

## Aflatoxin Detoxification in Rice using Citric Acid

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### Abstract

**Background:** Aflatoxins cause health hazards to human and animals and has also economical problems. Therefore, the detoxification effect of citric acid was investigated in rice as the main food of Iranian people.

**Methods:** Initially 275 samples of rice were examined for aflatoxins by HPLC. The aflatoxins contaminated samples were later treated by aqueous citric acid and detoxification of aflatoxins were quantified using HPLC.

**Results:** Among the 275 samples analyzed, aflatoxin B<sub>1</sub> and aflatoxin B<sub>2</sub> were detected in 211(76.72% of total) samples. Aflatoxin B<sub>1</sub> was detected in 203(73.82% of total) samples with a mean and standard deviation of 2.3±10.21ppb. Aflatoxin B<sub>2</sub> together with aflatoxin B<sub>1</sub> were detected in only 8(2.91% of total) samples with a mean and standard deviation of 1.38±2.7ppb of aflatoxin B<sub>2</sub> and 2.99±1.56 of aflatoxin B<sub>1</sub> respectively. Aflatoxin B<sub>1</sub> level in 5 samples (1.82%) was above the maximum tolerated level of aflatoxin B<sub>1</sub> in Iran (5ppb). However considering the Iranian maximum tolerated level for aflatoxins in rice (30ppb), only 3(1.09%) samples were above the 30ppb and also in regard to the European maximum tolerated level for aflatoxins in rice (4ppb), only 9(3.27%) samples were considered as higher than 4ppb.

**Conclusion:** The HPLC assay showed that although aflatoxins with a concentration of <30 and <4 ppb in the rice samples were completely degraded, but 97.22% degradation occurred in rice contaminated with ≥30 and ≥4ppb when treated with 1N citric acid. These results revealed the efficacy of 1N citric acid in reducing aflatoxins levels in rice.

**Keywords:** Detoxification, Aflatoxins, Aflatoxin B<sub>1</sub>, Aflatoxin B<sub>2</sub>, Citric Acid, Iran

### Introduction

Aflatoxins (AFT), belong to the secondary metabolites produced mainly by *Aspergillus flavus* (1), *A. parasiticus*, *A. nomius* and *A. tamaritii* on a wide spectrum of foods (2-3). This toxic substance, with four out of 20 different types which are much known and studied; including B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub> could lead to some health problems such as acute and chronic poisoning among animals and human beings; acute hepatic destruction, cirrhosis and some malignancies as well as immunodeficiency causing recurrent infections (1, 4-6). This has been disclosed that AFT contaminates about a quarter of foods and causes very prominent economic loss annually (4,6). Consequently, many of committees and institutes have defined standards for acceptable amounts of the

mentioned mycotoxin in foods because of its being harmful. For instance, a European committee named "Codex" has defined an acceptable maximum of 4 ppb of AFT in rice. This acceptable amount for aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) is 5-10 ppb in feedstuffs and 4 ppb in foodstuffs. The allowed amount which has already been defined in Iran is 30 ppb for AFT and 5 ppb for AFB<sub>1</sub> in rice (7).

Many studies have been performed in order to control the contamination of human and animal foods through physical, chemical and biological methods. They convert the toxin into less harmful materials with less mutagenic effects (8). These chemicals mainly include acids (9-10); bases (11-15); oxidizing agents (7, 16,17); bisulfites (18-22) and gases (5, 6).

This study was carried out to detect the aflatoxin contamination rate among different rice samples in

the market of Tehran Iran in addition to evaluate the decontaminative potential of aqueous citric acid (CA) on the contaminated samples. Rice, as the common foodstuffs over the world, especially in our country, Iran, was selected as a very effective field to study on.

## **Materials and Methods**

This study was conducted over the 19 mo from 1- Mar -2008 to 1-Nov-2009 in Mycology Laboratory, School of Public Health, Tehran University of Medical Sciences, and Scientific and Research Laboratory of Farogh, Tehran, Iran.

The sample size of 275 was calculated by considering a 95% confidence interval and 0.06 study accuracy based on the previous similar performances (23 and unpublished data).

This investigation was done on totally 275 imported rice samples (5kg each) that were randomly purchased and collected from the markets in Tehran, Iran according to method of the Iranian national standards of to assess aflatoxin existence rate and affect of CA treatment on the contaminated ones. Rice samples were stored in zip-locked plastic bags at 4°C until being sampling for aflatoxin analysis in agricultural products (24).

### ***Sample preparation***

For minimizing the sub-sampling error in AFT analysis, all the samples were ground with miller, thoroughly mixed and collected in a plastic bag. Finally, 50g of test portion from the ground samples was taken for analysis by high performance liquid chromatography (HPLC) method.

### ***Extraction and clean up***

Samples were analyzed using HPLC method (the AOAC official method 999.07 as the same as ISIRI 6872, national standard, 2004) (25) with some minor modifications. The test portion, regarding HPLC analysis, was extracted using 200 ml of methanol/water (80ml/20ml). After filtering, the extract was diluted by water before being filtered through a glass microfiber filter.

Aflatest immune-affinity columns (IACs) were used to clean up the samples. Initially, 10 ml of

phosphate buffer saline (PBS) passed through the IAC. Then, 75 ml of the filtrate passed through the IAC at a flow rate of ca.1 drop per second. The column was washed with 15 ml water and dried by applying little vacuum. Finally AFT was eluted using methanol through two following steps; 0.5 ml methanol was applied on the column and was allowed to pass by gravity, 1 ml additional methanol was poured on the column after a minute and eluate was later collected. The eluate was finally diluted by water before being analyzed by HPLC.

### ***HPLC procedure***

Reverse-phase HPLC was mainly applied to quantitate AFT along with fluorescence detector followed by post column derivatization (PCD) involving bromination using a water HPLC system (pump 1525; fluorescence detector 2475; analytical column Nova-pack-C18 250×4.6 mm: 4 µm). We used Kobra cell and adding bromide to the mobile phase to achieve PCD. 100 µl of diluted AF eluate was then injected into HPLC. Mobile phase included water, methanol, acetonitrile mixture with the 600:300:200 (V/V/V) ratio in addition to 350 µl of nitric acid 4 mol/l and 120 mg potassium bromide with a 1 ml/min flow rate (23).

The fluorescence detector was operated at wavelengths of 365 nm and 435 nm as excitation and emission, respectively. A five-point calibration curve was drawn daily for different types of AFT including AFB<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub> and AFG<sub>2</sub> (Sigma, USA) to compare and find linear correlation in rice samples. The LOD for AFB<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub>, AFG<sub>2</sub> and AFT were 0.1 ppb, 0.08 ppb, 0.15 ppb, 0.07 ppb and 0.4 ppb, respectively.

Acidification of the rice samples carried out after analyzing the rate of the contamination among them (1) is as follows:

Three 500g subsamples were picked up from each of ground rice samples with aflatoxin total level 1 (containing < 30 ppb and < 4 ppb) and aflatoxin total level 2 (AFT ≥ 30 ppb and ≥ 4 ppb) and were treated with 1N aqueous CA for 15 min (3 ml/g of contaminated rice) before being filtered through a micro-fiber filter to get rid of excess water and dried in a vacuum oven at 40°C for 48 h.

The treated samples were stored in labeled clean plastic bags at 4 °C for further analysis and evaluation of the effects of acidification later is being done. We analyzed and rechecked the contamination rate of the CA treated samples after 48 h by HPLC to evaluate the efficacy of rice detoxification with CA. All tests were carried out at least two times for each sample.

### Statistic analysis

The data were analyzed by pair *t*-test to compare the contamination rate before and after acidification of the rice samples. A significance value of ( $\alpha=0.05$ ) was used to distinguish significant differences between treatments. Student *t*-test was assessed for comparison of mean values for AFT and AFB<sub>1</sub> with Iranian and EU standards. Statistical significance was assured at  $P \leq 0.05$ .

### Safety

All handling of toxic compounds were done in the fume hood with protective wears. The glasswares were washed with hypochlorite and dilute acid before re-using and waste materials treated with hypochlorite before disposal.

### Results

Out of total 275 rice samples were analyzed, aflatoxin B<sub>1</sub> and/or B<sub>2</sub> were detected in 211 (76.72%) among which 203 (73.82%) had only AFB<sub>1</sub> with the mean and standard deviation of  $2.3 \pm 10.21$  ppb while other 8 (2.91%) were contaminated with AFB<sub>2</sub> and AFB<sub>1</sub> of  $1.38 \pm 2.7$  ppb and  $2.99 \pm 1.56$  ppb respectively. AFG<sub>1</sub> and AFG<sub>2</sub> were not detected in tested rice samples. None of the aflatoxins were found in 64 (23.27%) samples.

Regarding the Iranian and European standards for Maximum Tolerated Levels (MTL) (26), we adjusted two levels of AFT to level 1 which was for AFT concentration  $< 30$  ppb,  $< 4$  ppb and level 2 with  $\geq 30$  ppb,  $\geq 4$  ppb.

AFB<sub>1</sub> level of 5 samples (1.82%) were higher than the mentioned MTL of 5 ppb, while 3 of those samples (1.09%) had AFB<sub>1</sub> level higher than 30 ppb. The results of HPLC assay before and after treatment of rice samples with 1N CA are summarized in Table 1.

**Table 1:** Aflatoxin contents of rice samples before and after treatment with aqueous citric acid regarding to the MTL levels of Iranian (30 ppb) and EU (4 ppb) standards for AFT

Particulars	Aflatoxin fluorescence strength <sup>a</sup>			Detoxification rate (%) <sup>b</sup>
	No. of Samples	Total (ppb)	Post-reaction (ppb)	
Aflatoxin level 1 (<30)	208	$1.13 \pm 1.16$	ND <sup>c</sup>	100
Aflatoxin level 1 (<4)	202	$0.99 \pm 0.76$	ND <sup>c</sup>	100
Aflatoxin level 2 ( $\geq 30$ )	3	$86.33 \pm 5.73$	$2.85 \pm 0.28$	97.22
Aflatoxin level 2 ( $\geq 4$ )	9	$32.76 \pm 38.05$	$2.85 \pm 0.28$	97.22

a: Mean  $\pm$  Standard deviation, b: Measured as loss of fluorescence, c: Not detected

### Discussion

Comprehensive contamination control and treatment of agricultural foods would be vital to the human and domestic animals' health. Teratogenic and hepatotoxic effects of aflatoxin are important, especially on the mentioned animals because of their inevitable roles in the lives of the human beings. Several studies have been carried out to

assess the rate of contamination with AFT among many products as well as the performance of control methods to remove this dangerous alloy, one of which was performed by Mazaheri between 2006 to 2007 (23) that was similar to the present study. She enrolled 71 rice samples in her study and detected AFT (B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub>) contamination in 59 cases. Through the mentioned study she re-

ported the most rate of contamination for AFB<sub>1</sub>, followed by the equal rates for the others which are in agreement with our study except for AFG<sub>1</sub> and AFG<sub>2</sub> which have not been detected in our samples. One probable reason for this difference seems to be the origin of rice samples.

In spite of worldwide investigations about aflatoxin contamination and aflatoxin detoxification of other grains such as maize and wheat, little was known about rice as a main food of Iranian particularly in Iran. Therefore specific attempts have been made to find a way against aflatoxin as an important health hazard.

CA(2-hydroxy-1, 2, 3,-propanetricarboxylic acid) is a major commodity chemical manufactured by industrial fungal fermentation after ethanol. Among many uses of CA, about 70% is used in the food industry and 10% in cosmetics and pharmaceuticals. The remainder is employed for diverse industrial purposes, including and increasing use in liquid wash products. Due to its low toxicity and medical properties it is considered as a health drink. CA and other organic acids are used extensively to adjust the acid flavor of soft drinks, fruit and vegetable juices and candies. Being a natural ingredient in many fruits and juices, citric acid effectively brings out flavors and blends well with flavor systems. This important food additive also serves as an antimicrobial preservative, retarding the growth of spoilage organisms (27).

A research was carried out on maize in order to evaluate the detoxifying effects of chemicals (1). Like the present study they used 1N aqueous CA and got success rates of 100% and 97.22% in case of AL<sub>1</sub> and AL<sub>2</sub>, respectively. These rates of success were completely similar to our findings apart from our a bit non-significant better result in case of AL<sub>2</sub> (post-reaction AFT equal to 2.85±0.28 ppb in our study vs. 3 ppb (1).

Through another trial (8), 1N aqueous CA was affected on duckling feed contaminated with 110 ppb AFB<sub>1</sub>. The authors succeeded in 86% and showed a great reduction in mutagenic activity by Ames test on *Salmonella* TA100 strains (28). They also compared the effect of citric acid with lactic acid on sorghum in another work and showed

higher detoxification rate for the former against AFT (29).

It seems that CA might acts on AFT as lactic acid (10) which other detoxification ways also have been tried by many investigators (10). For instance, Jun-Ho Hwang *et al.* studied on Korean and US wheat and found that AFB<sub>1</sub> was better removed during heating than washing (30). In addition, wet wheat was decontaminated more than dried one during heating. Furthermore, McKenzie *et al.* obtained acceptable results with application of 20% ozone in AFT degradation (17).

Sodium hypochlorite is another chemical substance was used with commercial bleaches (31). The results showed a complete destruction of AFT in a very short time with high concentrations of 5-6% or 0.67 M to 0.81 M of NaOCl.

After a great deal of study, (29), the authors called aqueous citric acid a vital chemical in AFT detoxification, especially under appropriate extrusion conditions. They reclaimed that the mentioned chemical was effective against AFT without affecting the physicochemical functions and textural properties of the extrudates.

In conclusion, as agricultural products such as cereals make up the main part of pets' and other domestic animals' foods (32-34) and regarding 88% AFB<sub>1</sub> and 66.6% AFB<sub>2</sub> contamination rates in corn products in northern Iran based on Yazdanpanah's report (32), it would be a note worthy to prevent comprehensively any AFT contamination among them and treat the alloyed ones. More studies could also be done to offer a way of chemical control for the products at risk of contamination in the future.

### **Ethical Consideration**

All Ethical issues (such as informed consent, conflict of interest, plagiarism, misconduct, co-authorship, double submission, etc) have been considered carefully.

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