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Research Article
Analysis to Ascertain the Determination for Aflatoxin Contamination of Milk and Feeds from Gurage Zone, Ethiopia

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Abstract

Background and Objective: Aflatoxins are secondary metabolites which are naturally occurring contaminants of food and feeds. This toxigenic reduces animal productivity and highly carcinogenic. The level of contamination varies among different locations of Ethiopia. Hence, determination of the level of contamination at Gurage zone will pave the way to mitigate the impact of aflatoxin. The present study was initiated to examine the prevalence and quantify the amount of Aflatoxin M1 (AFM1) in raw cow's milk and Aflatoxin B1 (AFB1) in dairy feed samples. **Materials and Methods:** About ten milk and feed samples from dairy farmers were collected. Analysis for AFM1 and AFB1 were conducted by using High Performance Liquid Chromatography (HPLC). Statistical analysis done by using mean value and standard deviation. **Results:** It showed that the presence of AFM1 in milk samples and contamination level ranged between 0.02 and 0.31 μg L^{-1}. Overall, out of total 10 feed samples collected, only four (36%) contained total AFB1, AFB1, AFG1 and AFG2 at a level of undetectable. Furthermore, 4 (68%) milk samples exceeded 0.05 μg L^{-1}. About 60% of feed samples was contaminated with total AFB1, AFB1, AFG1 and AFG2 ranging between 4.22 and 10.54 μg kg^{-1}. This increase in aflatoxin caused by dairy farmers used mixed feed daily, which commonly included the mixture of wheat bran and Noug (*Guizotia abyssinica*) cake and cotton seed cake. **Conclusion:** Therefore, this suggest that risk mitigation should focus on Noug (*Guizotia abyssinica*) and cotton seed cake to effectively reduce aflatoxin contamination. Risk assessment of aflatoxins in large number of samples of milk and feed; noug will be the future line of study.

Key words: Aflatoxin B, aflatoxin G, aflatoxin M, HPLC, *Guizotia abyssinica*

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Aflatoxins are secondary metabolites produced by certain species of *Aspergillus*, specifically *Aspergillus flavus* and parasiticus fungi, which are naturally occurring contaminants of food and feeds[1]. Aflatoxin M1 (AFM1) is the principal hydroxylated AFB1 metabolite present in milk of cows feed with a diet contaminated with AFB1 and excreted within 12 h of administration of contaminated feeds[2]. AFM1 in milk has been shown to decline as contaminated feed is withdrawn, with no traces of aflatoxin in milk being detected after 3-4 days of withdrawal[3]. Fresh milk is regularly checked for aflatoxin M1; concentrations of M1 above 0.05 μg kg^{-1}, considered as undesirable and such milk cannot be used for products that go into the human food chain. Most frequently, aflatoxins are found in maize and oil seeds and of course in their by-products.

Previously, aflatoxin contamination in staple cereals like red chili pepper and ground peas is reported in Ethiopia[4,5]. Significant association between impaired child growth and aflatoxin exposure was reported from several countries in Sub-Saharan Africa including Benin[6] and Kenya[7]. The dairy sector in Ethiopia is commercial and uses specialized inputs such as improved genotypes of cattle, concentrate feeding, early weaning and production of improved forages[8]. Hence, the last Commission Regulation of the European Union (EU) No 165/2010 considered stringent parameters for aflatoxin regulation. The regulation forwarded maximum level of 8 μg kg^{-1} of AFB1 has been established in food subjected to sorting or physical treatment before human consumption and the maximum level of 0.05 μg L^{-1} has been set for AFM1 in milk.

The Food and Drug Administration in the USA (USFDA) sets action level for AFM1 in milk and total aflatoxin in animal feed to be 0.5 mg L^{-1} and 20 mg kg^{-1}, respectively[9]. Previously, high level of Aflatoxin M1 and aflatoxin B1 within milk and feed samples around Addis Ababa was reported[10]. The level of contamination of AFB1 and AFM1 is vary among different location of Ethiopia. Aflatoxins contamination is regulated in more than 80 countries, their legislation is harmonized at the international level[11].Therefore, this study tries to assess the contamination of aflatoxin in milk and feed on the other part of the country at Gurage zone. This study is an essential step towards exploration of the extent of aflatoxins contamination of milk. The toxigenic fungi cause yield loss, reduces animal productivity and hazardous from a human health perspective. This study will shade light towards designing mitigating mechanisms of aflatoxin contamination.

MATERIALS AND METHODS

Experimental samples were collected from Gurage Zone, Ethiopia from February-June, 2016. The data set includes raw milk sample collected at each dairy enterprise and household along with animal feeds sample for aflatoxin analysis. Five urban centers of Gurage zone (Butajira, Agena, Emdiber, Arekit and Wolkite) were purposively selected for this study based on Gurage Zone Agriculture Office report on amount of milk production. Therefore, two dairy enterprises were selected randomly from each town to collect the sample. Two raw milk sample of about 500 mL and two feed samples of about 500 g collected respectively from each town. Milk and animal feed samples analyzed for aflatoxin level at Bless Agri Food Laboratory Services PLC, (ISO 17025-2005 Accredited) in Addis Ababa, Ethiopia. Determination of aflatoxin was conducted using a very competent method of Higher Performance Liquid Chromatography (HPLC) techniques.

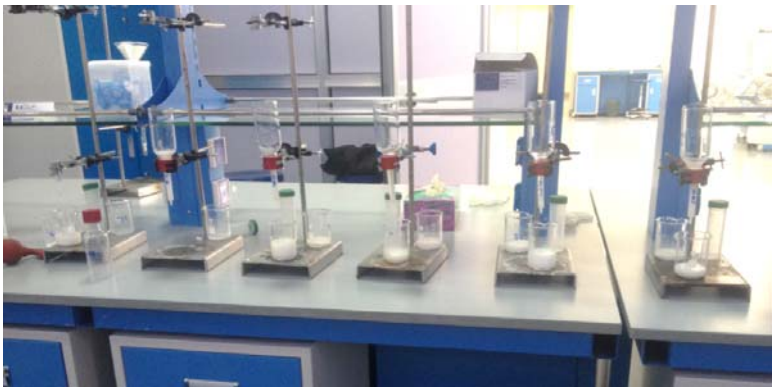
Determination of aflatoxin M1

Extraction procedure of milk samples: From each sample 100 mL of milk was taken in conical flask, incubated at 37°C in water bath, then centrifuged at 4000 ×g to separate fat layer, then lower phase was filtered and used for quantitative analysis. The prepared test portion of 50 mL was transferred into funnel attached with immunoaffinity column and passed at slow steady flow as shown on Appendix Fig. 1. The washing column was done with 10 mL distilled water and then it is blown to dryness and afterwards aflatoxin M1 was eluted with 3 mL pure acetonitrile by allowing it to be in contact with the column at least 60 sec.

Liquid chromatography determination with fluorescence detection for raw milk: The eluate was evaporated to dryness using gentle stream of nitrogen and at the time of LC (liquid chromatography) determination it is diluted with the mobile phase. The HPLC system of Agilent 1100 series (Agilent, USA), equipped with an auto sampler LAS G1313A and a fluorescence detector FLDG1321A was used for aflatoxin M1 determination as shown on Appendix Fig. 2. The retention time for aflatoxin M1 determination was used in the range of 8-10 min. The calibration report obtained is indicated in the following Fig.1.

Equation for the amount of aflatoxin are made according to the following Eq:

$$Wm = Wa \times \left(\frac{Vf}{Vi}\right) X \left[\frac{1}{Vs}\right]$$



Appendix Fig. 1: Extraction procedure of milk samples

Appendix Fig. 2: Liquid chromatography determination with fluorescence detection with HPLC

Where:

Wm = Amount of aflatoxin M1 in the test sample in μg L^{-1}

Wa = Amount of aflatoxin M1corresponding to area of aflatoxin M1 peak of the test extract (ng)

Vf = Final volume of re-dissolved eluate (μL)

Vi = Volume of injected eluate (μL)

Vs = Volume of test portion (milk) passing through the column (mL)

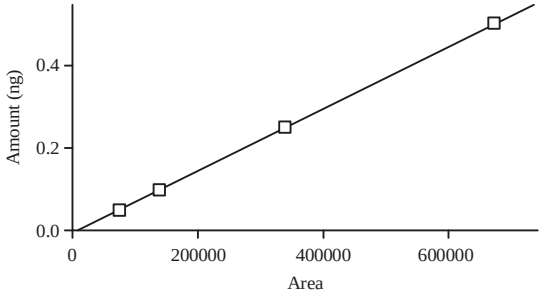


Fig. 1: Calibration report for aflatoxin M1 determination

Determination of aflatoxin B1, B2, G1 and G2

Extraction and clean-up procedure: A test portion of 20 g feed sample and extraction solution of 2 g NaCl with 80 mL methanol and 20 mL deionized water was used. Finally, 50 mL of hexane was added on prepared sample. Pressure pumper was used to extract and the final extract was collected in column reservoir and the solution was passed through filtration.

Aflatoxin derivatization: After adding n-hexane (200 μL) in the derivatization vial to re-dissolve aflatoxin, 50 μL of trifluoroacetic acid is added and it is mixed on vortex mixer for 30 sec. Layers are allowed to separate and aqueous layer (lower layer) containing aflatoxins is filtered and then injected onto LC column.

Liquid chromatography determination with fluorescence detection: The mobile phase (acetonitrile: methanol: deionized water in the ratio of 20:20:60) is degassed with sonicator before use. The retention time for aflatoxin B2, B1, G2 and G1 was near to 5.36 min or slightly different by changing conditions or instrument.

Aflatoxin B1 peak is identified in derivatized extract chromatogram by comparing its retention time with corresponding peak in the standard chromatogram as shown in Fig. 2.

The quantity of the aflatoxin was determined in the derivatized extract (injected) from the respective standard curves. The concentration of aflatoxin 'B2', 'B1', 'G2' and 'G1' is calculated in test sample as follows:

$$\text{Aflatoxins 'B2', 'B1', 'G2' and 'G1' ng g}^{-1} = C/W$$

Where:

W = Equivalent weight of test portion (in 10 µL) injected into LC

C = Aflatoxin (in 10 µL) injected into LC

Statistical analysis: The level of contamination of aflatoxin in all samples were calculated based on the level of aflatoxin <0.05 µg L^{-1} aflatoxin. Contamination for aflatoxin in feed was determined based on US regulatory limit, if the concentration of aflatoxin in feed is more than 20 µg kg^{-1} it will not be safe to feed animals. The geometric mean of aflatoxin level and concentration was determined using SPSS 20 statistical software package.

RESULTS AND DISCUSSION

The results for analysis of milk samples in all locations indicated on Table 1 and chromatographic results are given in Fig. 3a-7b. The Figures presented here are the standard report of aflatoxin detected under HPLC.

Table 1: Aflatoxin M1 contamination (µg L^{-1}) in milk sample

Sample location	Percentage	Mean±SD
Butajira	58	0.31±0.90
Agena	23	0.07±0.26
Emdibir	10	0.02±0.29
Arekit	9	0.04±0.31
Wolkite	-	ND

*ND: Not detectable

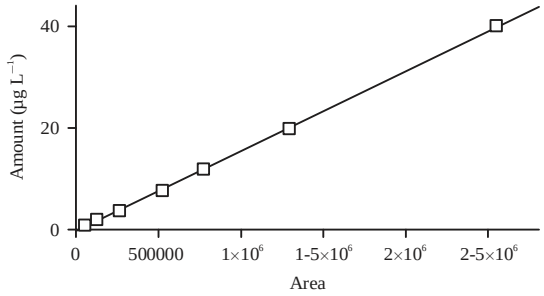


Fig. 2: Calibration report for aflatoxin determination in feed sample

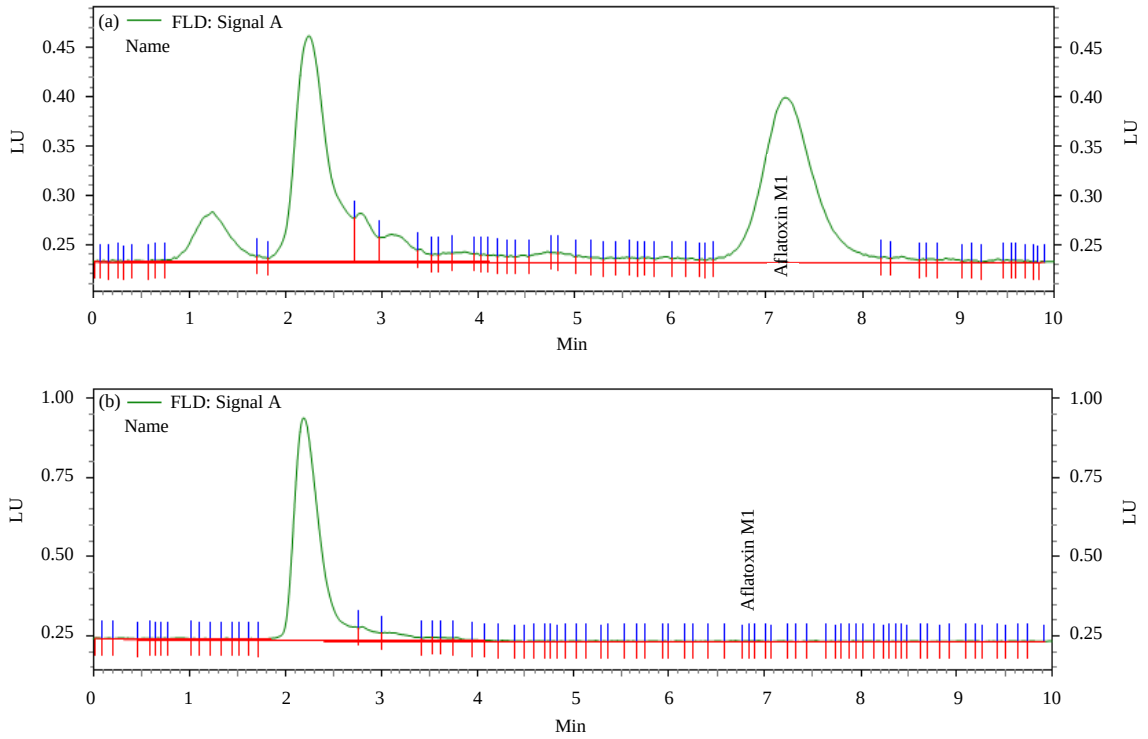


Fig. 3(a-b): Chromatographic result of milk sample from (a) Butajira location 1 and (b) Butajira location 2

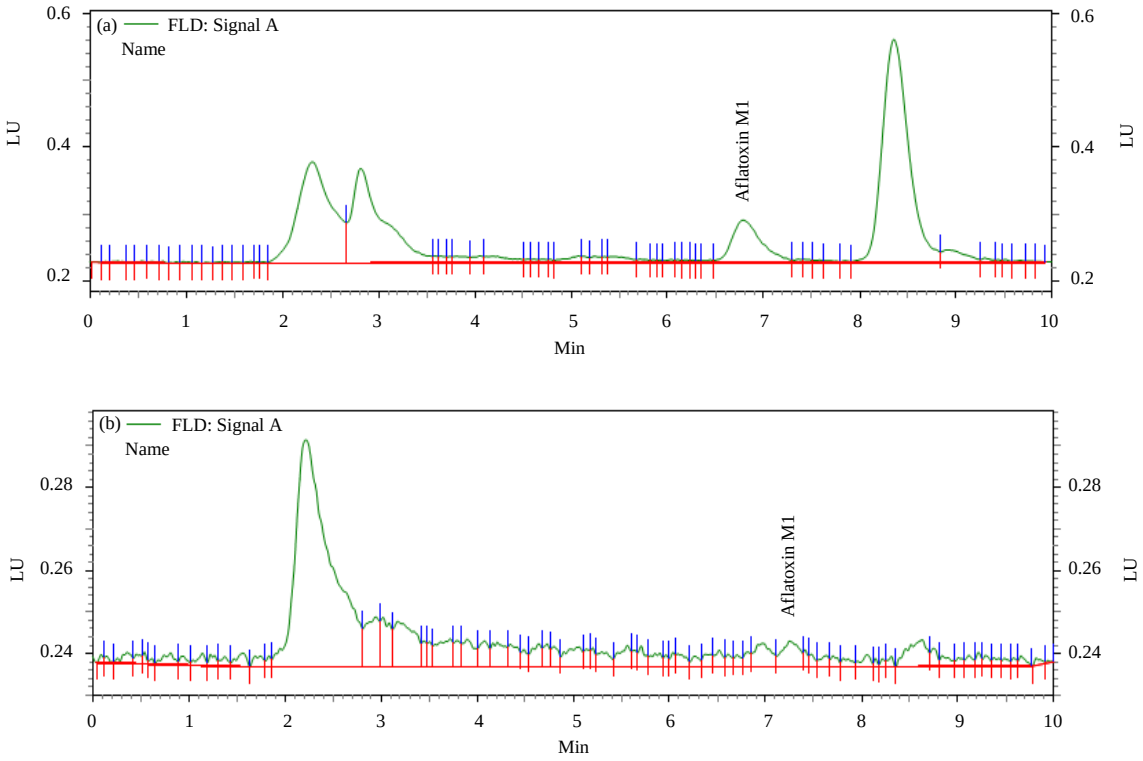


Fig. 4(a-b): Chromatographic result of milk sample from (a) Agena location 1 and (b) Agena location 2

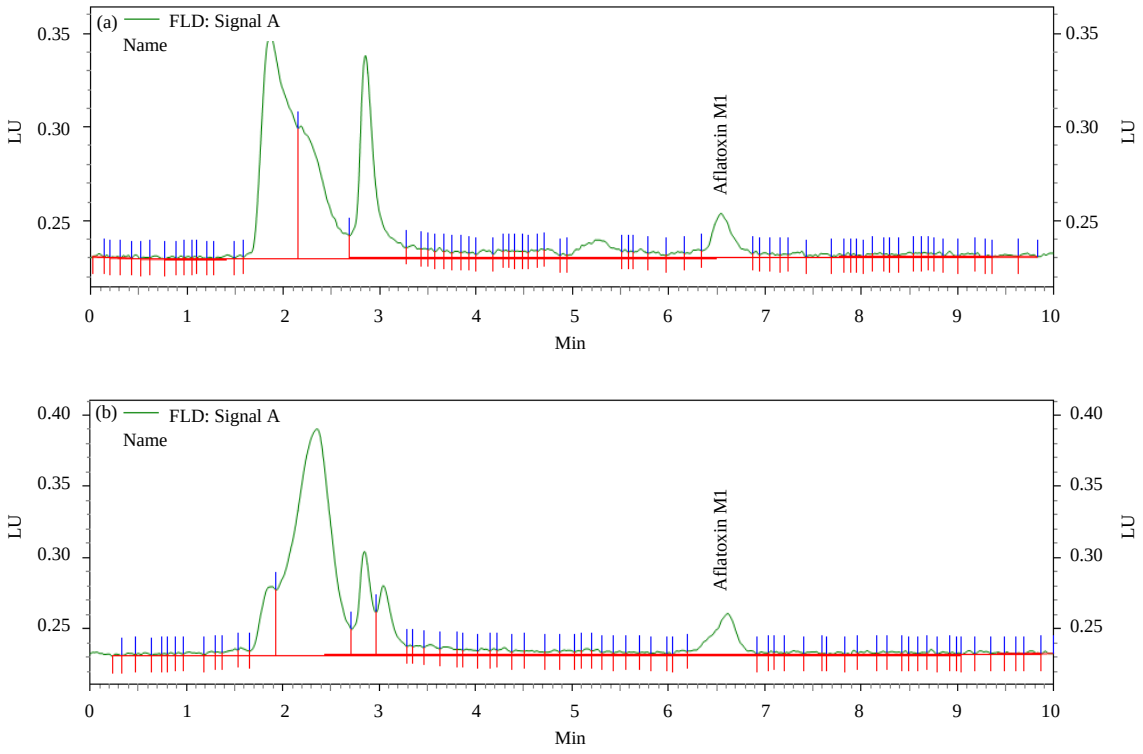


Fig. 5(a-b): Chromatographic result of milk sample from (a) Emdibir location 1 and (b) Emdibir location 2

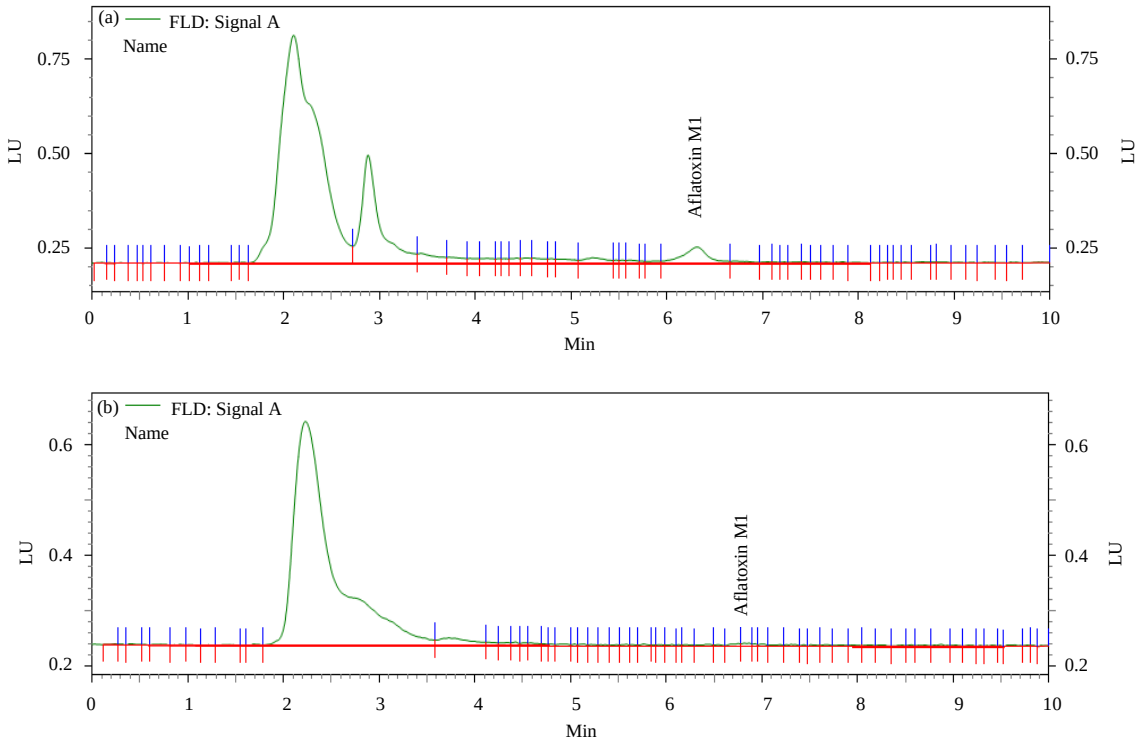


Fig. 6(a-b): Chromatographic result of milk sample from (a) Arekit location 1 and (b) Arekit location 2

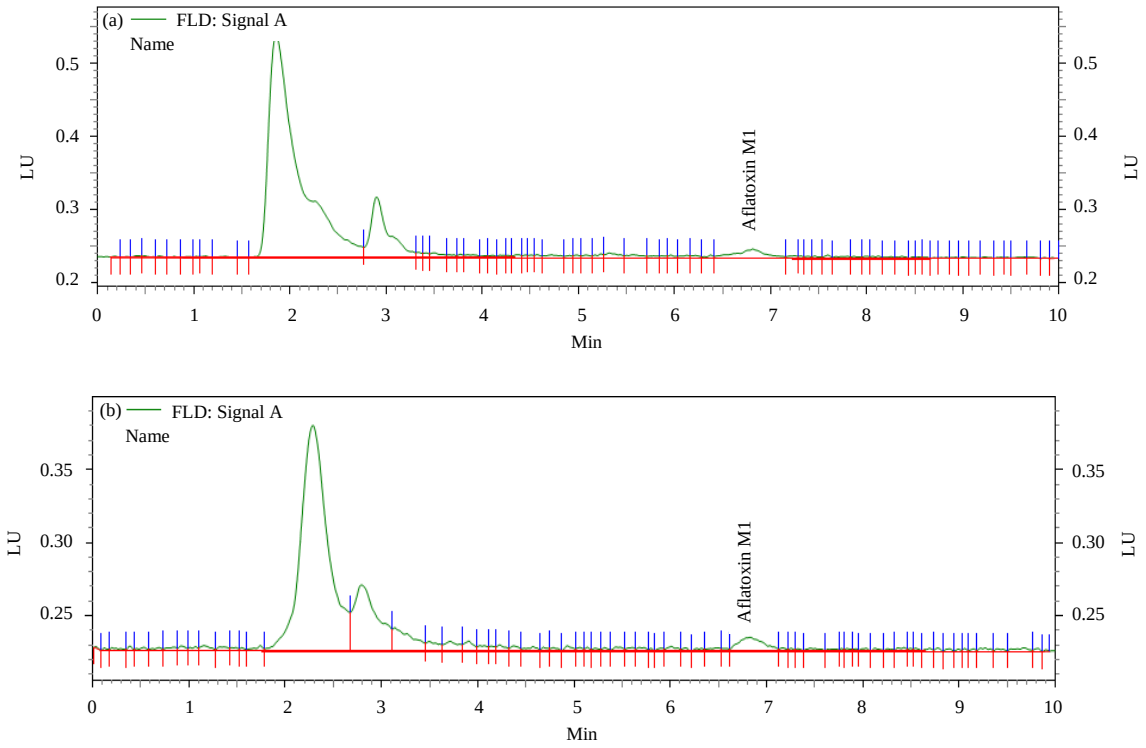


Fig. 7(a-b): Chromatographic result of milk sample from (a) Wolkite location 1 and (b) Wolkite location 2

The percentage contamination level in one location showed more than 50% and four locations resulted below the average as shown on Table 1. The level of aflatoxin contamination in all 10 milk sample ranged between 0.02-0.29 μg L^{-1}. About 58% contamination with aflatoxin obtained from Butajira location. Similarly, the mean value obtained from Butajira was 0.31 μg L^{-1} which was higher than 0.05 μg L^{-1} regulatory limit. Also one sample obtained from the location Agena and Arekit indicated a mean value of aflatoxin M1 0.07 and 0.04 μg L^{-1}, respectively, which was higher than 0.05 μg L^{-1} regulatory limit.

Previously, Gizachew *et al.*[10] reported highest level of aflatoxin contamination in milk sample collected from Bishoftu with contamination level of 4.98 μg L^{-1} and the lowest was 0.028 μg L^{-1} from Addis Ababa. Also samples collected from milk collector around Sululta reported about 0.002 μg L^{-1}. This result obtained suggested that the level of contamination is moderately low to high level in the study area. The level of contamination highest in the location indicated by chromatographic result on Fig. 3a and 4b as well.

Other studies from urban centers in Kenya have been reported AFM1 levels up to 0.68 μg L^{-1} [12]. In contrast to this result, the levels of AFM1 contamination found in raw milk collected from Khartoum state in Sudan, with an average concentration of 2.07 μg L^{-1} and maximum of 6.9 μg L^{-1}, were higher than what was found in this study[13].

The level of percentage of contamination could not be detected at Wolkite town. Also the concentration of aflatoxin is considerably varied among the samples. Therefore, the result report in this experiment is considerably concedes with previous reports.

In 10 feed samples total aflatoxins G2, G1, B2, B1 concentration were analyzed by HPLC result indicated on Table 2, while chromatographic result showed on Fig. 8-12. The average aflatoxins G2, G1, B2, B1 concentration was in a range of 10.54 -4.22 μg kg^{-1}, while six samples resulted under the detection limit as shown on Table 2.

The extent of aflatoxin contamination of feed varied among the type of feed analyzed at different locations. Higher value of contamination is obtained from Butajira with a value of contamination 52.72% and a mean value of contamination 10.54 μg kg^{-1} (Fig. 13). This value is the highest compared with regulatory limit which is aflatoxin content above 20 μg kg^{-1}; it will not be safe to feed milk cows. The other type of feed furshika is a composition of maize grain, semi grinded wheat grain. The dominant proportion of the mix was left over from edible oil distillers. Hence, the dominant type of feed from edible oil from the sample may be a source for contamination (Fig. 8).

The remaining two locations Agena and Emdibir indicated a level of contamination 6.61 and 9.08 μg kg^{-1}, respectively. Hence, the result in comparison with regulatory limit of 20 μg kg^{-1} is low so it's relatively safe to feed the milk cow. However, below the detection limit obtained from Wolkite and Arekit towns. This might be the feed sample containing for aflatoxin contaminant below the level of detection (Fig. 9-12).

The result showed that there was a clear association between AFM1 contamination in raw cow milk and the presence of mix type of feed that is particularly containing noug and cotton seed cake. Samples collected from town of Butajira had a higher level of AFM1 compared to Wolkite and Emdibir.

The correlation between feed samples contaminated with aflatoxin would be resulted contamination of milk with aflatoxin. As the level of aflatoxin in feed sample increased the milk sample contamination level increases (Fig. 13). The magnitude of association is indicated that contamination of milk from Butajira was around fourfold compared to milk sample from Emdibir, may be the presence of noug seed and cotton seed cake in feed caused a 58% increase of aflatoxin M1 in milk. Similarly, [10]reported the presence of AFM1 in raw cow's milk revealed that the contamination level of AFB1 for wheat bran was 9.31 μg kg^{-1}. By far the highest

Table 2: Aflatoxin G2, G1, B1, B2 contamination in feed sample (total aflatoxin by HPLC determination)

Sample	Feed type	Aflatoxin G2 (μg kg^{-1})	Aflatoxin G1 (μg kg^{-1})	Aflatoxin B2 (μg kg^{-1})	Aflatoxin B1 (μg kg^{-1})	Percentage	Mean±SD
Butajira	Mix	1.14	17.1	3.27	31.2	52.71	10.54±3.82
	Furshika	ND	ND	ND	ND		
Agena	Mix	ND	4.05	ND	2.56	6.61	4.56±8.05
	Furshika	ND	ND	ND	ND		
Emdibir	ArekeAtela	ND	1.13	ND	1.88	9.08	4.22±8.29
	Mix	ND	1.27	0.81	10.06		
Arekit	Furshika	ND	ND	ND	ND	-	-
	Furshika	ND	ND	ND	ND		
Wolkite	Mill powder	ND	ND	ND	ND	-	-
	Furshika	ND	ND	ND	ND		

*ND: Not detectable

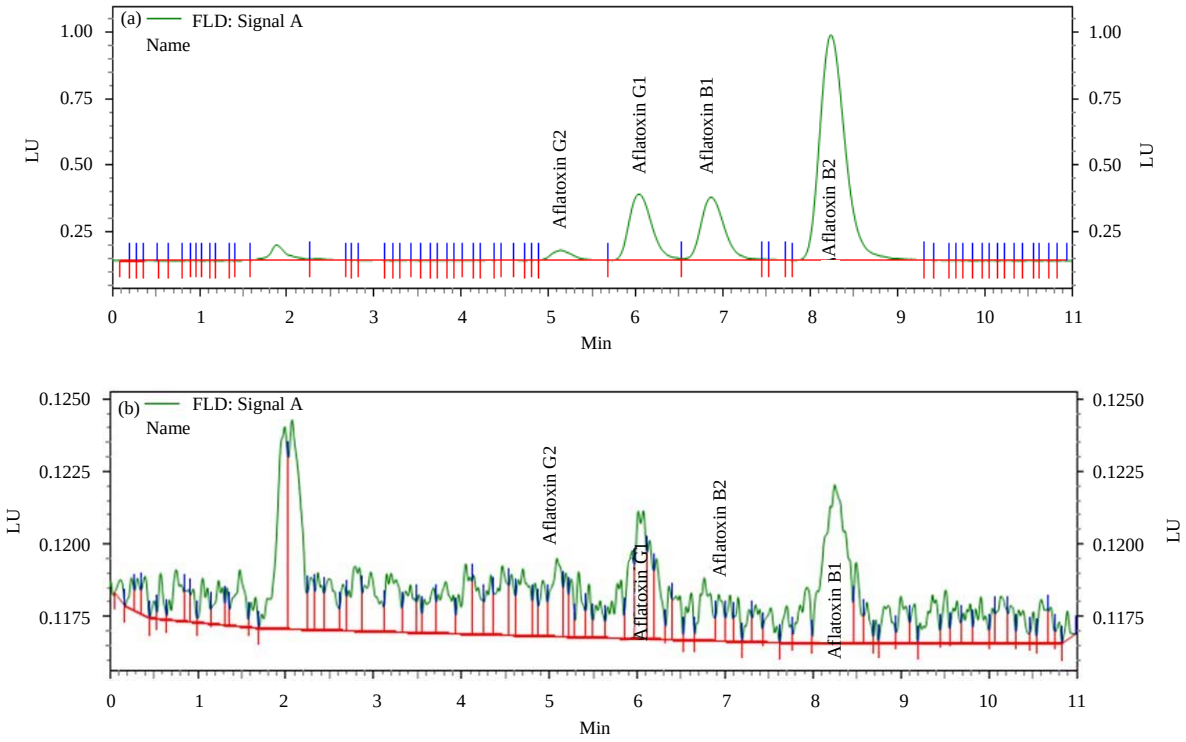


Fig. 8(a-b): Chromatographic result of feed sample from (a) Butajira location 1 and (b) Butajira location 2

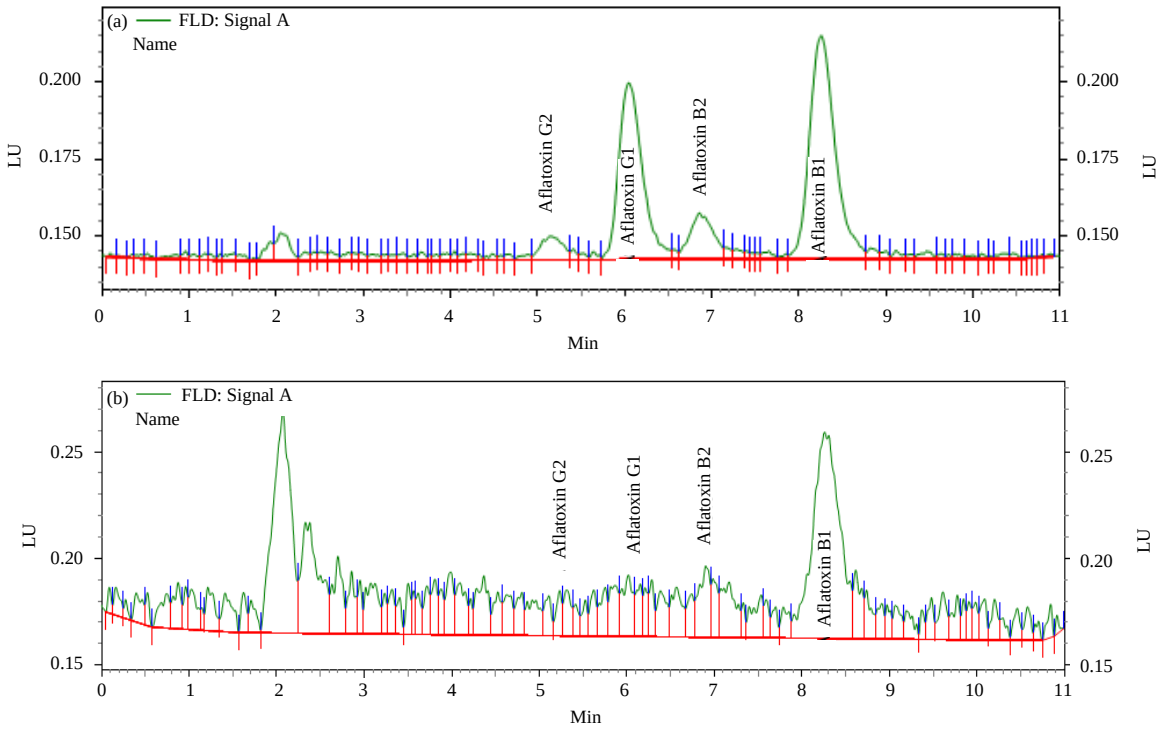


Fig. 9(a-b): Chromatographic result of feed sample from (a) Agena location 1 and (b) Agena location 2

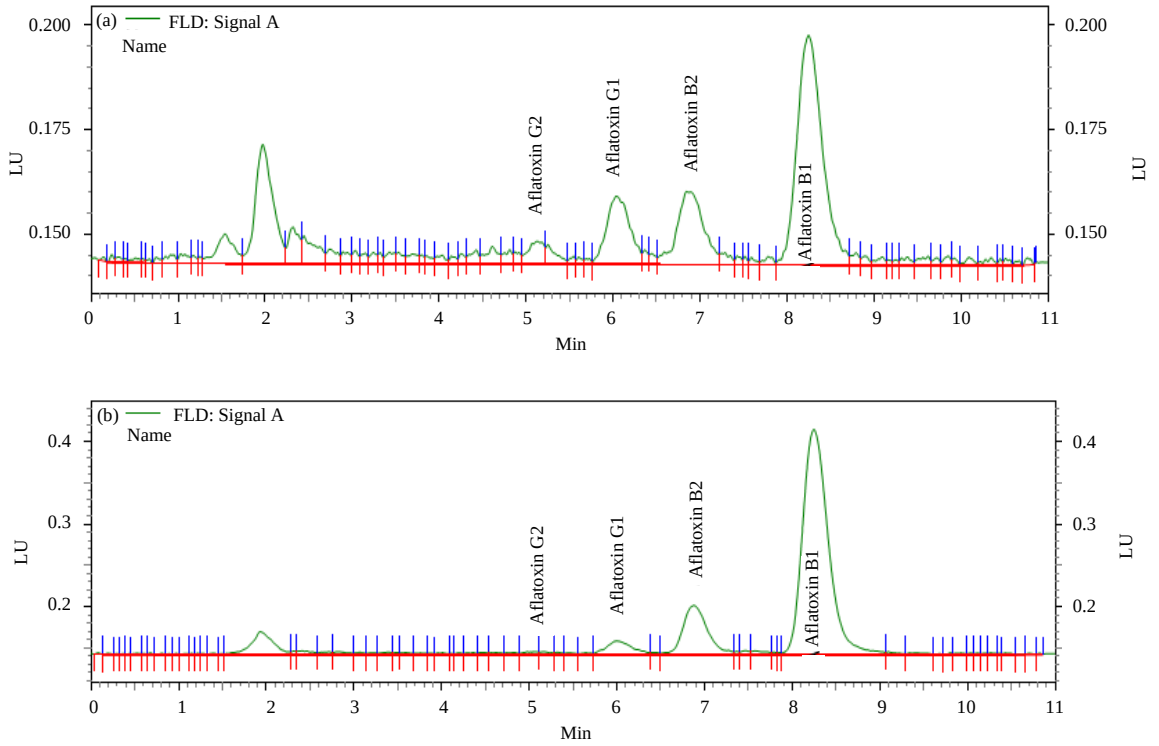


Fig. 10(a-b): Chromatographic result of feed sample from (a) Emdibir location 1 and (b) Emdibir location 2

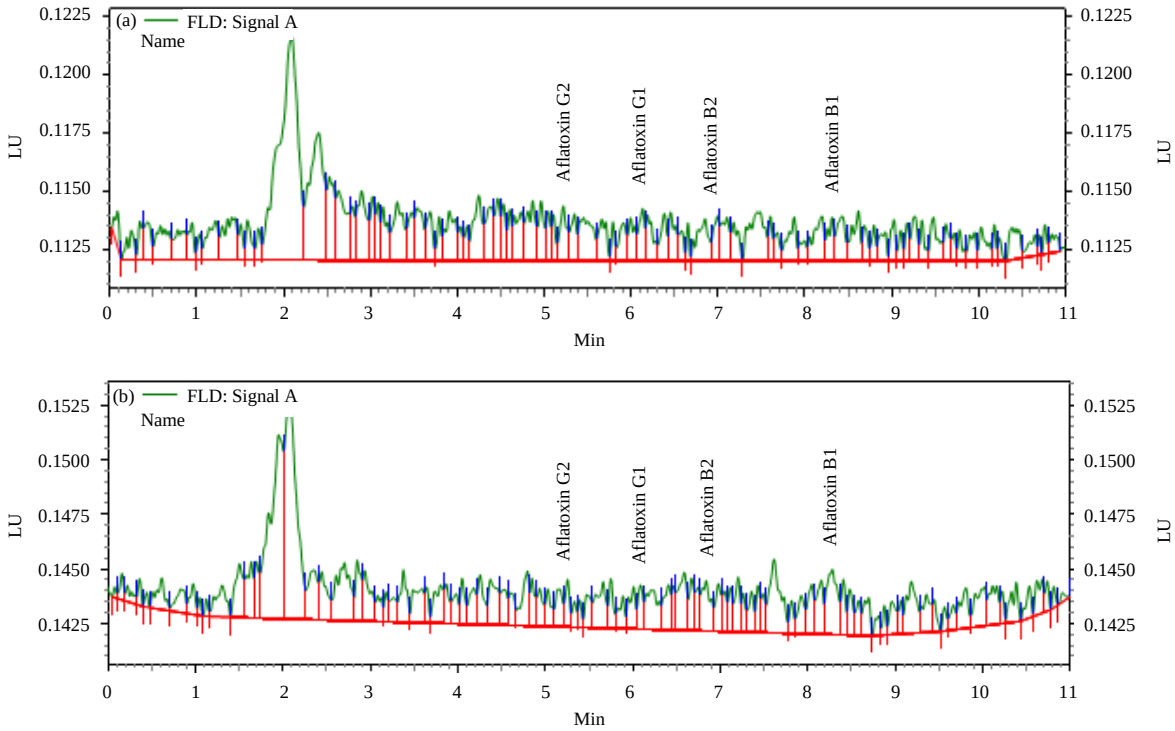


Fig. 11(a-b): Chromatographic result of feed sample from (a) Arekit location 1 and (b) Arekit location 2

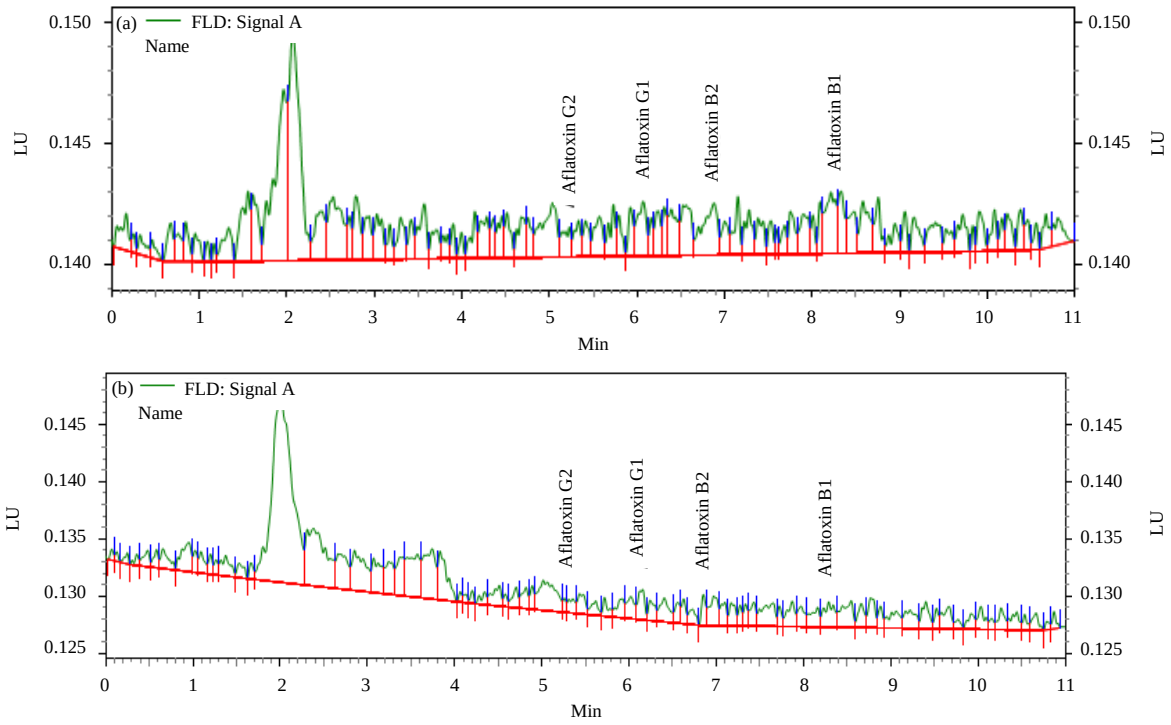


Fig. 12(a-b): Chromatographic result of feed sample from (a) Wolkite location 1 and (b) Wolkite location 2

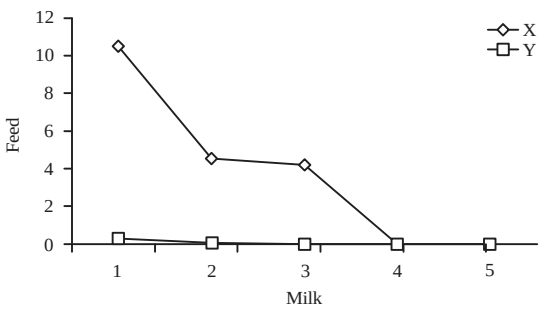


Fig. 13: Correlation between feed sample and milk sample

level of contamination was observed in the noug cake, ranging between 290 and 397 μg kg^{-1}.

Overall, the high levels of aflatoxin contamination of the feed should be a concern for the dairy sector, because aflatoxins can reduce livestock productivity. Limited studies have been conducted on aflatoxins in dairy feeds in Sub-Saharan Africa with the exception Kenya, where substantial analysis of aflatoxin contamination of maize has been carried out[14,15]. The results exhibited supported by Wilhad[16] which reported the concentration range of aflatoxin is achievable down to <0.02 ppb, with very good recoveries using HPLC. Previously, Herzallah[17] reported that the contamination of aflatoxin in milk obtained to be 0.56 μg L^{-1} AFM1 and 0.1 μg L^{-1} AFM2 whilst, the concentration of AFM1 and AFM2 was <0.05 μg L^{-1} for milk samples.

In Ethiopia, young calves are especially vulnerable to the harmful effects of aflatoxins before their rumen develops and they consume their mother's milk until weaning. Therefore, the economic losses due to chronic exposure of cattle to aflatoxins could significantly damage the dairy industry. Further studies on the feed handling and climatic conditions in these towns will provide insights in to practices that might mitigate the risk of exposure to aflatoxins.

CONCLUSION

This study revealed that dairy feeds and milk are moderately contaminated with aflatoxins. For example two samples of milk and four samples feed had non-detectable level of aflatoxin. About 60% of the milk samples exceeded the contamination with aflatoxin limit of 0.05 μg L^{-1}. The contamination of milk and feed with aflatoxin is a complex nature that is required a holistic and multi disciplinary approach to mitigate the risk of human and animal exposure. Further research should focus on risk mitigation targeted at mixed feed containing noug cakes as the primary source of aflatoxin contamination dairy. This study

also suggests that the levels of aflatoxin contamination in the Gurage Zone dairy require proper and adequate interventions to significantly reduce dairy cattle exposure to AFB1-contaminated feeds and human exposure to AFM1-contaminated milk.

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