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Incidence and Quantification of Fumonisins B_1 and B_2 at Harvest Stage in Sorghum

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Mycotoxins produced by *Fusarium* species particularly *Fusarium moniliforme* is a major concern in maize, millet, sorghum and agricultural products. A field experiment of sorghum with four different sowing dates with six replications was undertaken in the community of Tlayca (Morelos, Mexico) for the determination of fumonisins B_1 and B_2. Quantifications of these fumonisins in sorghum samples were done using reversed phase HPLC with fluorescence detection and OPA (*o*-phthaldialdehyde/2-mercaptoethanol) derivating method at λ_{ex} 370/λ_{em} 418 nm. Detection limit was 20 ppb for both 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 FB_1 and FB_2 in sorghum. Mean recoveries of FB_1 and FB_2 were 99.6 and 91.8% with relative standard deviation of < 4 and < 3 from spiked samples of sorghum respectively. In conclusion, total fumonisin levels do not exceed the recommended tolerance levels whereas temperature and weather conditions were critical factors affecting the fungal infection and fumonisin synthesis during the sorghum sowing dates.

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

INTRODUCTION

Fumonisins are a group of naturally occurring mycotoxins produced by the fungi *Fusarium moniliforme* and *Fusarium proliferatum.* Contamination by *Fusarium* species, particularly *Fusarium moniliforme*, is a major concern in maize, millet, sorghum and other agricultural products, intended for human and animal consumption. 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 causes leukoencephalomalasia (LEM) in horses, pulmonary edema in swine and cancer in rats. There is also evidence linking fumonisin with cancer in humans[1–4].

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More than ten fumonisins have been isolated and characterized, which are moderately heat stable and water soluble and are amino polyalcohol. By the use of TLC and HPLC (with fluorescence detection and precolumn derivatization of residues) techniques, these fumonisins can be detected at *ca.* 100 ng/g in corn, rice, milk and beer[5].

To the best of our knowledge there seems to be no report on the quantification of fumonisins in sorghum though there exist many reports which mention the contamination of sorghum by Fusarium species in continuation with our interest on mycotoxin problems related to food, feed and grains[6, 7]. This study was carried out to quantify the occurrence and sowing date infection of fumonisins FB_1 and FB_2 in sorghum by strong anion-exchange (SAX) of solvent extracts and the use of *o*-phthaldialdehyde as derivatizing agent at the harvest stage.

EXPERIMENTAL

Acetonitrile and methanol (HPLC grade) from J.T. Baker, glacial acetic acid (ACS grade), sodium dihydrogen phosphate and phosphoric acid (reactive grade). Fluoraldehyde (OPA reagent) was obtained from Pierce Chemicals Co. (Rockford, IL). The fumonisins B_1 and B_2 were obtained from Sigma Chemicals Co. (St. Louis, Mo, USA).

In the community of Tlayca, Morelos a field experiment with four sowing dates, six replications and a statistical design of random blocks was established. Weekly counts of sorghum ears with *F. moniliforme* were done in each plot beginning from the milky stage. At harvest the severity of the damage of each ear was determined with a scale of 1–5. After separating the sorghum grains of each plot, a sample of 100 g was taken for the determination of fusariotoxins[6].

Sample preparation and clean up: A 25 g ground sample of sorghum was extracted by blending for 2 min with 125 mL of methanol-water (4 : 1) (v/v). The supernatant was filtered through a Whatman filter paper no. 4. Filtrates were cleaned up on a 1 mL Bond-Elut strong anion exchange (SAX) column (Superclean™ LC-SAX, Supelco) which was previously conditioned with methanol (2 mL) followed by water (1 mL). With the first extraction solvent 1 mL filtrate (equivalent to 0.2 g) was added to the SAX column; with the second extraction solvent, 1 mL water was added to the column and then it was washed with 500 μL of methanol which was discarded, whereafter the fumonisins were eluted with 1 mL methanol-acetic acid (99 : 1) (v/v). Eluates containing the analytes were evaporated to dryness under a stream of nitrogen at *ca.* 60°C and residues were reconstituted in 100 μL of acetonitrile-water (50 : 50) (v/v) prior to derivatization. An aliquot of 25 μL reconstituted (equivalent to 0.02 g) residue was derivatized with *o*-phthaldialdehyde (OPA)/2-mercaptoethanol (MCE) reagent at room temperature. This reagent was prepared by dissolving 40 mg OPA in 1 mL methanol and 4 mL of 0.05 M sodium borate by adding 50 μL MCE. Derivatives were prepared immediately prior to injection, by the addition of 100 μL of OPA/MCE reagent to 25 μL of residue dissolved in acetonitrile-water (50 : 50) (v/v), mixed thoroughly and injected on to the HPLC loop.

Chromatographic analyses of FB_1 and FB_2: 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 were 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 fumonisin was performed by injecting sequentially sample extracts and comparing the peak area ratio and their retention times with the relevant standard curve and presence of fumonisins was confirmed by addition of pure FB_1 and FB_2 to selected prepared sample extracts.

Preparation of stock solutions: Fumonisin B_1 and B_2 stock solutions were prepared by transferring 1 mg of commercial standards (Sigma Chemicals Co.) into a 2 mL volumetric flask and made to volume with acetonitrile/water (50 : 50 v/v) to give a stock solution that was nominally 500 μg/mL. From this stock solution 100 μL of each fumonisin transferred to another vial and diluted with 900 μL of (50 μg/mL) acetonitrile/water. Dilutions made with acetonitrile/water (50 : 50 v/v) were 0.5, 1, 2, 3, 4, 5 μg/mL. Standard curves for fumonisin B_1 and B_2 were plotted using these dilutions and evaluated for linearity by determining the coefficient of determination (r^2).

Recovery experiment: Recoveries were determined by adding 3, 5, 7 and 9 μg/mL FB_1 and FB_2 stock solutions to the blank ground sorghum (Triplet) purchased from a retail store. Sample preparation and analysis were done as described.

Statistical analysis: Statistical analysis was done by Mann-Whitney Rank Test for all the processed samples.

RESULTS AND DISCUSSION

A total of 25 samples were analyzed for the detection of fumonisin B_1 and B_2 concentration in sorghum. These toxins were found in almost all the samples. Fumonisin B_1 was absent only in one sample (4%) in the fourth replication of the second sowing date, while FB_2 was absent in six samples (24%) in the 1st, 3rd, and 4th replication of first sowing date, 2nd replication in the second sowing date, 2nd and 6th replication in the third sowing date. The mean concentrations of 25 samples of FB_1 and FB_2 were found to be 0.53 and 0.47 μg/g respectively. Interestingly, the concentrations of both fumonisins (B_1 and B_2) were highest in the 6th replication of the second sowing date. A statistically significant difference ($p = 0.040$) was observed in the first and fourth sowing dates. It may be the weather conditions during the early sowing dates of sorghum favouring the growth of saprophytic fungi and production of the fumonisins. The sorghum plants were stressed by either excessive heat or lack of moisture and that stress

would appear to be an important factor in the production of these toxins but in contrast, in the later sowing dates, sorghum was contaminated by the presence of fungus mainly due to the cool and damp growing season; this implies that fumonisin levels were high in all the dates of experiment period. Similarly, Bacon and Williamson[8] also reported the effects of stress on the corn plant which was responsible for the fumonisin production.

In the range of 0.5–5 μg/mL, the standard curves were linear. In this study, the average fumonisin B_1 and B_2 recoveries for four tested levels were 99.6% and 91.8% respectively (Table-1). These results are comparable to those of Shephard *et al.*[9] who originally reported recoveries of 99.5% and 85.9% for FB_1 and FB_2, respectively. The detection limit for this method was 20 ppb for both FB_1 and FB_2 at a signal-to-noise ratio of 4 : 1. However, Stack and Eppley[10] reported detection limits of 10 ppb for both fumonisins while Hopmans and Murphy[11] reported 10 ppb for FB_1 and 20 ppb for FB_2. Derivatization of samples and/or standards with respect to time required for derivative formation can reduce the standard error associated with the instability of the fluorescent derivative. Rice *et al.*[12] and Thakur and Smith[13] also observed that a reaction time of 1 min before sample injection is best for reproducible results on the contrast longer reaction time (more than 1 min) showed greater variation and decreased fluorescence intensity. Derivation of analytes was carried out at room temperature (*ca.* 27°C) and OPA was kept at 4°C in the refrigerator, but the fluorescence stability of OPA adduct was not found different between these two different temperatures, which is because of OPA adduct containing primary amines having one hydrogen atom attached to the amino α-carbon[14].

TABLE-1
RECOVERIES AND R.S.DS OBTAINED FROM BLANK GROUND SORGHUM SPIKED WITH FUMONISINS B_1 AND B_2 AT DIFFERENT LEVELS

Spiking level (μg/mL)	Recovery ± S.D (%) of FB_1 and FB_2 (n = 3)		R.S.D.s of FB_1 and FB_2	
3	100.2 ± 1.9	93.8 ± 2.0	1.9	2.1
5	96.4 ± 2.3	91.6 ± 1.6	2.4	1.7
7	99.5 ± 3.2	89.0 ± 2.2	3.2	2.5
9	102.3 ± 1.2	92.7 ± 0.8	1.2	0.9

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

As fumonisins are polar molecules which are readily soluble in water and polar solvents, so they are ideally suited for their determination by reversed-phase liquid chromatography. They lack a significant ultraviolet chromophore and are not inherently fluorescent, so sensitive detection at low levels requires precolumn derivatization for the analysis of naturally contaminated food and feed sample extracts. Like corn, sorghum also produces more particulate pigments of intense red colour with other contaminant materials; therefore many problems occur with the liquid chromatography pumps.

In conclusion, temperature, moisture and weather conditions during the sowing dates of sorghum are critical factors affecting the fungal infection and fumonisin synthesis. In all the samples the total fumonisin levels do not exceed the recommended maximum limits in feed for horses and swine (5,000 and 10,000 μg/g, respectively)[1] and minimum safe level for fumonisin consumption for humans has not been set. There is need to study more on the levels and productions of fumonisins in sorghum.

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