Detection of aflatoxin B1, heavy metals and minerals from corn silage and mineral mixture (Wanda) of cows and buffalos.

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Abstract

Corn (*Zea mays* L), belongs to the family Poaceae is most widely distributed crop throughout the world. Corn is third important cereal in Pakistan after wheat and rice. It is an important crop cultivated over an area of 1 million hectares with a production of about 3.3 million tons in Pakistan. Silage is a dairy cattle feed and is produced from corn crop on a wide scale in Pakistan. Mineral mixture is another type of feed which can be used as replacement of corn silage. The major issue which influences the corn silage and mineral mixture is naturally occurring aflatoxins, aflatoxin B_1 (AFB1) and aflatoxin B_2 (AFB2) with AFB1 the most important, toxic and carcinogenic. Aflatoxins (AFB1 and AFB2) are toxins produced by *Aspergillus flavis* and *Aspergillus parasiticus* infecting the agricultural crops. Total of 40 samples of corn silage and mineral mixture (20 samples each) collected from different dairy farms and shops of Gujrat city and its surroundings were analyzed for detection of Aflatoxin B_1, heavy metals and minerals or crystalline earth metals. Thin layer chromatography, flame photometer and atomic absorption spectrophotometer were used respectively for their detection. 5 samples (25%) of corn silage were found positive for aflatoxin B_1 and none of the sample was found positive for aflatoxin B_2. 9 samples (45%) of mineral mixture were found positive for aflatoxin B_1 and 2 samples (10%) were found positive for aflatoxin B_2. Contamination level of aflatoxins, heavy metals and minerals were higher in mineral mixture as compared to corn silage. It is concluded that inappropriate handling and storage conditions can pose high levels of aflatoxins in corn silage and mineral mixture which can lead to impair child development, immune system suppression and cancer. It is also concluded that high levels of contaminations in mineral mixture are due to its composition.

Keywords: Aflatoxins, Heavy metals, TLC, Flame photometer, Atomic absorption spectrophotometer, Silage.

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Introduction

Corn (*Zea mays* L.) is the most extensively harvested crop in Pakistan cultivated over an area of 1 million hectares with a production of about 3.3 million tons. Because of its nutritional value, it is widely used for human consumption and animal feeding. A large percentage of corn production is meant for animal feeding especially cattle feeding. Corn silage making involves grinding and storage of whole corn plant and consists of grains, high percentage of stoves and stalks representing a new significant feed source for beef and dairy cattle [1]. Air and infiltration of rain lead to poor quality of silage production. Nutritional value of the resulting silage will be low and cows will avoid that silage to eat (low intake of dry matter). So infiltration of rain or water should be reduced for quality production of corn silage [2]. Mineral mixture is another type of feed which is used as replacement of corn silage. The major issue which influences the corn silage and mineral mixture is naturally occurring Aflatoxins (AFB1 and AFB2) with AFB1 the most important, toxic and carcinogenic. Aflatoxins (AFB1 and AFB2) are toxins produced by *Aspergillus flavis* and *Aspergillus parasiticus* infecting the agricultural crops [3]. AFB1 and AFB2 were named according to their fluorescence (blue or green) which they give under UV light [4]. Mycotoxins are the secondary metabolites of fungi that can cause a variety of health effects on humans as well as animals [5].

For dairy cattle mycotoxins not only induce diseases in animals or production losses but this can lead to the presence of their metabolites in dairy products like yoghurt, milk, butter which will eventually effect human health. AFB1 present in cattle feed results in appearance of AFM1 in the milk and milk products [6]. Aflatoxins, especially AFB1 have been found chronic as well as acute [7]. Today aflatoxins have become one of the most significant global issues concerning contamination of feed, food and food products [8]. Maximum permissible levels of aflatoxins in dairy cattle feed set by the food and drug administration are 20 ppb. Aflatoxins can be detected by various techniques like thin layer chromatography, Enzyme-linked Immunosorbant Assay (ELISA) and the more accurate, highly sensitive and advance technique is HPLC [9]. The results obtained from TLC are almost similar to that of HPLC and more reliable than ELISA [10].

The metals which have the densities higher than 5 gmL^{-1} are known as heavy metals, e.g. Cu, Hg, Mn, Cd, Fe, Ni, Zn and Pb [11]. Many of these heavy metals are essential micronutrients, Fe, Mn, Zn and Fe, but these heavy metals may become toxic when they exceed in concentration which is required for the normal growth. Some heavy metals like Pb, Cd and Hg are very toxic even when the lower concentration of them is present. Zinc and Cu are essential trace minerals which are required for the biological processes, especially for the normal functions of the enzymes and they are important for the normal growth and positive reproduction [12]. High concentration of these heavy metals in corn silage and mineral mixture can also pose health effects to animals as well as to human beings. Atomic absorption spectrophotometer and flame photometer are used for their detection [13].

Experimental Section

Present study was conducted to detect and quantify aflatoxins, heavy metals (Zn, Cr, Cu, Pb) and minerals or crystalline earth metals (Na, Ca, K) in corn silage and mineral mixture collected from Gujrat city and its surroundings. The research was conducted at the department of Environmental Sciences, University of Veterinary and Animal Sciences, Lahore and Food and Biotechnology Research centre PCSIR, Lahore. For this purpose 40 samples of dairy cattle feed (corn silage and mineral mixture) were collected. Total of 20 samples of corn silage were collected out of which 3 were collected from suppliers and 17 from dairy farms of Gujrat. Total of 20 samples of mineral mixture were collected out of which 11 samples were collected from dairy farms and 9 samples were collected from shops of Gujrat city. All the samples were collected by simple random collection technique. All the analytical grade chemicals were purchased from Merck (Darmstadt, Germany). These chemicals were included acetone, chloroform, methanol, diethyl ether, distilled water, perchloric acid and nitric acid. 50 g of grinded and mixed sample was weighed and then poured it to 500 ml conical flask. 25 g of diatomaceous earth plus 25 ml of distilled water and 250 ml of chloroform were added in the conical flask and shaken for 2 minutes. Aluminum foil was fixed on flask mouth and shaken it for 30 minutes on wrist action shaker. After that prepared solution was filtered through the filter papers. Then 50 ml of filtered solution was collected in a beaker and evaporated on a steam bath. Spots of 2.5, 5, 10 and 15 µL were spotted on TLC plate (approximately 1.5 cm from the base). Standard spot of 5 µL was also placed on TLC plate at one side. Then TLC plate was placed in TLC tank contained almost 100 ml of anhydrous ether in it. Sample spot and standard spot moved upward with anhydrous ether. Plate was removed from TLC tank 1 when flow reached near the top of the plate and dried it for some time. Plate was redeveloped in the same direction in TLC tank 2 which contained acetone-chloroform in 1:9 (v/v). After completion of upward flow plate was removed from TLC tank 2 and dried. Then developed plate was observed for presence or absence of spots originated from test solution that authentically represented aflatoxin B_1. Only presence or absence of aflatoxins was observed in tested solution spots. When plates showed the presence of aflatoxin B_1 or aflatoxin B_2 then related samples were further diluted for quantitative analysis. Preliminary plate showed that new tested solution was required (fluorescence of aflatoxin B_1 in some feed samples was more intense than standard of 5 µL). Evaporated mixture was diluted with the calculated volume of chloroform. After dilution, spots of 2.5, 5.0, 10.0 and 15.0 µL were spotted successively on new TLC plate. Size of all spots was almost same. Along with sample spots, standard spots of 2.5, 5.0 and 10.0 µL (related to aflatoxin B_1 observed on previous plates) were also spotted on TLC plates. TLC tank 1 and TLC tank 2 steps were repeated with new spotted TLC plates. The plates were again observed under UV light for quantitative determination of Aflatoxins [14]. Same samples were digested by using Di-acid digestion method at the Department of Environmental Sciences, UVAS, Lahore. Di-acid mixture solution was made by using HNO_3 and per choloric acid $HClO_4$:HNO_3 (1:3). In this method 1 g of sample was taken and 10 ml of Di-acid mixture solution was added in it. Then after heated it at 150°C for 30 minutes, the temperature was raised to 250°C until the completion of the reaction or end point which was vine green or water clear. After the digestion samples were analyzed on atomic absorption spectrophotometer and multi-channel flame photometer Model from Biotech Uk for the heavy metals (Zn, Cr, Cu and Pb) and alkaline earth metals (K, Na and Ca) detection respectively. Relative standards were used for detection [15]. All the sample results were analyzed by performing independent sample t-test by using SPSS software.

Results and Discussion

Pakistan is an agricultural country and corn crop is cultivated under different ecological conditions. Whole corn plant is used for the production of corn silage in Pakistan. Rainy season, adverse temperature, traditional practices of harvesting, insufficient and improper storage facilities stimulate the fungal infection. In this study an attempt has been made to find out the occurrence and concentration of aflatoxin B_1 in corn silage and mineral mixture samples collected from Gujrat region and its surroundings. In 20 samples of corn silage collected from dairy farms and suppliers, 5 (25%) were positive for aflatoxin B_1. None of the sample of corn silage was positive for aflatoxin B_2. In 20 samples of mineral mixture collected from dairy farms and shops, 9 (45%) were positive for Aflatoxin B_1 and 2 (10%) were positive for aflatoxin B_2 as shown in Table 1. All the positive samples of both corn silage and mineral mixture were above the maximum permissible levels set by the FDA and AFB1 was present more frequently in mineral mixture samples. Present study also showed that Zn, Cr and Cu concentration was more in mineral mixture than in corn silage while concentration of Pb was more in corn silage than in mineral mixture. Also concentration of Na, Ca and k was more in mineral mixture than in corn silage as shown in Tables 2 and 3. Present study will be supportive for the investigation of aflatoxins, heavy metals (Zn, Cr, Cu, Pb) and crystalline earth metals (Na, Ca, K) in corn silage and mineral mixture samples. Corn silage and mineral mixture is widely used as cattle feed all over the world and occurrence of aflatoxins in this commodity is a major concern to human health because humans consume meat and milk from them. The present situation is worse about the levels of aflatoxins which are higher than the prescribed limit by the regulatory authorities. It was observed that TLC technique is good for the determination of aflatoxins in developing countries where the facilities of sensitive instruments are not accessible. Furthermore to quantify levels of aflatoxins by using sensitive instruments like HPLC, GC-MS and LC-MS is required for accurate detection of Aflatoxins (B_1, B_2) in corn silage and mineral mixture samples available to protect the cattle from exposure of aflatoxins.

Table 1. *Aflatoxin contamination in corn silage and mineral mixture.*

Product	Aflatoxins	Total No. of Samples	Positive Samples	No. of samples exceeding FDA action levels of (20 ppb)	Non-toxic samples	Contamination Detected (%)
Corn silage	B1	20	5	5	15	25
	B2		0	0	20	0
Mineral mixture (Wanda)	B1	20	9	9	11	45
	B2		2	2	18	10

Table 2. *Heavy metals and minerals contamination in corn silage.*

Samples	Na (ppm)	Ca (ppm)	K (ppm)	Zn (ppm)	Cr (ppm)	Cu (ppm)	Pb (ppm)
Silage 1	138.33	2410	3885	5.83	4	0.88	10.5
Silage 2	50	506.67	1076.66	5.33	4.5	0.5	8.33
Silage3	105	805	1335	0.66	4	0.83	11.83
Silage 4	150	3065	3985	3.16	4.5	0.33	12
Silage 5	98.33	1725	3208.33	3.33	3.33	1.83	4.16
Silage 6	126.66	1781.66	3715	8.66	3.16	4.33	15.16
Silage 7	158.33	2338.33	3646.66	0	2.83	0.5	11.33
Silage 8	160	1476.66	2185	1.66	5.16	1.66	4.66
Silage 9	126.66	1853.33	3383.33	9	5.33	0.66	8.66
Silage 10	111.66	1141.66	2890	0	6.16	1.16	12.5
Silage 11	191.66	1048.33	1906.66	0.83	6.66	1.5	14.5
Silage 12	161.66	2413.33	3823.33	0	4.5	0.93	15.5
Silage 13	240	1175	1533	2.66	7.5	0.8	10.83
Silage 14	168.33	1370	1566.66	7.33	9.66	1.66	5.16
Silage 15	153.33	1903.33	1315	9.5	8.33	1	11.33
Silage 16	156.66	1068.33	1985	0	8.5	0.83	20.33
Silage 17	161.66	1306.66	1950	6.5	5.33	1.33	12
Silage 18	253.33	1758.33	3356.66	1	8.16	0.33	10.83
Silage 19	121.66	1718.33	3693.33	0.83	7.5	0.33	19.83
Silage 20	121.66	1701.66	2848.33	0.5	8.16	0.66	11.5

Table 3. *Heavy metals and minerals contamination in mineral mixture (Mix Wanda).*

Samples	Na (ppm)	Ca (ppm)	K (ppm)	Zn (ppm)	Cr (ppm)	Cu (ppm)	Pb (ppm)
Mineral mixture 1	1330	5033.33	5571	21.16	4.16	4	3.8
Mineral mixture 2	4746.66	6088	9326.6	25	5.66	4.66	3
Mineral mixture 3	2163.33	2843.33	7120	9.66	5.33	1.56	1.8
Mineral mixture 4	2066.66	3356.66	7170	16.16	8	2.5	2.3
Mineral mixture 5	1863.33	3433.33	8786.66	15.66	7	3.16	8.5
Mineral mixture 6	3000	4321	5766	14.66	7.83	3	5.55
Mineral mixture 7	1226.66	2896	8223.33	31.54	5	6.5	1.89
Mineral mixture 8	3306.66	4333	9053.33	22.66	5.33	5.5	5.33
Mineral mixture 9	4863.33	3520	7123.33	43	6.5	2.16	5
Mineral mixture 10	4233.33	3946.66	8073.33	16.33	5.66	2.16	4
Mineral mixture 11	1366.66	5945	7960	18.66	5.83	4.16	4.83
Mineral mixture 12	5340	4783.33	8363.33	26.33	6	5.5	6.16
Mineral mixture 13	2236.66	4426.66	7136.66	26.5	7.83	1.2	2.66
Mineral mixture 14	3140	3630	4222	22	7.66	4.33	5.16
Mineral mixture 15	4450	3850	8963.33	28.5	8.66	5.83	1.66
Mineral mixture 16	2836.6	3743.33	9020	15.83	9.33	0.33	1.98
Mineral mixture 17	4926.66	3596.66	8933.33	21.5	7.33	3.83	8.56
Mineral mixture 18	1500	3503.33	9293.33	21.33	8.66	3.66	3.55
Mineral mixture 19	3040	5676.66	9556.66	13.66	7.16	1.83	1.16
Mineral mixture 20	2306.66	3303.33	7753.33	15.33	8.66	2	1.23

Conclusion

Aflatoxin B_1 is carcinogenic and has acute as well as chronic effects even at low levels so it is mandatory to evaluate its current concentrations in food or feed before use. There is a need to provide better production and storage facilities for good quality of corn silage and mineral mixture. Corn silage was less contaminated with aflatoxin B_1, heavy metals and minerals as compared to mineral mixture so corn silage may become a healthy choice for farmers if good production or stored conditions are provided.

References

1. Molina AM, Roa LB, Alzate SR, et al. In Silage as a livestock feed source Rev LasallInvestig. 2004;66.
2. Johnson LM, Harrison JH, Davidson D, et al. Corn silage management: Effects of maturity inoculation and mechanical processing on pack density and aerobic stability. J Dairy Sci. 2002;85 434.
3. National Toxicology Program Department of Health and Human Services Reported Carcinogens - Aflatoxins CAS. 2011.
4. Bennett JW, Klich M. Mycotoxins Clin Microbio Rev. 2003:497.
5. Aliabadi MA, Alikhani FE, Mohammadi M, et al. Biological control of aflatoxins Eur. J Exp Biol. 2013;3:162.
6. Boudra H, Barnouin J, Dragacci S, et al. Ochratoxin A in raw bulk milk from French dairy herds. J Dairy Sci. 2007;90:3197.
7. Ehsani A, Barani A, Nasiri A. Occurrence of aflatoxin B1 contamination in dairy cows feed in Iran. J Toxin Rev. 2016;35:54.
8. Achakzai AK. Occurrence and health hazard status of aflatoxin in human food and animal feed of wheat from Pakistan: A review paper Pure Appl Biol. 2015;4:611.
9. Manetta AC. In Aflatoxins: Their Measure and Analysis Aflatoxins – Detection Measurement and Control Dr Irinco Torres-Pacheco InTech 94:2011.
10. Jaimez J, Fente C, Vazquez B, et al. Application of the assay of aflatoxins by liquid chromatography with fluorescence detection in food analysis. J Chromatogr. 2000.
11. Zhang F, Li Y, Yang M, et al. Content of Heavy Metals in Animal Feeds and Manures from Farms of Different Scales in Northeast China Internat. J Envir Res Pub Health. 2012;9:2658.
12. Li Y, Mccrory D, Powell J, et al. A Survey of Selected Heavy metal Concentrations in Wisconsin Dairy Feeds. J Dairy Sci. 2005;88:2911.
13. Nicholson F, Chambers B, Williams R. Unwin Heavy metal content of livestock feeds and animal manures in England and Wales. Biosource tech. 1999;70:23.
14. Association of Official Analytical Chemist AOAC 19th Ed AOAC Official method. 2012.
15. Nawaz R, Rehman US. Analysis of heavy metals in red meat in district Peshawar Khyber Pakhtunkhwa. J Med Sci. 2015;23:166.

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