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Determination of Levels of Aflatoxin in Maize Produced from Shebelle Zone, Somali Regional State, Eastern Ethiopia Using UHPLC

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Abstract

Aflatoxin producing substances are extremely dangerous to humans and can result in cancer when taken in foods contaminated with aflatoxin. In this study, the levels of aflatoxins (B1, B2, G1, and G2) in samples of maize collected from several Shebelle Zone districts were quantified. It was examined using a fluorescence detector and UHPLC. The extraction solvents (80:20 v/v percent) were methanol and water. Standard solutions of aflatoxins between 0.5 and 7 g/kg (ppb) demonstrated strong linearity from the calibration curve with regression coefficient (R^2) values of >0.9989. The spiked sample average percentage recoveries ranged from 89.78 to 97.87 percent. The average total aflatoxin values in the maize samples from K1, K2, and K3 were 0.14, 356.19, and 174.05 g/kg, respectively. Except for K1, all of the investigated maize samples had aflatoxin levels that above the upper tolerance limits established by international agencies like WHO, and the EU.

Keywords: Aflatoxins • *Aspergillus* fungi • Mycotoxins • Maize grain • UHPLC • Shebelle zone

Introduction

Following rice and wheat, maize is the world's third most significant crop. It is a staple food in Sub-Saharan Africa's diet [1]. Maize is Ethiopia's most widely grown cereal, with 6 million tons produced by 9 million farmers on 2 million hectares of land in 2012. Over half of Ethiopian farmers grow maize, primarily for subsistence, with the farming household consuming 75% of all maize produced. Maize is currently the cheapest source of calories in Ethiopia, accounting for 20.6 percent of total calorie consumption. Maize is thus a key crop for the country's overall food security and economic development [2]. However, mycotoxigenic fungi such as *Aspergillus, Fusarium*, and *Penicillium* are capable of degrading the grain [3]. Aflatoxin B1 (AFB1), B2 (AFB2), G1 (AFG1), and G2 (AFG2) are among the 18 various forms of aflatoxin. Aflatoxin B1 is found in higher amounts in foods such as maize, peanuts and peanut products, cotton seed and its extractions, and to a lesser extent, chillies, peppers, and pistachio nuts all over the world than other naturally occurring forms of aflatoxin [4]. The Food and Agricultural Organization (FAO), estimates that between 25% and 50% of agricultural crops worldwide is contaminated by mycotoxins [5]. The estimated value of maize lost to aflatoxin is $225 million per year, out of the $932 million due all the mycotoxins in the United States [6]. In recent years, data on maize mycotoxins in Africa has begun to accumulate, including reports from Kenya, Nigeria, Benin [7-10]. There are several reports from Ethiopia, although they are restricted in their information on the presence of *Aspergillus* species and aflatoxins in pre-harvest and post-harvest maize. Fuffa, H., and Urga, K. did research in several sections of Ethiopia on the most commonly eaten agricultural commodities, whereas conducted research in three cities on mycotoxins and surface and internal fungi of maize (Dire Dawa, Adama and Ambo). In general, only a few aflatoxin in maize research have been carried out over the world. In the present study, the aflatoxin levels in maize samples collected from Shebelle Zone was determined by using UHPLC method. As a result, the goal of this study is to determine the degree of *Aspergillus* and Aflatoxin contamination in post-harvest maize products in eastern Somalia region [11].

Materials and Methods

Description of the study area

The study was conducted in shabelle zone, gode, berano (berecano in Somali language) and kelafo district Somali Regional state, south east Ethiopia with latitude and longitude of 5º57 'N and 43º27 'E and it average elevation in this woreda is 358 meters above sea levels [12]. Gode was the capital of the Somali region until 1995

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when jijiga become the capital for political reason. It has a tropical climate and remains mostly hot and humid throughout the year. Gode is bordered on the south by the shabelle river which separates it from adadle, on the northwest by Imiberi, on the north by danan, on the north east by the korahe zone, and on the southeast by kelafo. Gode located in the shabelle Zone, far about 560 km and 1172 km distance from the captal city of Somali region-Jigjiga and Ethiopian Addis Ababa respectively [13]. The societies in gode, and kelafo districts are supported by agro-pastoral settings. Three woreda was selected purposively to collect raw maize sample. The study was conducted on maize harvested 2021/2022 production year. Figure 1 shows map of shebelle zone (gode, berano and kelafo).

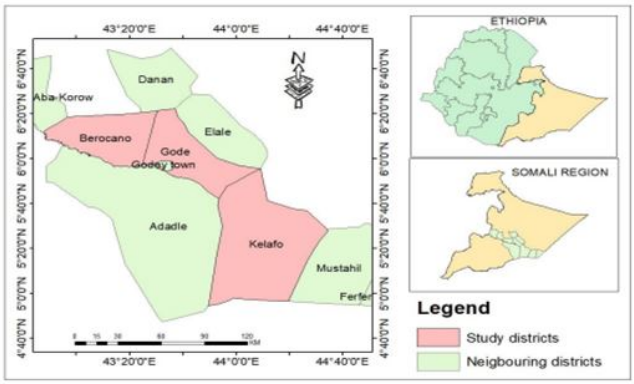

Figure 1. Sampling sites for Aflatoxin study in three district of shebelle zone, Somali Ethiopia.

Sample collection and the study area

A total of 30 maize grains were collected from farmers in the Shebelle zone woredas (namely gode (K1), Berano (K2) (berocano in Somali language) and kelafo (K3) district). In October 2021, these samples were collected at random from various maize producer farmers. The three samples were a combination of ten samples from the Gode district, ten sample from berano and ten samples from a maize producer in Kelafo. The entire experiment took place in Ethiopian Confomity Assessment Enterprises' aflatoxin analysis room, which is located near Megenagna in Addis Abeba, Ethiopia.

Instruments and apparatus

The apparatuses and instruments used for the experiment was mill grinding device (Kohinoor), amber glass bottle, centrifuge (sigma T:OII5982 III), analytical balance (Mettler Toledo), round bottom flasks volumetric flasks, micropipette, falcon tubes screwed type, beaker, syringes, syringe filters (0.2 and 0.45 µm), auto-sampler vials (4 mL), filtration apparatus, nitrogen evaporator (REACTI-VAP III#TS-18826) vortex, centrifuge, rotatory evaporator, homogenizer (polytron), immune-affinity column eclipsed plus C-18 column (4.6 × 250 mm), 5 µm and waters e2696 model UHPLC, Singapore.

Chemicals and reagents

Aflatoxin standard (B1, B2, G1 and G2), acetonitrile, methanol (HPLC grade and purity ≥ 99.9%), and acetone (HPLC grade and purity>99.8%) were used. All of chemicals used in this study was HPLC grade and supplied by Sigma Aldrich (St. Louis, MO, USA), used without any further purification. Deionized water (conductivity<0.06 µS/cm, HPLC-grade) were obtained from distilled water passed through a MilliQ water purification system (Millipore LTD, Bedford, MA, USA) and used in all experiments.

Sample preparation and extraction of aflatoxin

Sample preparation: The collected incremental maize samples were homogenized and a representative sample of 1 kg was taken and ground or milled to a desired particle size (1 mm) by grinding machene. To prevent aflatoxin cross-contamination, the grinder was cleaned with acetone before and after grinding. The ground maize was homogenized for 2 minutes using homogenizer and then, 25 g was taken and submitted for aflatoxin analysis. The rest of the ground maize sample was stored at -15°C.

Extraction of aflatoxin: The extraction solvents were methanol and de-ionized water. 15 mL of MeOH: H_2O (80:20) were added in each 25 g maize sample containing 250 mL beaker next, the three sample and spiked sample was mixed using polytron homogenizer for 6000 rpn for 10 min to facilitate the dissolution. Then centrifugation is followed, centrifugations were performed for 5 min at 50000 rpm and then quantitatively the supernatant transferred to 250 mL round bottom flask. Filter using fast fluted filiter paper and it was taken 15 mL of extract and it was added 30 ml PBS and mix well. Then it was taken 15 mL of dilute extract pass through immune-affinity column clean up. Then it was washed with 10 mL of deionized water pass air to dry and elute with 3 ml of methanol on 4 ml vial. The extractions were performed two times with the extraction solvent in order to enhance the extract of the analyte from the sample. The extracts were evaporated using nitrogen evaporator at (40°C, 772 mbar) and then reconstituted with 10 mL of mobile phase (60% H_2O: 25% ACN: and 15% MeOH followed by vortex 800 rpm for 30 sec. Finally, the solution was filtrated using 0.45 µm and 0.2 µm syringe filter paper consecutively and then the filtrate was transferred into an auto sampler vial for UHPLC analysis.

The chromatographic condition: Agilent technologies of liquid chromatography series coupled to a water e2696 model and florescent detector FLD (Excitation: 360 nm and Emission: 440 nm. Flow rate, 10 L/min, column temperature was 35°C. The chromatographic separation was performed on a reversed phase eclipsed plus C-18 (4.6 mm × 15 mm), 5 µm particle size, 4.6 × 250 nm) column, by injecting the sample extract. The column was eluted using a gradient flow (0.55 mL/min) of the mobile phases (60% H_2O, 25% ACN, 15% MeOH). The injection volume was maintained at 10 µl for both standard and sample solutions.

Preparation of the mobile phase: The mobile phase was prepared by mixing de-ionized water, acetonitrile, and methanol in v/v% of 60: 25: 15 respectively. Then the mixture sonicated for 30 sec, finally, the mixture was filtered using 0.45 µm filter paper. Similarly, equal volumes of mixture were prepared without ACN for standard solution preparations, which were used for calibration curve. The ACN in the mobile phase enhances ionization (gives clear chromatographic peaks).

Statistical analyses: Microsoft Office Excel 10 was used to compute descriptive statistics (such as mean and standard deviation) and create calibration graphs. Using a one-way analysis of variance (ANOVA) was performed to compare the differences between the samples. To create a graph for each maize in all samples (in mg/L), Microsoft excel,

Results and Discussion

Construction of calibration curve

The four major types of aflatoxins B1, B2, G1, and G2 were detected in Shebelle zone, Somali, Ethiopia, according to this study. The findings revealed that in the entire county, 20 out of 30 samples tested positive for aflatoxin at the market level the linearity of the calibration curves on the UHPLC system was investigated by injecting different concentrations of aflatoxin standards in mobile phase. Working solutions of standard aflatoxins in the range of 0.5-10 gμ/kg in a mobile phase of MeOH: ACN: H_2O were used to calibrate the system. The calibration curves for each standard aflatoxin were constructed by plotting the response or integrated peak-area of standard aflatoxin (y-axis) versus the concentration of standard aflatoxin in ppb (x-axis), and they demonstrated good linearity in the concentration ranges used, with coefficients of determination, R^2, were greater than 0.9989, which was considered as evidence of an acceptable fit of the data to the regression line [14]. Figure 2, indicating a good correlation of linearity across all concentrations used and a homostatic distribution of replicates.

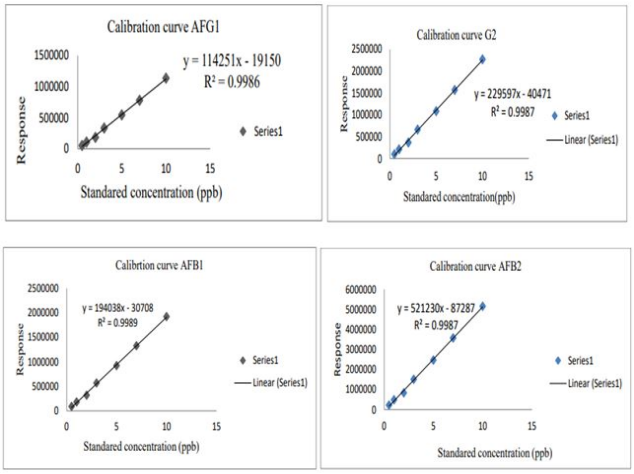

Figure 2. Calibration curves of aflatoxin (i) AFG1; (ii) AFG2; (iii) AFB1; and (iv) AFB2.

Limit of detection and quantification

The detection and quantification limits were calculated using equation 1 [15].

LOD=X+3SD and LOQ=X+10SD (1)

Where, X the mean concentration of the method blank and is the standard deviation of the method blank.

As shown in Table 1, limit of detection of the aflatoxins were 0.05, 002, 0.02 and 0.05 μg/kg for AFB1, AFB2, AFG1 and AFG2, respectively; and the limit of quantifications were 0.165, 0.066, 0.0.165 and 0.066 μg/kg for AFB1, AFB2, AFG1 and AFG2 respectively.

Aflatoxin	Linear equations	Slope	R^2	LOD	LOQ
AFB1	Y=194038x-30708	194038	0.9989	0.05	0.165
AFB2	Y=52130x-87287	52130	0.9987	0.02	0.066
AFG1	Y=114251x-19150	114251	0.9986	0.05	0.165
AFG2	Y=229597x-40471	229597	0.9987	0.02	0.065

Table 1. Parameters of linear regression measured for aflatoxins in UHPLC, LOD and LOQ.

The UHPLC chromatogram of the method blank, revealed a clear chromatogram with no specified visible chromatographic peaks, indicating that the chromatogram is free of any aflatoxin cross contamination from extraction solvents and equipment, indicating that the employed method is of high quality. The chromatogram also showed that the method was appropriate for determining aflatoxins in the sample.

As illustrated in Figure 3, all chromatograms of the aflatoxin standard mixture have clear and excellent chromatographic peaks for AFB1, AFB2, AFG1 and AFG2 with nearly the same retention time (min) at various standard aflatoxin concentrations (0.5, 1, 2, 5, 7, and 10 ppb). The retention time for all different types of aflatoxin standards was 11.772, 10.005, 9.424, and 8.167 min for AFB1,

AFB2, AFG1, and AFG2, respectively. Aflatoxin chromatographic peaks and retention times are used in sample analysis to identify aflatoxins.

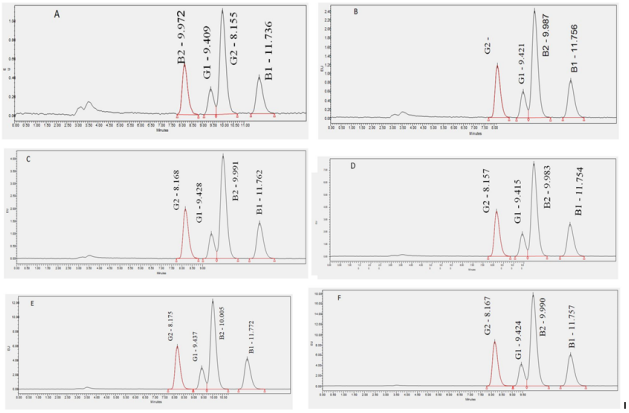

Figure 3. UHPLC chromatogram, counts versus retention time; for mixture of aflatoxin standards at 0.5 (A), 1 (B), 2 (C) 3 (D) 5 (E), and 7 µg/kg (F).

Chromatographic peak of aflatoxin contaminated maize

The purpose of this study was to use UHPLC to determine the level of aflatoxins contamination in maize grain samples. The concentration of each aflatoxin was determined using the peak area integration of a maize chromatogram. Aflatoxin B1, B2, G1, and G2 concentrations in Berano (k2) and kelafo (k3) maize samples were above the detection limit, but gode (k1) maize was below the detection limit. According to the UHPLC chromatogram, counts *vs.* retention time maize samples from Gode Berano and Kelafo districts, there are substantial chromatographic peaks observed in Gode maize samples within the range of retention time of standard aflatoxins, as shown in Figure 4. As there was no aflatoxin B1, B2, G1, or G2 identified above the limit of detection in maize samples, this means that. However, one large chromatographic peak was discovered that was beyond of the standard aflatoxin's acquisition time range. Because the retention period of this chromatographic peak differed significantly from that of aflatoxin, this chemical is not an aflatoxin (B1, B2, G1 and G).

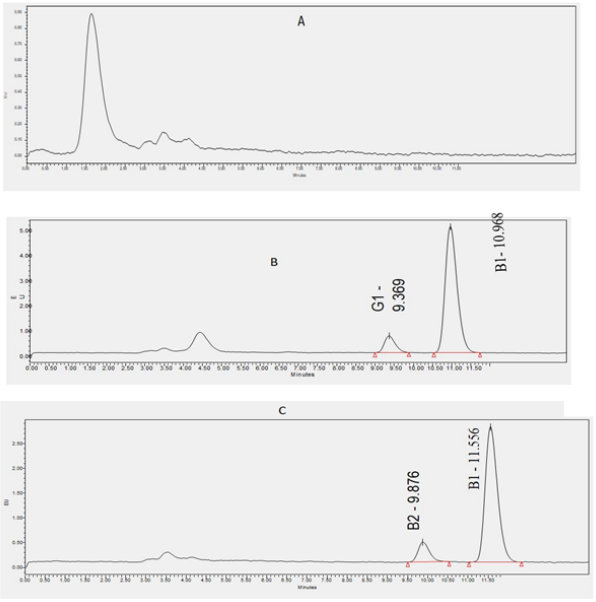

Figure 4. UHPLC chromatogram maize samples of gode (A) berano (B) and kelafo (C).

Aflatoxins can be identified qualitatively in samples by comparing their chromatogram and retention time to the standard aflatoxins. The UHPLC chromatograms for K1 samples were nearly identical, as shown in Figure 4. When compared to standard aflatoxin chromatograms with suspected ranges of aflatoxins retention time, there is no large chromatographic peak observed in sample chromatograms, but for k2 and k3 there are peaks indicating the presence of aflatoxin.

Recovery

The efficiency with which the analyte is separated from the sample is referred to as recovery. It is measured over values that cover the analytical range of the procedure and is expressed as a percentage of analyte determined experimentally after fortification of sample material at a known concentration. Both wheat samples were spiked inside 500 mL (0.5 mL) of 2 g/kg of aflatoxin standard to assess the% recovery of the procedure, and then the extraction was carried out. After that, the aflatoxins were extracted using the same process as in non-spiked samples, and the analysis was performed. The recovery % can be computed as follows.

% Recovery=(Concentration in spiked sample(S)-concentration(U))/(Amount spiked(C))*100

Where, S is the concentration of aflatoxin in spiked sample in µg/kg, U is the concentration of aflatoxin in non-spiked sample and C is the concentration standard added (Table 2).

Aflatoxin	Aflatoxins added (µg/kg)	Result found (µg/kg)	% Recovery	LOD	LOQ
AFB1	2	1.882602	94.13	0.05	0.165
AFB2	2	1.906379	97.87	0.02	0.165
AFG1	2	1.795659	89.78	0.05	0.066
AFG2	2	1.906373	89.4	0.02	0.066

Table 2. Recovery of the method performed by spiking 0.5 mL of 2 µg/kg aflatoxins.

The average recovery the spiked samples were ranging from 89.4%-97.87%. The recoveries obtained from this study were within the range between 70% and 125%, which were acceptable according to Association Officials of Analytical Chemistry (AOAC) international guidelines for method validation.

Levels of Aflatoxin in maize samples compared with international standard

When comparing the findings of this study to those of other similar studies on maize samples conducted in other countries, practically all investigations on maize samples show that the maize samples were infected with aflatoxins. Table 3 shows aflatoxin concentrations (AFB1, AFB2, AFG1, and AFG2) in the maize samples tested.

Sample	Aflatoxins	Concentration (µg/kg)
Gode (K1)	AFB1	ND
	AFB2	ND
	AFG1	ND
	AFG2	ND
	Total AF	<0.14
Berano (K2)	AFB1	288.93
	AFB2	<0.02
	AFG1	67.22
	AFG2	<0.02
	Total AF	356.19
Kelafo (K2)	AFB1	158.52
	AFB2	15.46
	AFG1	<0.05
	AFG2	<0.02
	Total AF	174.05

AF: Aflatoxins ND: Not Detected

Table 3. Concentrations (µg/kg) of aflatoxin in the collected maize samples.

As can be seen Table 3 average aflatoxin concentrations of the studied samples were ranging from<0.02-288.93 µg/kg (for AFB1); <0.02-15.46 µg/kg (for AFB2); and <0.05-67.22 µg/kg (for AFG1). AFG2 was detected in maize samples from K2 and K3, at relatively low concentration level, *i.e.,* <0.02 µg/kg. Among the studied aflatoxins, AFB1 was detected in K1 and K3 maize samples. The K1 sample was not completely detected, indicating that it is safe for society.

The average concentrations of each aflatoxins in maize samples collected from K2 was 288.93 µg/kg (for AFB1), 67.22 (µg/kg (AFG1) <0.02 µg/kg (AFB2) and 0.02 µg/kg (AFG2), but no AFB1 and AFG2 was below the UHPLC instrumental delectation limit. Similarly, the average concentrations of aflatoxin detected in the sample collected from K3 were 158.52 µg/kg (AFB1), 15.46 µg/kg (for AFB2) but no AFG1 and AFG2 was not detected in the sample maize. Similarly, AFB1 contamination in maize in south Ethiopia was 22.72 µg/kg [16].

From maize samples of K2 only AFB1 and AFG1 were detected at relatively higher concentration levels, *i.e.,* 288.93 µg/kg and 67.2-2 µg/kg, as well as K3 for AFB1 and AFB2 158.52 µg/kg and 15.46 µg/kg, respectively. The studied samples were showed that the total aflatoxin levels ranging from (<0.14-356.19 µg/kg) and specifically, <0.14, 356.19 and 174.05 µg/kg for maize sample of K1, K2, and K3, respectively. Similar study was reported in Ethiopia Eastern Ethiopia, aflatoxin levels ranging from 5-250 µg/kg were detected in groundnut samples another study also showed that 4.1 µg/kg of aflatoxin was detected from maize in Ethiopia.

The use of food and feed with high levels of aflatoxin has been identified as posing potential health concerns to both animals and humans. As a result, national and international regulatory authorities have established MRLs for individual and total aflatoxins in human food and feed (FDA, 2011-EU, 2010). The MRL for total aflatoxin in all cereals has been set at 20 g/kg by the United States Food and Drug Administration (USFDA) and the World Health Organization (WHO). Similarly, the EU set the MRL for all cereals at 2 µg/kg AFB1 and 4 µg/kg total aflatoxin. Based on this guideline, the concentrations of individual aflatoxin and total aflatoxins observed in the present study are far above the MRL set by USFDA/WHO and EU, indicating the safeness of the maize of the area in terms of the studied aflatoxins. The detected concentrations of the studied aflatoxins were also lower than the aflatoxin levels reported in cereals (barley, wheat, maize, millet, sorghum and teff) collected from different local markets of Ethiopia.

Conclusion

Aflatoxins are poisons produced by *Aspergillus* fungi that can contaminate a wide range of foods and crops. Aflatoxin poisoning can affect both humans and animals if they eat contaminated food. Maize samples were acquired from three wereda in Shebelle zone farmers for this study. Clear chromatograms for method blank and acceptable chromatographic peaks for standard aflatoxins were observed using the applicable method. As a result of these chromatograms, the approach was found to be suitable for determining aflatoxins in the sample. An excellent coefficient of regression (R^2) larger than 0.9989 was found from the examined working standard solution, which is considered evidence for a linear relationship between the concentration and its response. The recovery rates ranged from 89.78 to 97.87 percent, which was satisfactory according to the AOAC International method validation requirements. The LOD and LOQ were ranged between 0.02 and 0.05, and 0.066 and 0.165 µg/kg, respectively. The total aflatoxin contamination levels in samples obtained from K1, K2, and K3 were 0.14 g/kg, 356.19 g/kg, and 174.05 g/kg, respectively, according to the findings. The results obtained from Berano (K2) and Kelafo (K3) were far in excess of the maximum permissible limits set by the US Food and Drug Administration (FDA) and the World Health Organization (WHO) for total aflatoxin (20 g/kg) and the European Union (EU) for aflatoxin B1 (2 g/kg) and total aflatoxin (4 g/kg). As a result, the K2 and K3 samples were found to be unsafe for human and animal food, whereas the K1 sample was found to be safe.

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Conflicts of Interest

All reported work in this manuscript is original, and all authors have read and approved this version of the manuscript. Neither the entire paper nor any part of its content has been published, accepted, or submitted elsewhere. None of the authors declare any conflict of interest. All prevailing local, national and international regulations and conventions, and normal scientific ethical practices, have been respected, and consent is given for publication in food chemistry journal.

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