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Research Article

Occurrence of Aflatoxins G1, G2, B1 and B2in Chocolate Products Marketed in Karachi, Pakistan

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Abstract

Aflatoxins are toxic carcinogenic contaminants found in foodstuff as well as in feeds and are believed to be the primary health hazard. Determination of aflatoxins in food stuff and feeds is thus very important. Occurrence of Aflatoxins G1, G2, B1 and B2 in chocolate products purchased from various markets of Karachi, Pakistan, was investigated. Aflatoxins were estimated in thirty five dark chocolate, milk chocolate, and white chocolate samples by using HPLC analysis. A total of 80% chocolate samples were found to be contaminated by aflatoxins. Aflatoxin B1was detected in 28, B2 in 24 and G1in 4 out of 35 samples analyzed, whereas aflatoxin G2 was found less than the limits of detection in the entire chocolate samples. Level of aflatoxin B1was detected maximum among all mycotoxins as 2.98mg/kg for dark chocolate and aflatoxin G1 was detected at lowest among allaflatoxins as 0.22mg/kg in dark chocolate. Dark chocolate was found to contain the highest amount of aflatoxins whereas least amount was found in white chocolate. The high levels of aflatoxin in chocolate detected in the period of studies could be a serious risk to human health in the largest populated city of Pakistan, where limited resources are available for the prevention and controlling their levels in the food supply.

Keywords: Aflatoxin, food safety, HPLC, chocolate

Introduction

Aflatoxins are poisonous substances produced by two of the fungi, *Aspergillu sparasiticus* and *Apergillus flavus*; they are responsible to contaminate food crops. This contamination of aflatoxin has become a serious health threat to humans. These moulds generally contaminate foodstuff especially food crops under favourable conditions including high temperatures and high humidity particularly found in the equatorial and subtropical regions (Gourma and Bullerman, 1995). Toxic effects due to contamination of aflatoxinsuch asliver cancer and lowering in immune response in various animals and humans have already been reported earlier (Williams *et al.*, 2004, Jian *et al.*, 2005). Chocolates are now considered to be a necessary part of routine life and have become a tradition. This delicious dessert has been eaten as sweet course for over thousands of years worldwide and is favorite dessert of young generation (Serra Bonvehí, 2004, Brera *et al.*, 2011). Chocolate have very less amount of water to boost mycotoxin production, which might be taken place due to rapid multiplication of fungi (moulds) in the handling steps of the cocoa beans and other raw materials. Initially generated mycotoxininin raw material due to such mishandling in processing, unfavourable atmosphere and inappropriate storage conditions may give rise to the contamination in the final product (Kabak and Dobson, 2009). Detection of mycotoxins in cocoa beans in different regionshas already been reported in literature (Copetti *et al.*, 2010, Sánchez-Hervás *et al.*, 2008). Mycotoxins reported earlier were found as very stable compounds and cannot be terminated completely during further processing or even thermal treatment and contaminate chocolates (Copetti *et al.*, 2010, Ferraz *et al.*, 2010, Romani *et al.*, 2003)**.**

Keeping in view significant health hazards of aflatoxins and to ensure a sound and safe supply of food products, the aim and purpose of current study was to determine

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the level of aflatoxin in the chocolate samples marketed in Karachi, Pakistan. The expected results of this study will highlight the danger of such contamination and may cause to prevent accumulation of aflatoxin in the chocolate marketed in the city.

Material and methods

Samples Collection

Samples of three different categories of chocolates e.i. 20 samples of dark chocolate, 10 samples of milk chocolate and 05 samples of white chocolate were purchased from various super stores located at different areas of Karachi. The chocolate samples were grounded and were stored at −20^{o}C until analysis was carried out. The samples were extracted and were investigated in triplicate.

Chemicals and Reagents

Standards of Aflatoxins G1, G2, B1 and B2 (analytical grade) were stored at 4^{o}C prior to use. HPLC grade methanol and acetonitrile (99.9%) were used for analysis. ASC grade glacial acetic acid, potassium chloride, sodium chloride, dihydrogen phosphate and disodium hydrogen orthophosphate were purchased from Merck. Phosphate-buffered saline (PBS) was prepared according to procedure prescribed earlier (Sambrook, 1989). 8 g of NaCl was added to 0.2 g of KCl, 1.44 g of Na_2HPO_4 and 0.24 g of KH_2PO_4 in 1L ultrapure water and the pH was adjusted to 7.4 with HCl. Double distilled water was used for the preparation of solutions had a resistivity of >18 meq ohm-cm. All other reagents were reagent grade.

Aflatoxins Extraction

20g of sample was mashed along-with 2g NaCl and extraction was carried out in 8vol/2vol methanol:water solution. The mixture was mixed in a homogenizer for 30 minutes. The filtration was carried out by Whatman No. 1 filter paper and the filtrate was diluted to six times with the addition of already prepared phosphate buffered solution (pH 7.4). Immuno affinitycolumn with a flow rate of 2-3 mL/min was used to elute. Washing of the column was carried out with 30 mL distilled water, and elution of aflatoxins with 4 mL methanol. The Elute was then dried at 40^{o}C under N_2 atmosphere. The dry residue was re-dissolved in 1 mL 2:3 vol/vol mixture of methanol and water and then it was filtered through PVDF membrane having pore size 0.45 µm and was stored at -18^{o}C until HPLC analysis.

HPLC Analysis

Reverse-phase HPLC (model LC-10ADvp solvent delivery system; auto injection, Shimadzu, Japan) C18 Brownlee reverse phase column (220x4.6mm, particle size 5μm) with C18 guard column (Perkin Elmer) was used with a fluorescence detection set at 455 nm emission for aflatoxins G1 and G2 and 425 nm emission for aflatoxin B1 and B2. The mobile phase was water:acetonitrile:methanol (66:17:17, v/v/v) with 4M nitric acid and 119mg/LKBr. The oven temperature was maintained to 40^{o}C with a flow rate of 1mL/min and injection volume for standard and sample extracts was kept 30μL. Since aflatoxins are possible carcinogen, care has always been practiced to avoid exposure and 10% sodium hypochlorite was used for decontamination.

Retention Time

The retention time for Aflatoxins B_1 was obtained as 5.39 minutes, for B2 10.09 minutes, for G1 4.45minutes and G2 has retention time as 7.62 minutes, as shown in Figure 1.

Detection limit and calibration solutions

Limits of Detection LOD are the baseline to measure occurrence of aflatoxin in the chocolate. LOD of Aflatoxins for the chocolate samples was obtained and was estimated as three times signal-to-noise ratio. The calibration solution of aflatoxins B1, aflatoxins G1, aflatoxins B2 and aflatoxins G2 ranging from 0, 0.025, 0.05, 0.125, 0.25, 0.5, 1.25 ppb were prepared in 1 mL 2:3 vol/vol mixture of methanol and water and then it was filtered through PVDF membrane having pore size 0.45 µm. Seven point calibration curve of peak versus concentration mg/L was constructed for every standard solution. Calibration curve constructed for Aflatoxin B1 is shown as Figure-2.

Statistical Analysis

Standard deviation was estimated by using one way analysis of variance (ANOVA) according to AOAC guidelines. Clibration curves and linear regression curve showed r^2 values above 0.999 for each mycotoxin indicating good linearity.

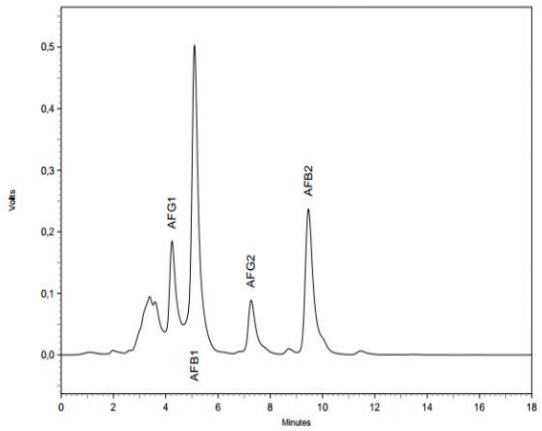

Figure 1: Retention times of standards of aflatoxins B1, B2, G1 and G2

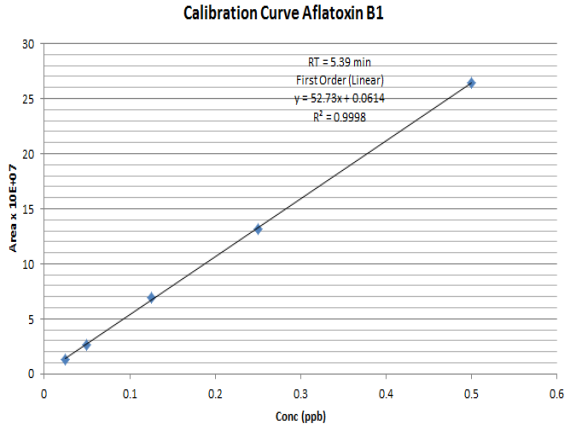

Figure 2: Calibration Curve of aflatoxins B1

Table 1: Aflatoxin B1, AflatoxinB2, AflatoxinG1 and Aflatoxin G2 in samples of chocolate

Products	Aflatoxins	Samples Analyzed	Samples Contaminated	Positive %	Mean ± SD	Concentration range (ppm)
Dark chocolate	B_1	20	19	95	2.48 ± 0.17	1.56 – 2.98
	B_2	20	17	85	1.63 ± 0.09	1.12 – 2.10
	G_1	20	2	10	0.34 ± 0.08	0.22 – 0.46
	G_2	20	0	00	<LOD	<LOD
Milk Chocolate	B_1	10	7	70	2.48 ± 0.17	1.32 – 2.62
	B_2	10	6	60	1.14 ± 0.07	0.98 – 1.64
	G_1	10	2	20	0.48 ± 0.02	0.42 – 0.54
	G_2	10	0	00	<LOD	<LOD
White Chocolate	B_1	05	2	40	0.82 ± 0.02	0.66 – 0.98
	B_2	05	1	20	0.48 ± 0.00	0.48
	G_1	05	0	00	<LOD	<LOD
	G_2	05	0	00	<LOD	<LOD

Results

Detection limit and Validation

HPLC method for the quantitative determination of aflatoxins B_1, B_2, G_1 and G_2 has been validated as described earlier (Muscarella *et al.*, 2009). The chromatographic separation of aflatoxins was accomplished using a C_{18} column eluted with an isocratic mobile phase consisting of water, methanol and acetonitrile. The sample preparation required a simple extraction of aflatoxins with CH_3OH/H_2O (80:20, v/v) and a purification step by immunoaffinity column cleanup. The total analysis time, including sample preparation and chromatographic separation, did not exceed 40 min with a run time of 10 min. The procedure for the determination of aflatoxins was extensively validated following Regulation (EC) No. 882/2004 of the European Parliament and of the Council. Detection limits for aflatoxins B1 was found to be 0.015μg/kg, aflatoxinsB2, 0.020μg/kg, aflatoxinsG1, 0.020μg/kg, aflatoxinsG2 0.020 μg/kg.

The analytical results of occurrence of aflatoxin are summarized in Table-1. Occurrence of aflatoxin was detected for the most of the samples evaluated. A total of 35 chocolate samples were analysed for contamination of aflatoxinout of which 28 (80%) samples were found positive. Aflatoxin $B_1$was detected in 28, B_2 in 24 and G_1in 4 out of 35 samples analysed. Aflatoxin G_2 was not detected in any of the chocolate samples. Maximum level of aflatoxin $B_1$was detected as 2.98mg/kg for dark chocolate and minimum level 0.66mg/kg in milk chocolate. Dark chocolate was found to contain maximum concentration of aflatoxin B_2 i.e.2.10mg/kg and minimum concentration 0.48mg/kg was found in white chocolate. Aflatoxin G_1 was detected as 0.54mg/kg in milk chocolate and minimum amount of $G_1$was found in dark chocolate as 0.22mg/kg.

Discussion

The incidence of aflatoxins in chocolate has already been reported. The results of current study are comparable to the study carried out in Brazil in which 125 samples of different variety of chocolate were evaluated for aflatoxins. The aflatoxins reported to be detected in 80% of all samples evaluated (Copetti *et al.*, 2014). Current results are found to be comparable to the result reported for a study carried out for samples in Lahore where 83% of the analysed samples found to contain aflatoxin. It is also pertinent to mention that the highest level of aflatoxins detected in the dark chocolate samples in current study is comparable to that study (Naz *et al.*, 2017). Current results are also comparable to the study carried out in Italy and Canada, where aflatoxins were observed in 80% of the sample analysed (Brera *et al.*, 2011, Copetti *et al.*, 2011, Turcotte *et al.*, 2013).

Current study reveals that chocolate marketed in the city of Karachi contains quite high levels of aflatoxins. Several factors of mishandling during post-harvest processing of cocoa beans initiated mycotoxins contamination have already been discussed in literature (Brera *et al.*, 2011, Copetti *et al.*, 2011, Copetti *et al.*, 2014)**.** It is not safe to make deduction on results from a limited sampling in this study. However the current study pointing out the incidence of presence of aflatoxins G1, G2, B1 and B2 in chocolate products purchased from various markets of Karachi, during the period of study may be regarded as hazardous to human health.

Conclusion

It can be concluded from the current study that the chocolate products marketed in the city of Karachi are heavily contaminated with aflatoxins B1, aflatoxins G1, aflatoxins B2and aflatoxins G2. The Study provides important evidence of contamination of chocolate with aflatoxin but at a very limited sampling and in the period of study. A detailed study is required to be carried out to reduce the hazard of aflatoxin through the chocolate mostly consumed in Karachi. The high levels of aflatoxin in chocolate could be a serious problem for the largest populated city of Karachi, where limited resources are available for the prevention and controlling their levels in the food supply. It is very much needed to establish maximum limits of aflatoxins for all food products marketed in the city which unfortunately is not available. The authorities should also take steps for strict implication of FDA and Codex Alimentarius standards for the imported chocolate marketed in the city of Karachi. It has also become necessary to carryout monitoring of aflatoxin contamination in the locally produced chocolate products aiming to reduce the toxic effects of such toxins to human health.

Conflicts of Interest

Authors have declared no conflicts of interest regarding publication of this paper. It is also confirmed that no funding has been acquired from any of the organization.

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