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TOXIGENIC *FUSARIUM* SPECIES AND FUMONISIN B_1 AND B_2 ASSOCIATED WITH FRESHLY HARVESTED SORGHUM AND MAIZE GRAINS PRODUCED IN KARNATAKA, INDIA

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Abstract

Fumonisins are toxic secondary metabolites produced by the species of Fusarium. Accurate detection of these toxins in cereals is essential for a reliable evaluation of human exposure to these carcinogenic mycotoxins. In the present study, maize and sorghum samples were randomly collected from different regions of Karnataka (India) used for the analysis of fumonisin contamination. Preliminary mycological studies of samples confirmed the occurrence of mycotoxigenic Fusarium species such as F. verticillioides (18 strains), F. proliferatum (2 strains) and F. anthophilum (2 strains). The method used for the analysis of fumonisins was solvent extraction, C_{18} Sep-Pak reverse phase clean-up and ortho-phthalaldehyde plus 2-mercaptoethanol derivatization followed by high-performance liquid chromatography with fluorescence detector. The FB_1 and FB_2 concentration in maize patties inoculated with three different Fusarium species ranged between 0.065 to 121.42 µg/g. Fumonisin concentrations in 13 of 15 natural maize samples were found to be 0.003-23.43 µg/g. similarly FB_1 and FB_2 concentrations in 07 of 13 natural sorghum samples were found to be 0.001 to 17.09 µg/g. The average yield of FB_1 produced was much higher concentration to that of FB_2 in all the samples analyzed. Further, FB_2 was always found in association with FB_1 in all the samples analyzed by HPLC. This is first report on the natural occurrence of fumonisins in cereals produced in Karnataka (India).

Keywords: Fumonisin, *Fusarium verticillioides*, *Fusarium proliferatum*, maize, mycotoxin, mycotoxigenic fungi, sorghum.

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1. INTRODUCTION

Fumonisins are a family of mycotoxins produced primarily by *F. verticillioides*, *F. proliferatum* and other *Fusarium* species, which are common contaminants of maize, sorghum and other related grains throughout the world (Da Silva et al., 2004; Morales-Rodriguez et al., 2007). Chemically they are long chain polyhydroxyl alkylamines containing two propane tricarboxylic acid moieties that are esterified to hydroxyl groups on adjacent carbon atoms. There are more than 10 types of fumonisins among which fumonisin B_1 (FB_1) and fumonisin B_2 (FB_2) is the most important representative (Lerda et al., 2005; Gelderblom et al., 2007). The FAO estimated that each year, 25% to 50% of the world's food crops are contaminated by mycotoxins (Fandohan et al., 2003). Contamination of food commodities by fumonisins has become a serious food safety problem throughout the world (Ghali et al., 2009). Fumonisins are often found as natural contaminants of maize, sorghum and other grains intended for animal/poultry feeds and maize or sorghum-based foods destined for human consumption. Fumonisin can occur in maize kernels with little or no symptoms (Desjardins and Plattner, 2000), although concentrations of the same have varied greatly with geographic location and climatic conditions (Ross et al., 1991). Few mycotoxicological studies from India have reported contamination of cereals and cereal based products with fumonisins (Chhatterjee

and Mukherjee, 1994; Shetty and Bhat, 1997; Jindal et al., 1999). Karnataka, Andhra Pradesh, Bihar, Punjab, Uttar Pradesh and Madhya Pradesh states are major producers of maize and sorghum in India (Joshi et al., 2005). A major share of maize and sorghum produced in India comes from Karnataka and Andhra Pradesh states. Karnataka is the leading producer of maize in India and produces around 15% of India's total produce.

Despite maize and sorghum being staple food for majority of population that fall below poverty line in Karnataka, India, no studies have been conducted to understand the magnitude of the "fumonisins" problem. Data on the occurrence and level of fumonisins in cereal grains are needed for use in fumonisin exposure assessments that will provide information on the extent of fumonisin intake among people of Karnataka and facilitate decision-making for appropriate preventive actions. In view of this, maize and sorghum grains intended for human and animal consumption in Karnataka was collected from main crop production areas. The production of fumonisins by *Fusarium* species isolated as internal mycoflora from these cereals was examined. Also, the extent of fumonisin contamination in natural samples was investigated.

2. MATERIALS AND METHODS

Study area and Sample collection

Karnataka is one of the four southern states of India and is the 8th largest Indian state by area and 9th largest by population. It covers an area of about 1,91,791 km and forms 5.83% of the total area of the country (Anonymous, 2007). The state has three principal regions such as Northern Karnataka Plateau, Central Karnataka Plateau and Southern Karnataka Plateau. The Deccan Plateau, comprising the main inland region of the state, is drier and verging on the semi-arid, having an average temperature of 35-40 °C during the hottest and 26-28 °C during the coldest months. The humidity in the plains never exceeds 50%. The coastal strip between the Western Ghats and the Arabian Sea is lowland with moderate to high rainfall levels (Mallappa, 2004).

A total of fifteen (15) freshly harvested maize and thirteen (13) sorghum samples (1kg each) were collected from markets, local stores, agricultural co-operatives and farm fields (after the harvest) from three principal zones of Karnataka such as Northern Karnataka Plateau (Districts of Bagalkot, Belgaum, Bijapur, Dharwad, Gadag and Gulbarga), Central Karnataka Plateau (Bellary, Chitradurga, Davangere, Haveri, Koppala and Shimoga) and Southern Karnataka Plateau (Hassan, Mysore and Tumkur). Samples were brought to the laboratory in sterile plastic bags and kept at 4 °C. All the samples were subjected to mycological and fumonisin analysis.

Mycological analysis

Maize and sorghum kernels/grains (200 No. each) were surface sterilized with 2% sodium-hypochlorite solution for 3 min. and rinsed twice with distilled water. Samples were then plated on Malachite green agar 2.5 (MGA 2.5) plates at the rate of 10 grains per plate. The plates were incubated under alternating periods of 12h darkness and 12h of light at 25±2 °C for 7 days. The incubated plates were visualized under the stereo binocular microscope for the presence of diverse *Fusarium* species after 7 days of incubation. These isolates were identified up to the species level based on the micro-morphological features using fungal keys and manuals (Leslie and Summerell, 2006) and confirmed with molecular markers (Sreenivasa et al., 2008).

Maize patty cultures

Maize patties were prepared in 18-cm diameter Pyrex petri-dishes by autoclaving 30 g maize kernels in 30ml distilled water for 1 h at 121 °C on two consecutive days (Alberts et al., 1993) followed by the inoculation with 1ml spore suspension (4 day old culture prepared in distilled water) of each isolate. Patty cultures were incubated at 28 ± 2 °C for 4 weeks in darkness, oven-dried overnight at 60 °C. Dry patty cultures were harvested and ground in a mixer grinder (Kenstar Classique, MG-9605A) to fine meal. The ground meal was packed,

labeled and stored at -20 °C until the conduction of fumonisin analysis.

Extraction of fumonisins

Fumonisins were extracted from samples (maize/sorghum/patty cultures) by the method described by Rice et al., (1995). Samples (10 g) were extracted with 50 ml of acetonitrile/water (50:50, v/v) for 30 min using laboratory shaker (Heavy rotary Shaker, Kemi, India). The extract was filtered through Whatman No. 4 filter paper. An aliquot (2 ml) of extract was added to 6 ml of 1% Potassium chloride (KCl) and mixture was passed through Sep-Pak C_{18} cartridge (Waters, Division Millipore Carp., Milford, MA) at a flow rate of 1 ml/min, pretreated with 2 ml acetonitrile (ACN) and 5 ml 1% KCl. Fumonisins were eluted with 2 ml of ACN:H_2O (70:30, v/v) after washing the column with 2 ml each of 1% KCl and ACN:H_2O (15:85, v/v). The final elutant was evaporated to dryness under a gentle stream of nitrogen and reconstituted in 100 µl of ACN:H_2O (50:50, v/v) for HPLC analysis.

Analysis of fumonisin

An aliquot (50 µl) of reconstituted residue was mixed with 50 µl borate buffer (450 ml, 100 mM boric acid and 50 ml sodium borate pH adjusted between 8.0-8.5 made up to 1 L with milli-Q water), 50 µl OPA solution (20 mg O-pthalaldehyde dissolved in 10 ml ACN with 20 µl β-mercaptoethanol) and 50 µl water in a micro centrifuge tube covered with aluminum foil. The tube was shaken briefly and allowed to react for 10 min at room temperature. An aliquot of (20 µl) derivatized solution was injected into HPLC system. The analysis was carried out with Shimadzu LC 10AVP- HPLC system equipped with a fluorescence detector and Phenominix C_{18}, column (250 × 4.60 mm, 5 µ). Excitation and emission wavelengths were set at 335 nm and 440 nm respectively. Isocratic elution program was employed using the mobile phase containing MeOH: 0.1M KH_2PO_4 (75:25, v/v) pH was adjusted to 3.3 with phosphoric acid. The flow rate was 1.0 ml/min. Individual fumonisin homologues were calculated based on the calibration curve of standards of FB_1 and FB_2. The determinations were carried out in duplicate.

Working stocks of 0.1, 0.2, 0.4, 0.8, 2, 4 and 8 µg/ml of FB_1 and FB_2 standard solutions were prepared. An aliquot of 50 µl of each standard solution was derivatized and 20 µl was injected to HPLC system. Calibration curve was constructed for both FB_1 and FB_2 by plotting the peak areas against the concentrations from 0.5 ng (0.025µg/ml) to 40 ng (2µg/ml).
The recovery rate of toxins from ground samples was analyzed after spiking the maize and sorghum meal (10 g) with FB_1 (5, 10, 15 µg) and FB_2 (10, 15, 25 µg).

3. RESULTS

The mycological study revealed the occurrence of three different mycotoxigenic species of *Fusarium* on maize and sorghum collected from different regions of Karnataka. *Fusarium verticillioides*, *F. proliferatum* and *F. anthophilum* were the species identified among the 22 isolates. The dominant species of *Fusarium* recorded on maize and sorghum samples was *F. verticillioide* (18) *followed by F. proliferatum* (2) *and F. anthophilum* (2) isolates.
A representative example of typical chromatogram of FB_1 and FB_2 standards is depicted in fig. 1. The calibration curve was obtained using the linear least squares regression procedure for the peak area plotted versus the concentration. The linearity for FB_1 and FB_2, in the working standard solutions at four determinations of seven concentration levels, between 0.025 and 2.0 µg per ml which corresponds to 0.5 ng and 40 ng injected was good as shown by the fact that the correlation coefficients (r^2) were 0.996 and 0.997 for FB_1 and FB_2 respectively (Fig. 2).
Maize patty cultures inoculated with three different *Fusarium* species revealed that 17 of 18 (94.4%) *F. verticillioides* isolates produced fumonisins (FB_1+FB_2) at concentrations ranging from 0.07-121.45µg/g (Table1). The concentration of FB_1detected in maize patties inoculated with *F. verticillioides* was between 0.07 to 116.54 µg/g followed by *F.*

proliferatum (0.62 to 0.79 µg/g) and *F. anthophilum* (0.06 µg/g), while the concentration of FB_2 detected in maize patties inoculated with *F. verticillioides* ranged between 0.21- 5.33µg/g. An isolate of *F. verticillioides* from central Karnataka plateau produced highest amount of FB_1 (116.54 µg/g) and FB_2 (4.91µg/g). *F. verticillioides* (11 isolates) produced FB_1 as well as FB_2 and six isolates of *F. verticillioides* produced only FB_1. The concentration of FB_1 produced was much higher than that of FB_2 in all the *Fusarium* isolates tested.

Levels of FB_1 and FB_2 in maize samples collected from different geographical locations of Karnataka are shown in table 2. 12 out of 15 (80%) maize samples were naturally contaminated with fumonisins. The concentration of fumonisins (FB_1+FB_2) ranged from 0 - 23.43µg/g. The level of FB_1 detected was 0.02 to 23.43µg/g whereas levels of FB_2 detected were between 0.18 to 1.39µg/g. As expected, the levels of FB_1 detected in samples were much higher than FB_2. The chromatogram of maize sample with 22.83µg/g of FB_1 and 0.6 µg/g of FB_2 from southern Karnataka plateau. Among the 12 contaminated samples only 3 samples were detected with FB_1 as well as FB_2. Maize samples collected from the southern Karnataka plateau showed highest incidence of contamination (100%). Also the amount of fumonisin detected (upto 23.43µg/g) was higher in comparison to samples collected from to other geographical regions.

Table 2, summarizes the levels of FB_1 and FB_2 present in freshly harvested sorghum samples. The incidence of contamination (46.15%) was less in sorghum than maize samples. Among the 13 samples analyzed 06 samples were contaminated with fumonisins. The total concentration of (FB_1+ FB_2) was in the range of 0 - 17.09µg/g. The amount of FB_1 and FB_2 detected ranged from 0.05 to 14.51µg/g and 0.01- 2.58µg/g respectively. Among the 6 contaminated samples 4 samples were detected with FB_1 as well as FB_2. An amount of 14.51µg/g of FB_1 and 2.58 µg/g of FB_2 was detected in sorghum samples collected from central Karnataka plateau.

Table 1: Fumonisin B_1 and B_2 (µg/g) produced by the species of *Fusarium* isolated from maize and sorghum samples

Sl. No	Place of collection	Substrate	Name of *Fusarium* species	FB_1 (µg/g)	FB_2 (µg/g)	Total (µg/g)
1	Northern Karnataka Plateau	Maize	*F. verticillioides*	27.83	0.00	27.83
2		Maize	*F. anthophilum*	0.00	0.00	0.00
3		Sorghum	*F. verticillioides*	19.75	0.00	19.75
4		Sorghum	*F. verticillioides*	6.66	0.21	6.87
5		Maize	*F. verticillioides*	0.41	0.26	0.67
6		Maize	*F. verticillioides*	0.00	0.00	0.00
7	Central Karnataka Plateau	Maize	*F. verticillioides*	2.38	0.21	2.59
8		Maize	*F. verticillioides*	0.87	0.00	0.87
9		Maize	*F. verticillioides*	116.54	4.91	121.45
10		Maize	*F. proliferatum*	0.79	0.00	0.79
11		Sorghum	*F. verticillioides*	1.12	0.00	1.12
12		Sorghum	*F. verticillioides*	17.32	0.61	17.93
13		Sorghum	*F. verticillioides*	14.99	5.33	20.32
14		Maize	*F. proliferatum*	0.62	0.00	0.62
15		Maize	*F. verticillioides*	31.15	0.00	31.15
16	Southern Karnataka Plateau	Maize	*F. verticillioides*	35.30	0.38	35.68
17		Sorghum	*F. verticillioides*	3.57	0.10	3.67
18		Maize	*F. verticillioides*	87.82	1.82	89.64
19		Maize	*F. verticillioides*	25.22	0.89	26.11
20		Maize	*F. anthophilum*	0.06	0.00	0.06
21		Sorghum	*F. verticillioides*	12.39	0.21	12.60
22		Sorghum	*F. verticillioides*	0.07	0.00	0.07

Note: Values given are average of two HPLC readings

Table 2: occurrence of fumonisin B_1 and B_2 (µg/g) in freshly harvested maize and sorghum samples collected from the different regions of Karnataka

Sl No	Place of collection	Maize			Sorghum		
		FB_1 (µg/g)	FB_2 (µg/g)	Total (µg/g)	FB_1 (µg/g)	FB_2 (µg/g)	Total (µg/g)
1	Northern Karnataka Plateau	0.05	0.00	0.05	0.05	1.12	1.17
2		0.03	0.00	0.03	3.81	0.18	3.99
3		0.02	0.00	0.02	0.00	0.00	0.00
4		0.00	0.00	0.00	0.00	0.00	0.00
5		0.08	0.00	0.08	0.00	0.00	0.00
6	Central Karnataka Plateau	1.11	0.00	1.12	0.00	0.00	0.00
7		6.54	1.39	7.93	0.58	0.00	0.58
8		0.95	0.00	0.95	12.17	0.24	12.41
9		0.00	0.00	0.00	14.51	2.58	17.09
10		0.00	0.00	0.00	0.00	0.00	0.00
11	Southern Karnataka Plateau	1.00	0.00	1.00	0.00	0.00	0.00
12		1.58	0.00	1.58	0.00	0.00	0.00
13		2.40	0.00	2.40	0.46	0.00	0.46
14		6.86	0.18	7.04	NA	NA	NA
15		22.83	0.60	23.43	NA	NA	NA

Note: NA: Not analyzed, Values given are average of two HPLC readings

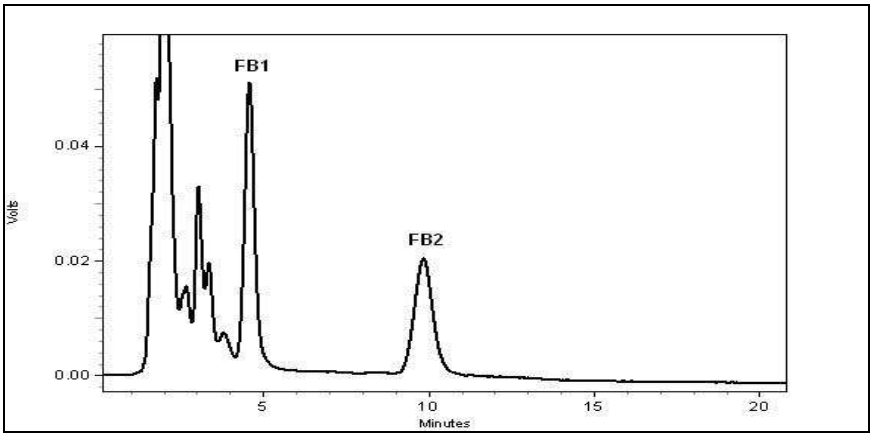

Fig. 1: HPLC Chromatogram of standard Fumonisins B_1 and B_2 4 ng (0.2µg/ml))

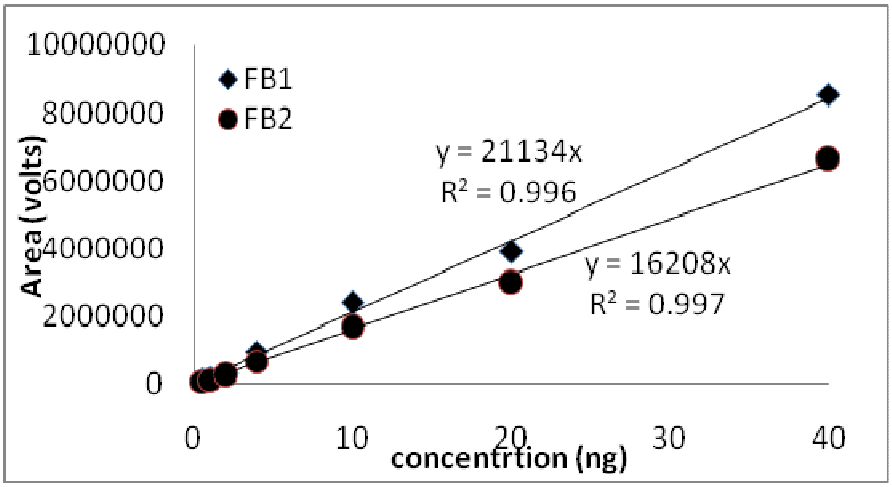

Fig. 2: Standard curve for Fumonisin B_1 and B_2 0.5 ng (0.025µg/ml) to 40ng (2µg/ml)

4. DISCUSSION

Fumonisins are a relatively novel class of *Fusarium* toxins, and have attracted the attention of both social experts and scientist's because of their high health risk potentials (Nelson et al., 1993). In some regions of Africa, Asia and other parts of the world, high levels of fumonisins have been constantly detected and recorded in maize. Isolates of *F. verticillioides* and *F. proliferatum* from maize, sorghum and other substrates from different geographic locations of North America, Africa, Asia and Australia have been reported (Desjardins et al., 2000; Ghiasian et al., 2005). The two species of *Fusarium* viz., *F. verticillioides* and *F. proliferatum* are the most prolific fumonisin producers (Shephard et al., 1996) and are known to produce fumonisins from 17,900µg/g to 31,000µg/g of FB_1 (Rheeder et al., 2002). The production of fumonisins by different strains of *F. proliferatum* varies widely. Some studies have shown very low or even no fumonisin production (Ross et al., 1992; Da silva et al., 2004), while other study has shown that, *F. verticillioides* can produce high levels of fumonisins (Nelson et al., 1992). Out of 22 *Fusarium* species analyzed in the present investigation, *F. verticillioides*, *F. proliferatum* and *F. anthophilum* were producers of fumonisins on maize patties. The concentration of fumonisin FB_1 (0.07 - 116.54 µg/g) produced under laboratory conditions is usually higher than fumonisins produced under natural conditions. The FB_2 concentrations in maize patties inoculated with *F. verticillioides* is 0.21- 5.33µg/g whereas it was not detected in patties inoculated with F. *proliferatum* and *F. anthophilum*. Similar results have been reported from the United States, Europe, Asia and Africa (Alberts et al., 1993; Visconti and Doko, 1994). In contrast to some other previous studies, low productions of FB_1 that of FB_2 have been reported (Ross et al., 1992; Musser et al., 1997).

The proposed analytical method was optimized in order to obtain good accuracy and precision. The average recovery rate of FB_1 was 82% and 79% whereas the average recovery of FB_2 was 94% and 90% for maize and sorghum meals respectively. Recovery study values are in accordance with the performance characteristics for FB_1 and FB_2 established by the European Commission (2005). Recoveries obtained for each analytical method fell within the acceptable range throughout the study (79–94%) and all results presented are corrected for recovery. The FB_1 and FB_2 concentrations in maize samples ranged from 0.02-22.83 and 0.18 2 1.39 µg/g and in sorghum samples the concentration of FB_1 and FB_2 ranged from 0.02 to 14.51µg/g and 0.18 to 2.58 µg/g respectively. The FB_1 concentration is much higher in samples than FB_2 in all but one sample analyzed. Further, FB_2 is always found in association with FB_1 in samples analyzed by HPLC. The levels of fumonisins in maize and sorghum in the present study agree with previous reports indicating that the incidence of fumonisins in sorghum is generally low in comparision to maize (Leslie and Marasas, 2001; Da silva et al., 2004). The natural occurrence of fumonisin B_1 in Indian sorghum with the contamination levels ranging from 0.01-5.0 mg/kg and 0.15-0.51 mg/kg has been reported by Shetty and Bhat (1997). Ghali et al., (2009) reported lower levels (20 to 260 µg/kg) of fumonisins in samples collected from India than the cereal samples collected from Europe and Africa. Although the data are from a limited survey, the high incidence of fumonisin contamination is consistent with what has been reported for maize in USA, South Africa and some European countries (Placinta et al., 1999; Pietri et al., 2009). Consumption of maize highly contaminated with fumonisins has been associated with increased risk of human esophageal cancer in some regions of South Africa (Rheeder et al., 1992) and China (Chu and Li, 1994). Studies on the prevalence of esophageal cancer in regions of South Africa, China, Italy and Iran, revealed an association between this disease and the consumption of maize contaminated by *Fusarium* species (Shephard et al., 2000; Wang et al., 2000). The International Agency for Research on Cancer (IARC) evaluated the

toxins derived from *F. verticillioides* as possible carcinogens to humans (class 2B) (Fandohan et al., 2003). There is a possibility that fumonisins are connected with infant neural tube defects in South Texas, USA (Hendricks, 1999). In addition to their adverse affect on the brain, liver, and lungs, fumonisins also affect the kidneys, pancreas, testis, thymus, gastrointestinal tract, and blood cells. There is also concern that consumption of fumonisins during early pregnancy could result in elevated risk of neural tube defect in the developing fetus. The biological effects of the fumonisins result from their ability to alter sphingolipid metabolism. They are potent inhibitors of key enzymes involved in the formation and turnover of sphingosine-based lipids, which, in turn, are involved in multiple structural and regulatory aspects of cell functioning (Wang et al., 1991).

5. CONCLUSIONS

The occurrence of fumonisins in food grains has been the subject of many investigations all over the world. In Karnataka (India), information on the incidence and levels of fumonisins in cereals is very limited, whereas reports on fumonisins in maize and sorghum are so far not been available. This study confirms that fumonisins are widespread contaminants of maize and sorghum intended for human consumption in Karnataka, India. It shows that populations in rural areas of Karnataka are at a risk of exposure to unacceptably high levels of fumonisins. Based on their consumption of maize and sorghum and the high fumonisin contamination determined in samples from the Central Karnataka region, people in that region are at a relatively higher risk of exposure to fumonisins. The amount of FB_1 produced was much higher to that of FB_2 in both the samples analyzed. Further, FB_2 was always found in association with FB_1 in all the samples analyzed. This is first report on the natural occurrence of fumonisins in cereals produced in Karnataka (India).

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