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# Detection of Hydrolyzed Fumonisins $B_1$ and $B_2$ by Use of High Performance Liquid Chromatography in Sorghum

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Fumonisins are a family of mycotoxins produced by *Fusarium moniliforme* and *F. Proliferatum,* two of the most prevalent fungal contaminants of maize, sorghum, millet and many other agricultural products. The most readily identifiable and toxicologically active metabolites of fumonisins $B_1$ and $B_2$ are hydrolyzed fumonisins $B_1$ ($HFB_1$) and $B_2$ ($HFB_2$). Quantification of these fumonisins in sorghum samples was done using reversed phase HPLC with fluorescence detection and OPA (*o*-phthaldialdehyde/2-mercaptoethanol) derivation method at $\lambda_{ex}$ 370/$\lambda_{ex}$ 418 nm. Detection limit was 20 ppb for both hydrolyzed fumonisins at a signal-to-noise ratio of 4 : 1. To the best of our knowledge this report appears to be the first study on the quantitative determination of $HFB_1$ and $HFB_2$ in naturally contaminated sorghum. $HFB_2$ was absent in all the samples. In this study, the average hydrolyzed fumonisin $B_1$ and $B_2$ recoveries for four tested levels were 95.4% and 69.3% respectively. In conclusion, as corn is one of the major dietary staples for both animals and humans worldwide and many workers have reported their work on it, so there is need to study on the levels and production of fumonisins and their hydrolyzed moieties in sorghum also.

**Key Words:** ***Fusarium*** **spp., High performance liquid chromatography, Hydrolyzed fumonisins $FB_1$ and $FB_2$, Mycotoxins, Sorghum.**

## INTRODUCTION

Fumonisin $B_1$ ($FB_1$) is the most abundant toxin in fungal cultures and in naturally contaminated corn and other human and animal dietary staples[1–3]. The most studied mycotoxins in *Fusarium* are toxic to both plants and animals and sometimes it can be lethal to animals. Contamination by *Fusarium* spp., particularly by *Fusarium moniliforme* and *F. Proliferatum,* two of the most prevalent moulds are a major concern in maize, sorghum, millet and other agricultural products[4]. Chemically, fumonisins are diesters of propane-1,2,3-tricarboxylic acid and either 2-(acetyl amino) or 2-amino-12,16-dimethyl-3,5,10,14,15-pentahydroxycosane (Fig. 1). The occurrence of these mycotoxins in agricultural commodities is a major health concern for livestock and humans. More than ten fumonisins have been isolated and characterized; of these, fumonisin $B_1$ ($FB_1$), fumonisin $B_2$ ($FB_2$) and fumonisin $B_3$ ($FB_3$) are the major fumonisins which are

most abundant in naturally contaminated foods and feeds. Fumonisin $B_1$ generally comprises 75% of the total content of these homologs, which is most cancer-promoting and causes equine leukoencephalomalasia (ELEM) in horses, porcine pulmonary edema (PPE) in swine and cancer in rats. There is also evidence linking fumonisin with cancer in humans[5–8].

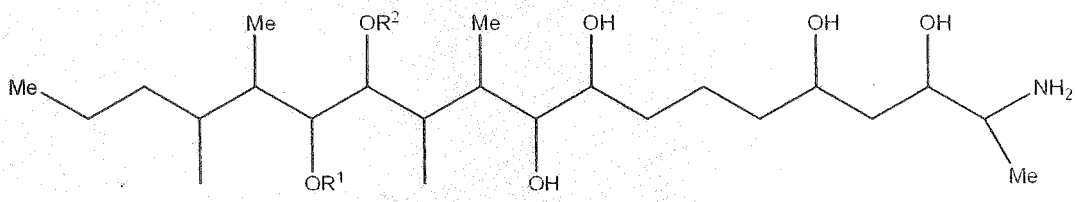


Fig. 1. (1) Chemical structure of $FB_1$ 721.84 and (2) the aminopentol moiety 405.61 (a) $R_1$ and $R_2$ = CO·$CH_2$·CH($CO_2H$)—$CH_2$—$CO_2H$, (b) $R_1$ and $R_2$ = H

Fumonisins are moderately heat-stable and water-soluble and are amino-poly-alcohols. The most readily identifiable and toxicologically active metabolites of fumonisin $B_1$ and $B_2$ are hydrolyzed fumonisin $B_1$ ($HFB_1$) and $B_2$ ($HFB_2$)[6, 9]. Hydrolyzed $FB_1$ is produced when corn is nixtamalized and is toxicologically more active on rats than $FB_1$. A rat feeding study in which rats were fed corn containing 50 ppm of $FB_1$ or 10 ppm of $HFB_1$ showed almost equal toxicity signs in both groups, indicating that $HFB_1$ could be more toxic than $FB_1$ itself[10]. To the best of our knowledge there seems to be no report on the quantification of hydrolyzed forms of fumonisins $B_1$ and $B_2$ in sorghum though there exist many reports which mention the presence of these metabolites of fumonisin in several commodities like corn, cereal products, animal excreta and beer etc[1, 6, 11]. In continuation of our interest, this study was carried out to quantify the metabolites of fumonisins $B_1$ and $B_2$ after base hydrolysis of naturally contaminated sorghum by *Fusarium* species by the use of high performance liquid chromatography technique with fluorescence detection and precolumn derivatization of residues.

## EXPERIMENTAL

Acetonitrile and methanol (HPLC grade) from J.T. Baker, glacial acetic acid (ACS grade), sodium dihydrogen phosphate and phosphoric acid (reactive grade) were used. Fluoraldehyde (OPA reagent) was obtained from Pierce Chemicals Co. (Rockford, IL). The fumonisin $B_1$ and $B_2$ were obtained from Sigma Chemicals Co. (St. Louis, Mo, USA). HPLC analyses were done using a Perkin-Elmer S-400 HPLC fitted with a Perkin-Elmer 5 ports injector and having a 20 μL loop. Chromatographic separation was achieved using a Phenomenex C-18 reverse phase column (250 × 4.00 mm i.d.), 5 μ micron packing material; detection was performed with a fluorescence detector (Perkin-Elmer) set at an excitation wavelength of 370 nm and emission cut-off at 418 nm. Mobile phase mixture was methanol-0.05 M sodium dihydrogen phosphate (55 : 45) (v/v); pH 3.3 was adjusted with phosphoric acid, with a linear gradient of 0–95% acetonitrile-water (80 : 20) (v/v) over 10 min eluted at a flow rate of 1.0 mL/min. Extracts were injected strictly within 1 min of their derivatization into the

chromatographic apparatus by full loop injection system (equivalent to 0.016 g). Quantification and identification of each hydrolyzed fumonisin were performed by injecting sequentially sample extracts and comparing the peak area ratio and their retention times with the relevant standard curve.

**Sample preparation:** A 25 g naturally contaminated ground sorghum sample was extracted by blending for 2 min with 125 mL of methanol-water (4 : 1) (v/v). The supernatant was filtered through a Whatmann filter paper no. 4. Filtrates were cleaned up on a 1 mL Bond-Elute strong anion exchange (SAX) column (Supelclean$^{TM}$ LC-SAX, Supelco). The eluate was hydrolyzed by mixing 4 mL of 1 N potassium hydroxide (KOH) and incubating the mixture in a closed vial at 70°C for 1 h. The mixture was titrated to pH 4.5 by 2 N HCl very slowly and cleaned up on a C-18 solid phase extraction (SPE) column (Supelclean$^{TM}$, Supelco) preconditioned with 2 mL methanol and 1 mL water.

**Isolation of $HFB_1$ and $HFB_2$:** Hydrolyzed fumonisins $B_1$ and $B_2$ were eluted with 1 mL methanol after washing the column with 500 µL methanol-water (50 : 50) (v/v). The eluate was evoparated to dryness under nitrogen and reconstituted in 100 µL of acetonitrile/water (50 : 50) (v/v). An aliquot of 25 µL reconstituted residue was derivatized with 100 µL of *o*-pthaldialdehyde (OPA)/2-mercaptoethanol (MCE) reagent at room temperature and derivatives were prepared immediately prior to injection.

**Base hydrolysis of the fumonisins:** $HFB_1$ and $HFB_2$ were prepared from 20 µg/mL of stock $FB_1$ and $FB_2$ solutions using base hydrolysis with controlled time and temperature of the reaction. Complete hydrolysis was obtained by mixing 4 mL of 20 µg/mL of stock fumonisin $B_1$ and $B_2$ solutions with 5 mL of 1 N KOH each and heating the mixture in a closed vial at 70°C for 1 h. After clean-up, the dilutions of different concentrations (0.5, 1, 2, 3, 4, 5 µg/mL) were made with acetonitrile/water (50 : 50 v/v). Standard curves for $HFB_1$ and $HFB_2$ were plotted using these dilutions and evaluated for linearity by determining the coefficient of determination ($r^2$).

**Recoveries:** Recoveries were determined by adding 1, 3, 5 and 7 µg/mL of $HFB_1$ and $HFB_2$ solutions to the blank ground sorghum (triplet) purchased from a retail store. Sample preparation and analysis were done as described.

## RESULTS AND DISCUSSION

*Fusarium* mycotoxins have traditionally been associated with temperate cereals, since these fungi require somewhat lower temperatures for growth and mycotoxin production. These mycotoxins found in food and feed are generated primarily in the field although some toxin synthesis may occur during storage also. Environmental stresses such as heat and drought may be factors that increase the colonization of the *Fusarium* species and their production of fumonisins. In our previous report, the mean concentrations of $FB_1$ and $FB_2$ were found to be 0.53 and 0.47 µg/g respectively which has been reported earlier[4], communicated). Hydrolyzed moieties of fumonisin mycotoxins are polar molecules which are soluble in water and polar solvents and are ideally suited for determination by

reversed phase HPLC. *o*-Pthaldialdhyde/2-mercaptoethanol (MCE) reagent derivatives were added to isolated $HFB_1$ and $HFB_2$ just before the injection and injected in less than 1 min for best reproducible results. Thakur and Smith[12] also suggested 1 min reaction time of analytes for better resolution and fluorescence intensity. In this study fumonisins $B_1$ and $B_2$ (aminopentols) were analyzed in 25 naturally contaminated sorghum samples, since the aim of this study was to determine what concentrations of $HFB_1$ and $HFB_2$ could occur in contaminated sorghum samples. The hydrolyzed fumonisin $B_2$ moiety was absent in all the samples while analysis of $HFB_1$ could be possible for most of the samples; in contrast, HPLC peaks for some samples were interfered by other co-eluting components as being a low concentration of $HFB_1$. Statistical analysis were done by Mann-Whitney Rank Test for all the processed samples but it was not significant for all samples. Absence of $HFB_2$ may be attributed to the deficiency of reliable method of its analysis which needs more efficient and modified methodology. Use of 1 N KOH for the hydrolysis took less time for their complete hydrolysis and these were heat stable at high temperature. Jackson *et al.*[13] observed that both fumonisins $B_1$ and $B_2$ are fairly heat-stable in an aqueous medium. Complete hydrolysis of fumonisins was confirmed by MS; chemical reactions such as base hydrolysis reduced fumonisin levels but produced hydrolyzed FBs which are almost as toxic as unmodified fumonisins. Similarly, Sydenham *et al.*[1, 14] also suggested that the process which involves a step with high pH and heat would destroy or reduce $FB_1$ significantly; it might be due to $FB_1$ losses in its two carboxylic acid groups (TCA moieties) to form aminopentol ($AP_1$), which appears to be more toxic for animals as well as for humans also. The American Association of Veterinary Laboratory Diagnosticians has recommended maximum levels of 5, 10, 50 ppm fumonosin $B_1$ in feed for horses, swine, beef and poultry respectively. A minimum safe level for humans has not been determined because the risk to humans is still being assessed. This method gives good recoveries of fumonisins and their hydrolyzed moieties from spiked samples. In the range of 0.5–5 μg/mL, the standard curves were linear (Fig. 2). In this study, the average $HFB_1$ and $HFB_2$ recoveries for four tested levels were 95.4 and 69.3% respectively (Table-1).

TABLE-1
RECOVERIES OF AMINOPENTOLS ($AP_1$ AND $AP_2$) ADDED TO BLANK SORGHUM

| Concentration added (μg/mL $AP_1$/$AP_2$) | Recovery ± SD% (n = 3) | |
|---|---|---|
| | $AP_1$ | $AP_2$ |
| 1 | 83.0 ± 1.0 | 71.5 ± 0.9 |
| 3 | 98.2 ± 1.2 | 69.3 ± 1.2 |
| 5 | 100.4 ± 1.8 | 68.0 ± 0.6 |
| 7 | 100.1 ± 1.3 | 68.6 ± 1.1 |

S.D. = Standard deviation (n = 3 replicates)

Sydenham *et al.*[1, 14] also reported 96.3% recoveries of $AP_1$. The detection limit for this method was 20 ppb for both $HFB_1$ and $HFB_2$ at a signal-to-noise ratio of 4 : 1, while Scott and Lawrence reported their detection limits 10 ppb for $AP_1$ with OPA/MCE derivatization.

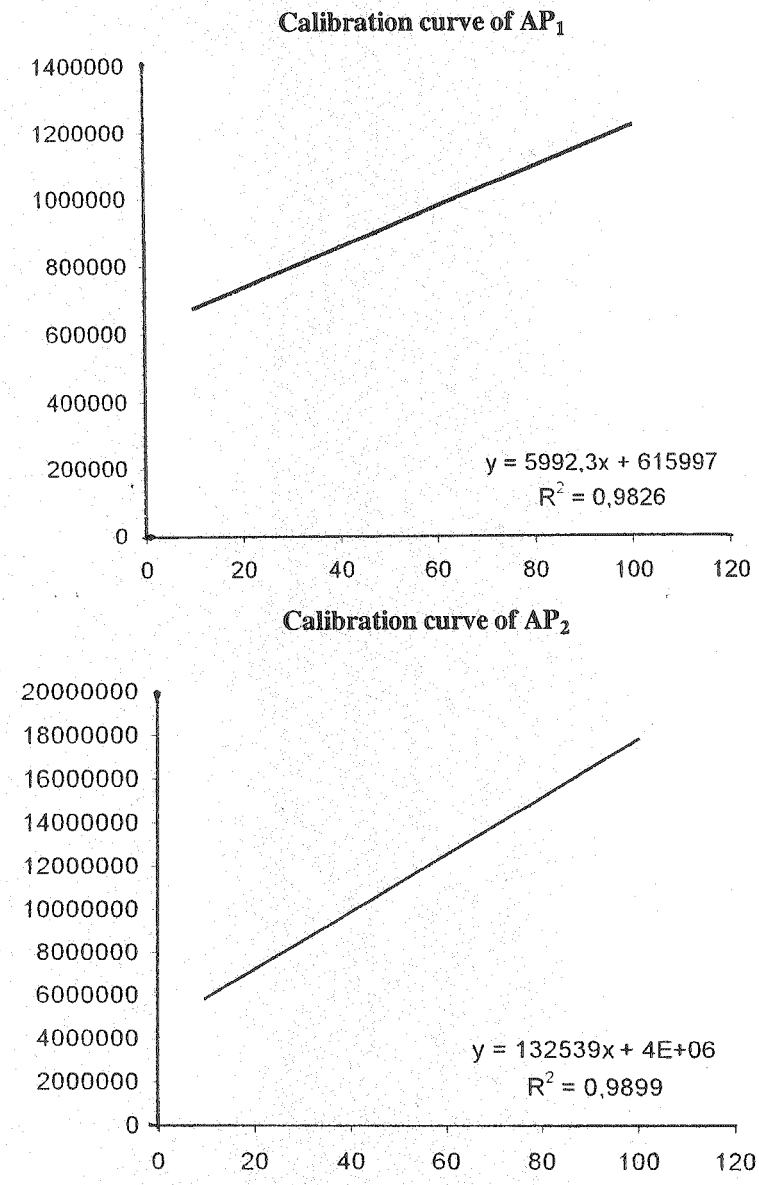


Fig. 2. Calibration curves of $AP_1$ and $AP_2$

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