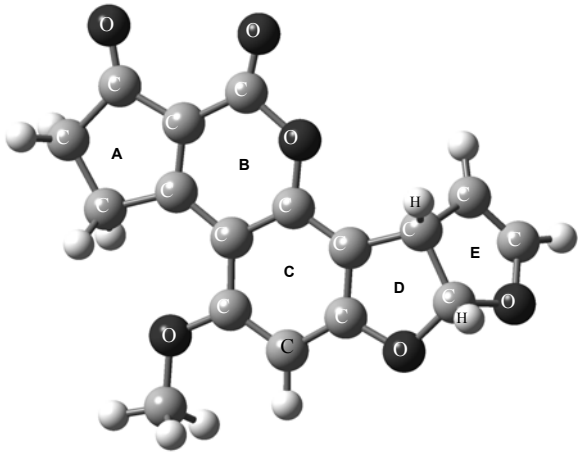

Fig. 5: Optimized structure of AFB_1, determined by B3LYP/6-31G (d,p)

The charge transference in the lactone ring and on some carbon atoms of benzene, indicates the existence of a conjugation among them. The charge transfer observed between the ground and the excited singlet state provided an indication of the fluorescence, an increase and a decrease in the electronic charge of the atoms involved in the lactone ring. Therefore, the fluorescence phenomenon diminishes when the aflatoxin structure is hydrolysed.

In conclusion, the reaction of citric acid dissolutions and AFB_1 leads to the hydrolysis of the lactone ring of the aflatoxin molecule. The proposed reaction mechanism of the AFB_1 acidification was also confirmed by both MS/MS and computational studies. Moreover, theoretical calculations indicate that the hydrolysis of the carbonylic carbon atom of the aflatoxin molecule is possible and could therefore

interact with nucleophiles present in aqueous citric acid conditions.

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