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INFLUENCE OF HIGH PRESSURE AMMONIATION PROCEDURE ON THE DETOXIFICATION OF MYCOTOXIN (AFLATOXINS)

By

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ABSTRACT

Ammoniation represents the best technique to detoxify aflatoxin contaminated grain and it is considered as economically practicable for commercial applications. Aspergillus parasiticus was used to contaminate yellow corn to produce the final concentration reached 4000 ug/Kg corn total aflatoxin. Two procedures of ammoniation (in aqueous ammonia concentrations, 0.25, 0.5, 1, 1.5 and 2%) were adopted for aflatoxin destruction. The first procedure was under atmospheric pressure at ambient temperature (AP/AT) for 24 hrs, and the second procedure was under high pressure (2 bar) at high temperature (121℃) (HP/HT) for 15 min. Aflatoxin concentrations were determined by HPLC using fluorescence detection. The results revealed that high pressure treatment was more destructive to aflatoxins than the treatment under atmospheric pressure. Moreover, high pressure ammoniation required minimum level of ammonia with less processing time.

INTRODUCTION

Every year a significant percentage of the world's grain and oilseed is contaminated with hazardous mycotoxins including the aflatoxins. Unfortunately, discontinuing the feeding of aflatoxins contaminated grain is not always practical, especially when alternative feedstuffs are not readily available or affordable (*Park et al, 1990).* Aflatoxins are potent hepatotoxins as well as potent carcinogens. The Food and Agriculture Organization (FAO) estimates that 25% of the world's food crops are affected by mycotoxins *(Mannon et al, 1985).* Significant aflatoxin contamination levels in corn and corn-based commodities have been reported in Latin America and the Caribbean. Aflatoxins were detected in many corn-based commodities such as corn, corn on cob, corn drink, Torilla corn kernel corn gluten raw, corn gluten feed, yellow corn, white corn, corn flour and flakes

(Park and Liang, 1993). Sodium hydroxide, methylamine. Hydrogen preoxide, ozone and other chemical reagents were used as inactivation treatments of aflatoxin. These chemical reagents were used as inactivation treatments of aflatoxin. And achieved some degree of success, but generally were not economically practicable for commercial application *(Kolton et al 1979 and Park et al, 1993).* In the United states: Texas, North Carolina, Georgia, and Alabama have approved the ammoniation procedure for aflatoxin-contaminated corn. Mexico has approved ammoniation for corn, also, many countries such as France, Brazil, Senegal, South Africa India and several countries of the European Economic Community use some ammonia-treated crops *(Park and Lee, 1990).* The toxicity from ammonia aflatoxin reaction products was several orders of magnitude lower than that of aflatoxin B_1. Even the formation of these decontaminated reaction products in the feed matrix is usually < 1% of the original aflatoxin contamination level. A large portion of the reaction products is bound to feed components such as protein and is potentially not biologically available to animals *(Park, 1993).* On the other hand, *(Phillips et al, 1994)* reported that if the reaction between aflatoxin and ammonia is allowed to proceed sufficiently, the process is irreversible. The first step in the reaction is reversible, if the ammoniation process is carried out under mild conditions. However, when the reaction is allowed to proceed, the products formed do not revert back to aflatoxin B_1, The reaction products of ammoniation are dependent on temperature, pressure and the source of ammonia. Human exposure to aflatoxins and other my cotoxins can result from direct consumption of contaminated commodities, or from the consumption of animal- derived foods. Therefore, our study aimed to compare between the efficiency of hight pressure and atmospheric pressure ammoniation in the destruction of relatively high level of aflatoxins (400 ug/ Kg) in contaminated yellow corn.

MATERIALS AND METHODS

Aspergillus parasiticus NRRL 3145 strain was subcultured on potato dextrose agar (PDA) for 7 days at 25 C° and stored at 4 C° until utilization. This fungal strain was activated on (PDA) media.

Yellow corn was used as a model for an important component in different animal feeds which recorded frequent incidents of high levels of aflatoxin contamination.

Preparation of high concentration of aflatatoxin contaminated corn

Yellow corn was artificially infected with the *Aspergillus parasiticus* stain according to *(Codner et al, 1963 and Stubblefiled et al 1967).*

Preparation of final concentration of aflatoxin contaminated corn

The highly contaminated corn was diluted to the desired concentration by adding aflatoxin free corn. To ensure the homogeneity of sample both the contaminated corn and aflatoxin free corn were milled to the final particle size.

Ammoniation procedure for the 4000-ug-level aflatoxin

Two procedures of ammoniation were adopted for the destruction of 4000-ug-level aflatoxin. The main difference of the two procedures is the use of hight pressure and temperature (HP/HT) along with ammonia for one procedure and using the ammonia under the atmospheric pressure and ambient temperature (AP. AT), in the second one.

The moisture content of 40 Kg contaminated corn was adjusted to 18% wet basis. Then ammonia was sprayed to provide a level of 0.25, 0.5, 1.0, 1.5, and 2% ammonia on dry matter basis. Each ammonia concentration was used to spray 10 Kg contaminated corn to be used for the 2 ammoniation procedures (5 Kg each).

a. Atmospheric pressure and ambient temperatures (AP/AT)

A total of 25 sample weighed 25 Kg (5 samples for each ammonia concentration) were packed in polyethylene bages (1 Kg each) and stored for 24 hrs. The aflatoxin residues were determined by HPLC.

b. High pressure and high temperatures (HP/HT)

Another 25 contaminated corn samples were packed in autoclavable polyethylene bags (1 Kg each) and autoclaved. The corn was directly extracted to determine the aflatoxin residue by HPLC.

Extraction and determination of aflatoxins

The extraction and clean up of aflatoxins in all samples were performed according to CB method AOAC, (1990).

HPIC analysis was carried out with Waters Liquid Chromatography equipped with solvent delivery systems (model 6000A), system controlled (model 720), data module (model 730), U6K injector and fluorescence detractor (modle 420) with excitation 338 nm and emission 455 nm. Econospher c18 reverse phase column (5, 250 mm XID 4.6 mm) (Alltech) was used.

Derivatization

To the final extract (residue), an amount of 200 ul hexane was added followed by 50 ul trifiouroacetic acid (TFA) and mixed well by avortex shaker for exactly 30 sec.; the mixture was left to stand for 5 min. A mixture of 1.95 ml H_2O + acetonitrile (9 + 1 vIv) was added and mixed well for exactly 30 sec. And the mixture was left to stand for 10 min. Then the hexane layer was discarded *(Park et al, 1990).*

Preparation of aflatoxin standard

Different concentrations of B_1 (0.76 uM) B_2 (47.9 uM) G_1 (0.55µM), and G_2 (0.75 uM) (Sigma Co.) were dissolved and mixed using methanol (HPLC grade). The methanol was then evaporated under a stream of nitrogen and the derivatization procedure was. The same derivatization procedure was applied on aflatoxin standard B_1, B_2, G_1 and G_2.

Chromatographic conditions

Mobile phases included solvent A[mixture of acetonitrile + water (23 + 77 v/v)] and solvent B (methanol). The linear gradient program was illustrated in Table (1).

LC determination

Only 20 µl of derivatized standard solutions was injected to prepare standard curve to check linearity of responses. A20 µl of TF A treated sample solution was injected, the aflatoxin concentration (µg/ Kg) of corn was calculated using standard curves for each toxin (B_1, B_2, G_1 and G_2.)

Table (1). The HPLC gradient program used for aflatoxin separation.

Time (Min)	Flow rate (ml/Min)	% Solvent A	% Solvent B
0	1	100	0
5	1	60	40
10	1	40	60
15	1	0	100
20	1	100	0
25	1	100	0

Statistical analysis

The effect of different ammoniation treatments on the 4000 ug aflatoxin contaminated corn was statistically analyzed using two way analysis of variance. The significancy of difference between high and low pressure under different ammonition level was tested according to the following model :

$$X_{IJK} : = u + aI + Bj + aibj + E$$

where u : General mean.

Xijk : Sample (K) of treatment (I) and concentration (J).

ai : Treatment (high & low) effect.

Bj : NH_3 concentration effect.

aibj : Interaction between pressure and concentration.

Eijk : Residual.

Main effect was used to detect the significancy of difference between each 2 treatments in the matrix of the different treatment levels. Regression analysis was performed to determine the slope and the regression to identify the type and shape of the relationship between percent aflatoxin destruction and the 2 ammoniation treatments under different ammonia concentrations *(Sas, 1990 and Winer, 1971).*

Table (2) Effect of ammoniation treatment under low pressure on aflatoxins destruction.

Toxin	0.25%	0.5%	1%	1.5%	2%
	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
G_1	58.74 ± 1.14	78.76 ± 2.59	84.54 ± 1.20	94.44 ± 8.34	96.92 ± 1.30
B_1	39.52 ± 5.22	66.94 ± 1.27	77.44 ± 0.40	80.74 ± 1.54	88.02 ± 2.81
G_2	29.70 ± 0.85	74.80 ± 3.67	83.90 ± 2.69	89.40 1.85	93.02 ± 1.03
B_2	34.52 ± 3.26	63.18 ± 3.43	73.14 ± 3.20	78.72 ± 2.14	85.40 ± 1.64
Total	40.78 ± 2.43	70.94 ± 2.71	79.78 ± 1.81	85.84 ± 1.19	90.02 ± 1.36

SE = Standard error. Values have the same letters are not significant.

RESULTS AND DISCUSSIONS

1- Effect of ammonia concentration on the stability of aflatoxins

a- Atmospheric pressure . Data presented on Table (2) showed the effect of ammoina concentrations on the stability of aflatoxins (G_1, B_1, C_2 and B_2) under atmospheric pressure. A proportional increase on destruction of aflatoxin was noted with the increase of ammoina concentrations.

This incline relationship was come to a plateau (no obvious increase in the aflatoxins percent destruction) with the use of 1.5% ammoina concentration (Figure 1) Regression analysis confirmed this relationship which was significant at the second order (Table 6) Significant differences

were observed among the effects of 0.25, 0.5, and 1.0% ammonia concentration on aflatoxins destruction. No significant differences were noticed upon application of 1.5 and 2.0% ammonia concentration.

Concerning the destruction of aflatoxin different ammonia concentration under atmospheric pressure ranging form 40.3% (with 0.25% ammonia) to 90% with 2.0% ammonia), total aflatoxins percent destruction was similar to those reported by *(Koltun et al, 1979)*, who found that increasing ammonia concentration from 3% to 5% at 180 F for 15 minutes, increased total aflatoxins percent destruction, from 45% to 86% Similarly, Bagley (1979), confirmed this relation when reported that aflatoxin B_1 percent destruction was increased from 83% to 89% when ammonia concentration increased from 0.5% to 1.5%.

On the other hand, Jorgensen and Ralph (1981) reported that 2% ammonia and 43°C for 15 days resulted in 98.8% aflatoxin B_1 destruction in naturally contaminated whole cottonseed.

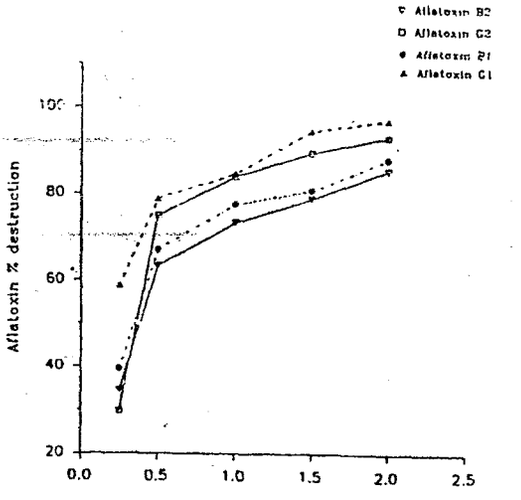

Fig. 1. Effect of different ammonia concentrations under atmospheric pressure on aflatoxins π destruction.

Table (3) Effect of ammoniation treatment under high pressure on aflatoxins destruction percentage

Toxin	0.25%	0.5%	1%	1.5%	2%
	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
G_1	87.14 ± 1.77	96.78 ± 1.09	99.54 ± 0.08	99.80 ± 0.06	100.0 ± 0.0b
B_1	23.72 ± 5.18	76.64 ± 5.32	94.28 ± 1.00	97.14 ± 0.25	99.90 ± 0.08
G_2	81.16 ± 3.15	94.58 ± 0.96	99.52 ± 0.55b	99.64 ± 0.90	100.0 ± 0.00
B_2	81.88 ± 2.42	91.28 ± 0.63	98.04 ± 0.37	98.84 ± 0.11	00.76 ± 0.09
Total	68.90 ± 2.95	93.08 ± 1.52	97.74 ± 0.37	98.64 ± 0.013	99.92 ± 0.05

Table (4). Effect of ammoniation pressure regardless ammonia concentration on aflatoxins destruction percentage

Toxin	High	Low	P
G_1	96.56±1.07	82.68±2.84	0.0001
B_1	80.61±5.72	70.53±3.63	0.0002
G_2	94.78±1.56	74.16±4.79	0.0001
B_2	93.96±1.45	66.99±3.81	0.0001
Total	92.60±2.32	73.63±3.70	0.0001

Table (5) Effect of ammonia concentration regardless ammoniation pressureon aflatoxins destruction percentage

Toxin	0.25%	0.5%	1%	1.5%	2%
	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
G_1	72.94 ± 4.84	87.77 ± 3.29	92.04 ± 2.56	97.12 ± 0.91	98.46 ± 0.53
B_1	32.50 ± 4.45	71.79 ± 3.05	85.86 ± 2.85	88.94 ± 2.75	93.96 ± 2.38
G_2	55.43 ± 8.72	84.69 ± 3.75	91.21 ± 2.77	94.52 ± 1.92	96.51 ± 1.26
B_2	58.20 ± 8.12	77.23 ± 4.96	85.59 ± 4.41	88.70 ± 3.50	92.50 ± 2.51
Total	53.28 ± 5.25	82.01 ± 3.97	88.76 ± 3.12	92.24 ± 3.21	95.37 ± 1.65

Similarly, Narred (1982), reported that atmospheric ammoniation of contaminated corn with 100 ppb total aflatoxins resulted in destruction of 99%. Also *(Park et al, 1988)*, reported that aflatoxin corn was inactivated by more than 96% by ammoniation procedure. Comparable results were reported by *(Mahalingam et al, 1990)*, who found that Ap/AT ammoniation treatment reduced aflatoxin content from 35 µg/g to a undetectable level. In the same respect *(Phillips et al, 1994)*, reported 1-5% ammonia under atmospheric pressure at ambient temperature for 14-42 days reduced the aflatoxin levels in corn to equal or below 20 ppb.

b. High pressure

Table (3) showed that ammoniation under high pressure resulted a similar trend in aflatoxin destruction with the increase of ammonia concentration.

However, the incliner relationships was confirmed by regression analysis which proved to be significant at the second order (Table 6).

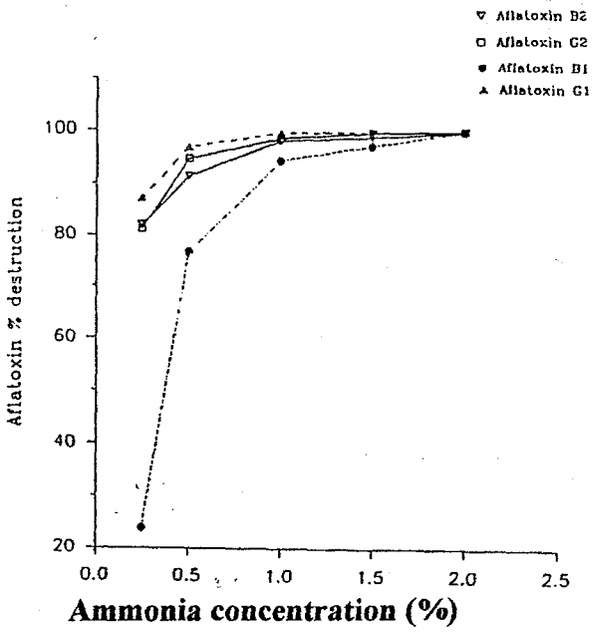

Fig. 2. Effect of different concentrations of ammonia under high pressure on aflatoxins % destruction.

Table (6) . Effect of different types of aflatoxins on the stability of aflatoxin under different treatment pressure.

Toxin	Regression equation
	Atmoshperic pressure
G_1	50.9 + NH_3 Conc 19.9 – NH_3 $Conc^2$ x 13.6
B_1	30.0 + NH3 Conc 66.6 - NH3 $Conc^2$ x 19.4
G_2	16.9 + NH_3 Conc 103.2 NH_3 $Conc^2$ x 33.2
B_2	24.5 + NH_3 Conc69.2 - NH_3 $Conc^2$ x 19.9
Total	30.5 + NH_3 Conc 72.8 - NH_3 $Conc^2$ x 21.5
	High pressure
G_1	83.1 + NH_3 Conc 25.2 – NH_3 $Conc^2$ x 8.6
B_1	6.6 + NH3 Conc 131.4 - NH3 $Conc^2$ x 43.4
G_2	74.6+ NH_3 Conc 33.0 NH_3 $Conc^2$ x10.5
B_2	75.1 + NH_3 Conc 32.9 - NH_3 $Conc^2$ x 10.5
Total	63.3+ NH_3 Conc 52.7 - NH_3 $Conc^2$ x 17.7

Significant differences were observed between aflatoxins destruction at 0.25 and 0.5% ammonia concentration. However, no significant differences were observed between 1.0, and 2.0%. In this respect *(Gardener et al, 1971)*, noted that ammoniation of cottonseed aflatoxin by more than 99%.

These results were in agreement with those of *(Park et al, (1984), Samarajeewa et al, (1990), and Phillips et al, (1990).*

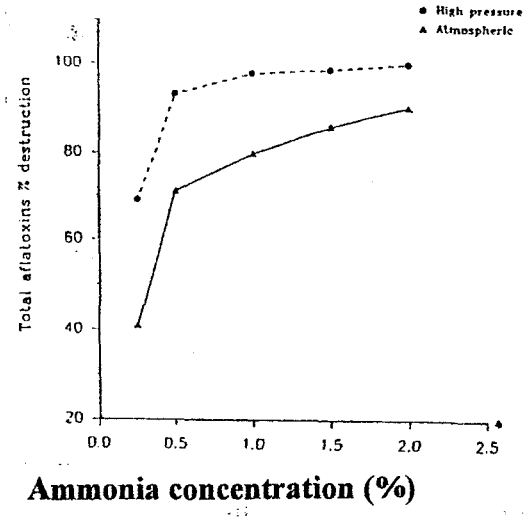

Fig. 3. Effect of different ammoniation pressures at different ammonia concentrations on total aflatoxins % destruction.

2- Effect of treatment pressure

Comparing Table (2) and Table (3), it becomes evident ammoniation under high pressure increase aflatoxins destruction for all types of tested aflatoxins except for aflatoxins B_1 at 0.25% ammonia.

Regardless the ammonia concentration, Table (4) illustrated that G_1, B_1, G_2, B_2 and B_2 destruction percentage were higher under HP/HT treatment compared with treatment under AP/AT.

Data in Table (4) showed a highly significant differences ($p>0.001$) between high and low pressure for the tested aflatoxins. The effect of high pressure ammoniation was faster (plateau at 1%) than atmospheric pressure ammoniation (plateau at 1.5%) in reaching the maximum aflatoxins destruction, (Fig. 3).

Similar results were reported by *Brekke et al, (1977), Bagley (1979), Mashaly et al, (1983), and Frayssinet (1990).*

3- Effect of different types of aflatoxins

Table (2) illustrated that under atmospheric pressure (AP/AT) aflatoxin B_2 showed higher stability ammonia treatments compared with the other types of aflatoxins. Figure (1) also confirmed this trend of the higher stability of group B compared with group G aflatoxins when ammoniated under atmospheric pressure.

Table (3) indicates that treatment under high pressure and at the 0.25% ammonia concentration B_1 recorded the lowest rate of destruction while G_1, G_2, and B_2 recorded higher destruction rate. Also at 0.5% ammonia B_1 was reduced by only 76% where more than 91% of the other types of aflatoxins were desintegrated. Figure (2) illustrates that the higher stability of aflatoxin B_1 compared with the other types of aflatoxin was distinct at 0.25% and 50% ammonia concentration while at higher concentrations, these differences were getting closer.

Regardless of the treatment pressure (Table 5), aflatoxin B_1 recorded the minimum destruction rate at 0.25 and 0.50% ammonia. At the same time aflatoxins B_1 and B_2 were found to be more stable at 1.0, 1.5 and 2.0% ammonia compared with aflatoxins G_1 and G_2.

Regardless of the ammonia concentration, Table (4) indicated that aflatoxin B_2 is more stable (66.99% destruction) under low pressure treatment while aflatoxins B_1 is more stable (80.61% destruction) under high pressure treatment. On the other hand, aflatoxin G_1 recorded the maximum destruction percent (82.68% and 96.65%) at low and high pressure respectively.

These results indicating the higher stability of group B aflatoxins compared with group G aflaotoxins were confirmed by *Roegner (1976) and Moerck et al, (1980).*

In general, the high pressure treatment was more destructive to aflatoxins than the treatment under atmospheric pressure. Moreover, high pressure ammoniation required minimum level of ammonia with less processing time.

The obtained results revealed that :The effect of HP/HT procedure at the different ammonia concentrations was more destructive on aflatoxins than the AP/AT was Faster (plateau at 1%) than the AP/AT (plateau at 1%) than the AP/AT (plateau at 1.5%) to come to the maximum aflatoxins destruction. Aflatoxin B_2 showed higher stability for ammonia treatment (0.5, 1, 1.5 and 2%) compared with the other types of aflatoxins. The higher stability of group B compared with group G aflatoxin when ammoniated under AP/AT. Regardless treatment pressure, aflatoxin B_1 recorded the minimum destruction percent (32.5 and 71.79) at 0.25% ammonia, respectively. At the same time, aflatoxins B_1, and B_2 were found to be more stable at 1.0, 1.5 and 2.0% ammonia compared with aflatoxins G_1 and G_2. Regardless ammonia concentration aflatoxin B_2 is more stable under AP/AT, while aflatoxin B_1 is more stable under HP/HT treatment. On the other hand, aflatoxin G1 recorded the maximum destruction percent (82.68% and 96.65% at low and high pressure, respectively).

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تأثير الأمونيا ذات الضغط العالى على إزالة سمية السموم النظرية (الأفلاتوكسينات)

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يعتبر استخدام الأمونيا أفضل لإزالة سمية الذرة الملوث بسموم الأفلاتوكسينات، وكذلك تعتبر كوسيلة إجرائية صالحة لأستخدامها وتطبيقها على النظام التجارى.

وقد أستخدم فطر أسبرجلس بارازيتيكس فى تلوث الذرة الأصفر وذلك لإنتاج تركيزات من سموم الأفلاتوكسين وقد وصل التركيز النهائى إلى ٤٠٠٠ ميكرو جرام / كيلو جرام ذرة. وكانت التركيزات المستخدمة من محلول الأمونيا هى ٠,٢٥- ٠,٥ - ١,٠ - ١,٥ و ٢٪ وقد أستخدمت الأمونيا على طريقتين الأولى تحت الضغط الجوى العادى على درجة حرارة الغرفة لمدة ٢٤ ساعة، والثانية عند ضغط جوى عالى (٢ بار) على درجة حرارة ١٢٠م° لمدة ١٥ دقيقة.

وقد تم تقدير تركيزات الأفلاتوكسينات بأستخدام جهاز HPLC وقد أظهرت النتائج أن المعاملة ذات الضغط العادى كانت أكثر تدميراً لسموم الأفلاتوكسينات عن معاملة الأمونيا تحت الضغط الجوى العالى هذا بالإضافة إلى أن أستخدام الأمونيا تحت الضغط العالى تتطلب مستويات أقل من تركيزات الأمونيا مع وقت أقل لإتمام المعاملة.