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DETERMINATION OF AFLATOXIN IN COMMERCIALLY AVAILABLE WHEAT SAMPLES FROM MARKETS OF GONDAR TOWN USING LC-MS/MS

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Abstract: Wheat is one of the major cereal crops of the world ranking second after rice both in area coverage and production among cereal grains. It is also an important cereal crop in Ethiopia in terms of cultivated area, production and consumption, which is the fourth most widely grown crop after teff, maize, and sorghum. It has great importance in the human diet, contributing as a direct source of energy, protein, vitamins, minerals and fiber. However, this cereal crop is vulnerable to degradation by aflatoxins mainly by Aspergillus flavus, Aspergillus Parasiticus and Aspergillus nomius, which have adverse health effects on humans and livestock that ingest aflatoxin contaminated food products and feeds. The contamination of aflatoxins can occur through its growth, harvest, transport and storage. Aflatoxins are carcinogenic substances and are extremely toxic to humans when consuming aflatoxin contaminated foods. Therefore, this study has been undertaken to determine aflatoxin (B1, B2, G1 and G2) in wheat samples collected from two market places (namely, Arada and Azezo) of Gondar town, by using LC-MS/MS. The extraction solvents were acetonitrile and water (84:16 v/v %). Good linearity from the calibration curve was obtained in standard solution of aflatoxins in range between 0.1 and 15 ng/mL, with regression coefficient (R^2) values of > 0.999. Limit of detection of the aflatoxins were 0.1208, 0.0302, 0.0328 and 0.1272 µg/kg for aflatoxin B1, B2, G1 and G2, respectively. The average percentage recoveries of spiked samples were range between 70.80 and 77.23 %. Aflatoxins were not detected in both wheat samples from Arada and Azezo.

Keywords: Aflatoxin, *Triticum aestivum* L., Chromatographic peak, LC-MS/MS

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Introduction

Wheat (*Triticum aestivum* L.) is a member of *Poaceae (Gramineae*) family and *Triticae* tribe. It is an important staple food crop for more than one third of the world population and ranking second after rice both in area coverage and production among cereal crops.[1-2] Wheat supplies carbohydrate, protein, minerals and vitamins and is more preferable over rice for its higher seed protein content about 12 %, fat 1.72 %, carbohydrates 69.60 % and mineral matter 27.20 %.[3] Wheat is the most important cereal crop in terms of cultivated area, production, and consumption in Ethiopia. It is the fourth most widely grown crop in the country after teff, maize, and sorghum.[4] Owing to the abundant production and the main role of wheat and its flour products in the diet of humans, they can play a very important role in endangering human health in case of contamination with health-threatening microorganisms, especially mycotoxins. Wheat contamination and damage by aflatoxin may occur in the field during kernel maturation, in harvest, transportation and storage processes, if conditions of high moisture and temperature are present.[5]

The word “aflatoxin” is the combination of three words; “A” for the *Aspergillus* genus and “fla” for the species *flavus*, and toxin meaning poison.[6] Aflatoxins are acute and chronic toxicant, immunosuppressive, mutagenic, teratogenic, genotoxic and carcinogenic compounds produced in agricultural commodities by *Aspergillus* species; *Aspergillus flavus* which produces aflatoxin B1 and B2, while *Aspergillus parasiticus* and *Aspergillus nomius* produces aflatoxin B1, B2, G1 and G2.[7-8]

Aflatoxins are a group of mycotoxins produced as secondary metabolites by fungi and are a family of structurally related mycotoxins. Aflatoxins include aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1

(AFG1), and aflatoxin G2 (AFG2), in addition aflatoxin M1 and M2 known as milk-aflatoxins which are the metabolites of AFB1 and AFB2, respectively. The "B" and "G" refer to the blue and green fluorescent colors produced under UV light on thin layer chromatography plates, and the numbers 1 and 2 indicate major and minor compounds respectively.[9] Among the known aflatoxins, AFB1 is the most commonly produced aflatoxin and the most potent and it has been reported to be the most powerful natural carcinogen in mammals. The order of aflatoxin toxicity is AFB1 >AFG1 >AFB2 >AFG2. These are the most widely detected in food and have been classified in group 1 as human carcinogens by the International Agency for Research on Cancer.[10-11] Maximum levels of aflatoxin contamination have been established by different countries to protect public health. In particular, the European Commission regulated 2 µg/kg for AFB1 and 4 µg/kg for total aflatoxins (sum of AFB1, AFB2, AFG1 and AFG2) in cereals and derived products.[12]

Regarding to the detection and quantification of aflatoxins, analytical methods involving chromatography have been developed, for instance TLC, HPLC with different detectors such as UV and FLD, LC/MS or LC-MS/MS have been employed. LC/MS/MS has been shown to be suitable for the analysis of mycotoxins in cereals; it enables simultaneous qualification and quantification.[13]

In Ethiopia, the problem of aflatoxin contamination in agricultural commodities is much more serious and considered to provide a favorable condition for aflatoxigenic mold production of agricultural products.[14] For instance study on *Aspergillus species* and aflatoxin levels in sorghum stored at different period and storage system in Northern Shewa indicated that about 56.7 and 23.3 % of the sorghum samples were found to be

contaminated with *Aspergillus flavus* and *Aspergillus parasiticus*, respectively; the level of aflatoxin B1, B2, G1 and G2 were in the range of 3.95 to 153.72 µg/kg, 1.17 to 91.82 µg/kg, 9.87 to 139.64 µg/kg and 3.22 to 52.02 µg/kg, respectively.[15] Research was conducted for determining the natural occurrence of toxigenic fungi species and aflatoxins in freshly harvested groundnut kernels in Tigray, Northern Ethiopia. The results showed that all samples were found 100 percent positive for *Aspergillus* species, with the detected aflatoxin concentrations ranging from 0.1 to 397.8 ppb.[16] Besides in west Gojam, Northern Ethiopia, a research was conducted on *Aspergillus* species and aflatoxin contamination of pre and post-harvest maize grain. Results from 15 pre-harvest and 15 post-harvest maize samples indicated that 77.7 % of pre-harvest and 80 % of post-harvest samples were contaminated by total aflatoxin within the range from 3.13 to 63.66 µg/kg and 9.02 to 139.8 µg/kg, respectively. Aflatoxin B1 was detected in 66.7 % of pre-harvest maize with the mean level of 5.00 µg/kg and in 87.7 % of post-harvest maize with the mean level of 9.86 µg/kg.[17] On a study conducted by Tefera Adisie, for determination of aflatoxins in maize samples from Dera and Fogera Districts of South Gondar, The average recoveries were range between 65.95 and 97.60 %. The mean level of aflatoxins were below limit of detection of AFB1 (0.0253 ppb), AFB2 (0.0255 ppb), AFG1 (0.0257 ppb) and AFG2 (0.0258 ppb).[18] This author's result is comparable with the current study.

Wheat at all stages of growth and storage subject to numerous problem of deterioration due to the mycotoxin colonization, which is hazardous to human and animal when consumed as food or feed.[19] This study interested to determine aflatoxin levels and compare the results with reported literatures.

Results and Discussion

The working range of standard solution

Linearity is the capacity of the method to obtain results directly proportional to the concentration or amount of analyte in a defined range. The LC-MS/MS system's linearity was evaluated by assessing the signal responses of the calibration standards. The Linearity of the calibration curves on LC-MS/MS system was studied by injecting different concentrations of the aflatoxin standards in mobile phase. The system was calibrated by using the working solutions of standard aflatoxins in the range of 0.1-15 ng/mL in a mobile phase of H_2O: ACN: MeOH.

The calibration curves for each standard aflatoxin were constructed from the response or integrated peak-area of standard aflatoxin (y-axis) versus the concentration of standard aflatoxin in ppb (x-axis). The calibration curves for aflatoxin (B1, B2, G1 and G2) as shown in Figure 1. The analyzed working standard solution gives excellent values of regression coefficient for each of aflatoxins. Regression coefficient (R^2) values were greater than 0.999, which was considered as evidence of an acceptable fit of the data to the regression line. Higher regression coefficient shows high correlation between concentration of standard aflatoxins and their responses (peak area). The chromatogram, mass spectra, and chromatographic peaks were generated using Origin 8 and Agilent mass hunter workstation software-data acquisition for the 6460 Series of triple quadrupoles, while the calibration curves were created using Microsoft Excel.

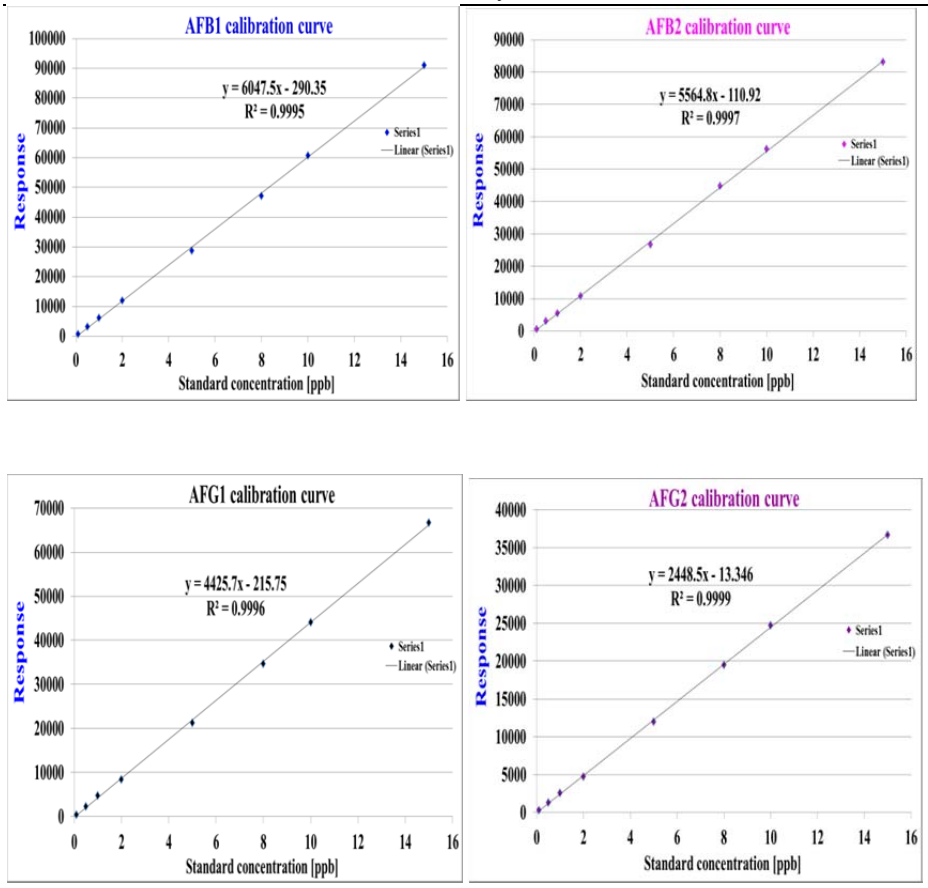


Figure 1. The calibration curves of standard aflatoxin B1, B2, G1 and G2.

Limit of detection and quantification

Limit of detection (LOD) is the lowest amount of analyte in a sample which can be detected, but not necessarily quantitated as an exact value. LOD is the point at which a measured value is larger than the uncertainty associated with it. Limit of quantitation (LOQ) is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions of the test. The LOQ is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of

impurities or degradation products. The limit of detection and limit of quantification were calculated by using equation given below.[20-21]

$$\text{LOD} = \overline{\text{X}} + 3\text{SD} \text{ and } \text{LOQ} = \overline{\text{X}} + 10\text{SD}$$

Where $\overline{\mathbf{X}}$ the mean concentration of the method blank and **SD** is the standard deviation of the method blank.

As shown in Table 1, Limit of detection of the aflatoxins were 0.1208, 0.0302, 0.0328 and 0.1272 µg/kg for aflatoxin B1, B2, G1 and G2, respectively; and the limit of quantifications were 0.2608, 0.0738, 0.0790 and 0.3232 µg/kg for aflatoxin B1, B2, G1 and G2, respectively.

Table 1: LOD and LOQ of the aflatoxins.

Aflatoxin	LOD (µg/kg) ± SD	%RSD	LOQ (µg/kg)
B1	0.1208 ± 0.006	4.97	0.2608
B2	0.0302 ± 0.001	3.31	0.0738
G1	0.0328 ± 0.002	6.10	0.0790
G2	0.1272 ± 0.007	5.50	0.3232

As can be seen in Figure 2, LC-MS/MS chromatogram of the method blank, counts versus acquisition time showed that clear chromatogram, the chromatogram doesn't have any specified visible chromatographic peaks, which means, free of any aflatoxin cross contamination, from extraction solvents and equipment's, so, this depicts that the quality of the employed method. The chromatogram also demonstrated that the procedure was suitable for the determination of aflatoxins in the sample.

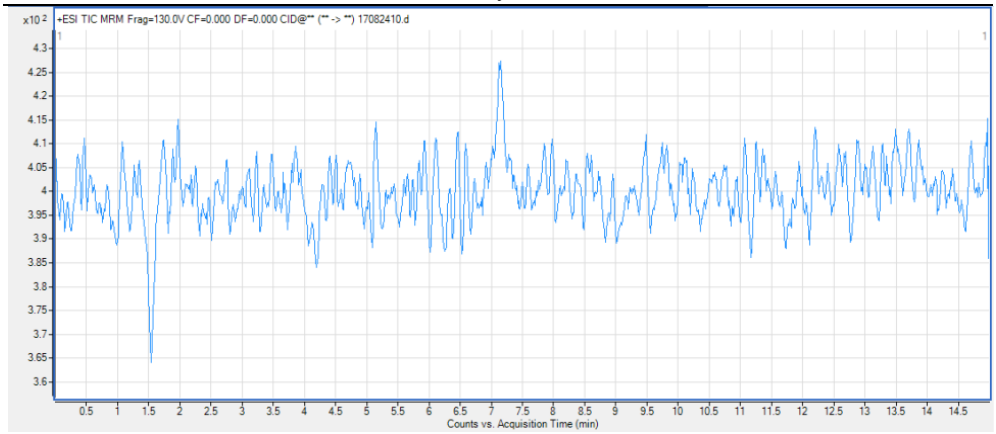
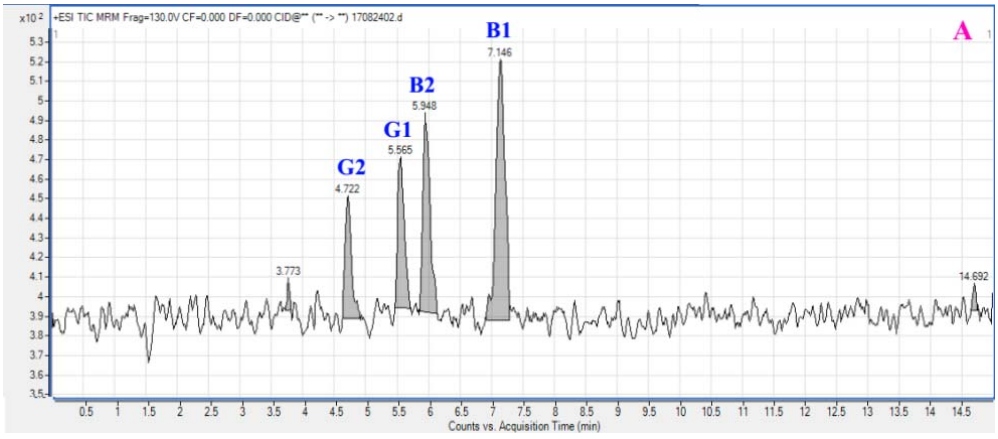


Figure 2. LC-MS/MS chromatogram of method blank.

As shown in Figure 3, all chromatograms of the mixture of aflatoxin standards have clear and excellent chromatographic peaks for aflatoxin B1, B2, G1 and G2 with almost the same retention time (min) at various standard aflatoxin concentration (0.1, 0.5, 1, 2, 5, 8, 10 and 15 ng/mL). The retention time for all different types of concentration of aflatoxin standards were slightly vary at 7.1, 5.9, 5.5 and 4.7 min for aflatoxin B1, B2, G1 and G2, respectively.



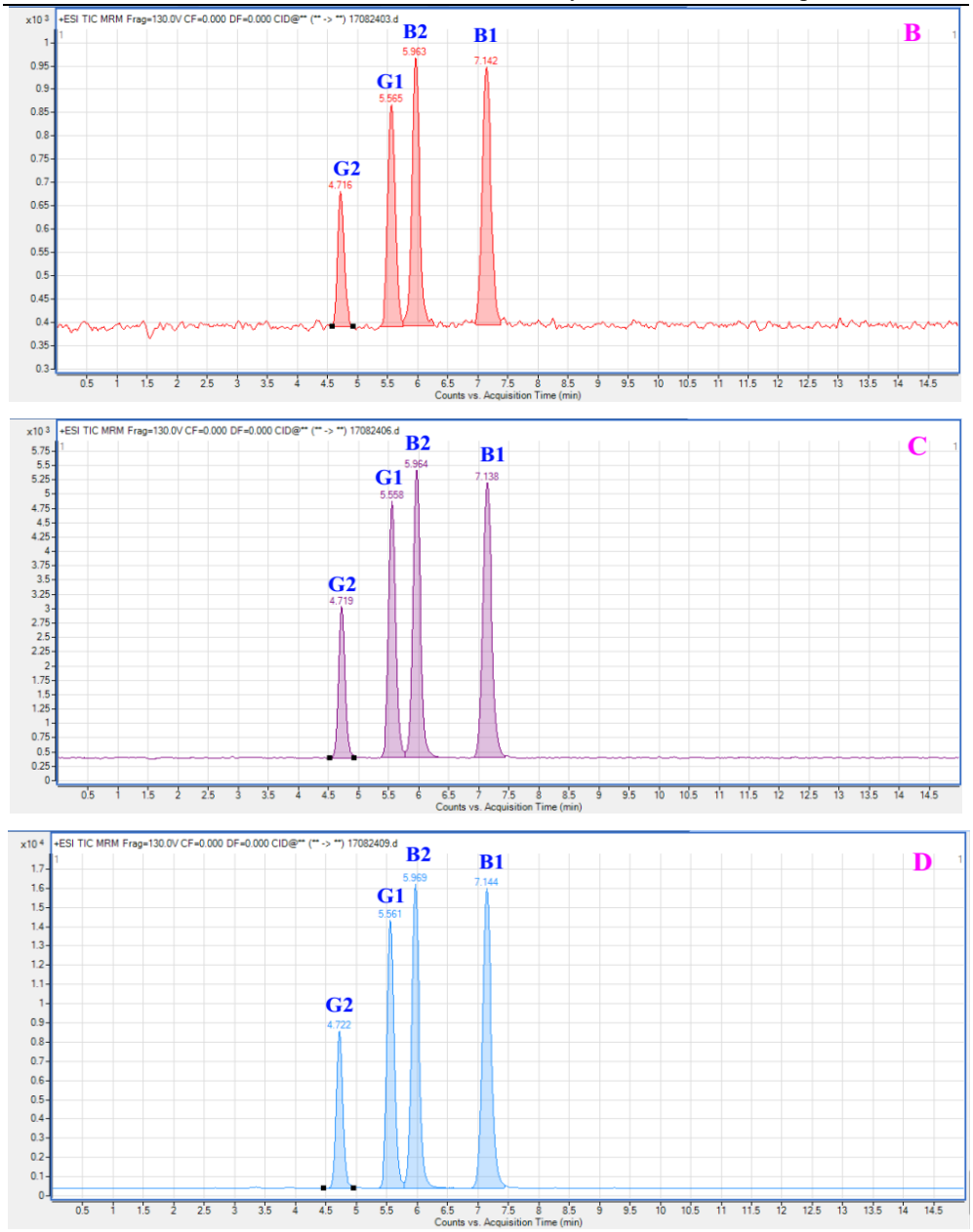
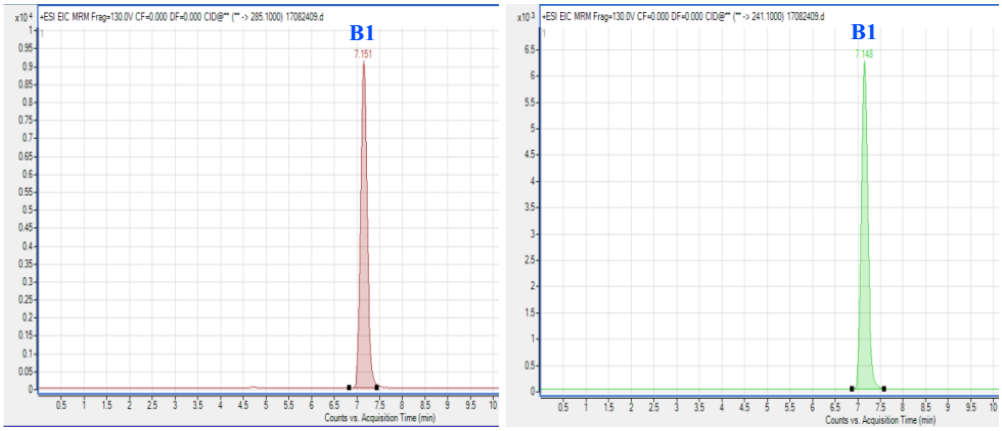


Figure 3. LC-MS/MS chromatogram, counts versus retention time; for mixture of aflatoxin standards at 0.1 (A), 0.5 (B), 5 (C) and 15 µg/kg (D).

The results of LC-MS/MS chromatogram of standard aflatoxins; retention time, peak response; peak sharpness and peak broadening describes the sensitivity and separation efficiency of the method for

aflatoxin determination. Chromatogram of standard aflatoxins, mass spectral and retention time of precursor $[M+H]^+$ to primary product ions and secondary product ion (m/z) were given in Figure 4. The chromatographic peaks observed in Figure 4 are molecular ion fragments of primary product ions (left hand side peaks) and secondary product ions (right hand side peaks). The primary product ions were (285.1, 287.1, 311.1 and 313.1) which were used as quantifiers, and secondary product ions were (241.1, 259.1, 243.1 and 245.1) which were used as qualifiers, for AFB1, AFB2, AFG1 and AFG2 respectively. The precursor ions were 313.1, 315.1, 329.1 and 331.1 for AFB1, AFB2, AFG1 and AFG2 respectively.

Standard aflatoxin chromatographic peaks and mass spectrometry spectral analysis (MRM mode) and retention times used for identification of aflatoxins in sample analysis. The ion fragments were evaluated by their retention time to the most abundant m/z and the ion with the uppermost intensity was selected as the basic ion for quantification.



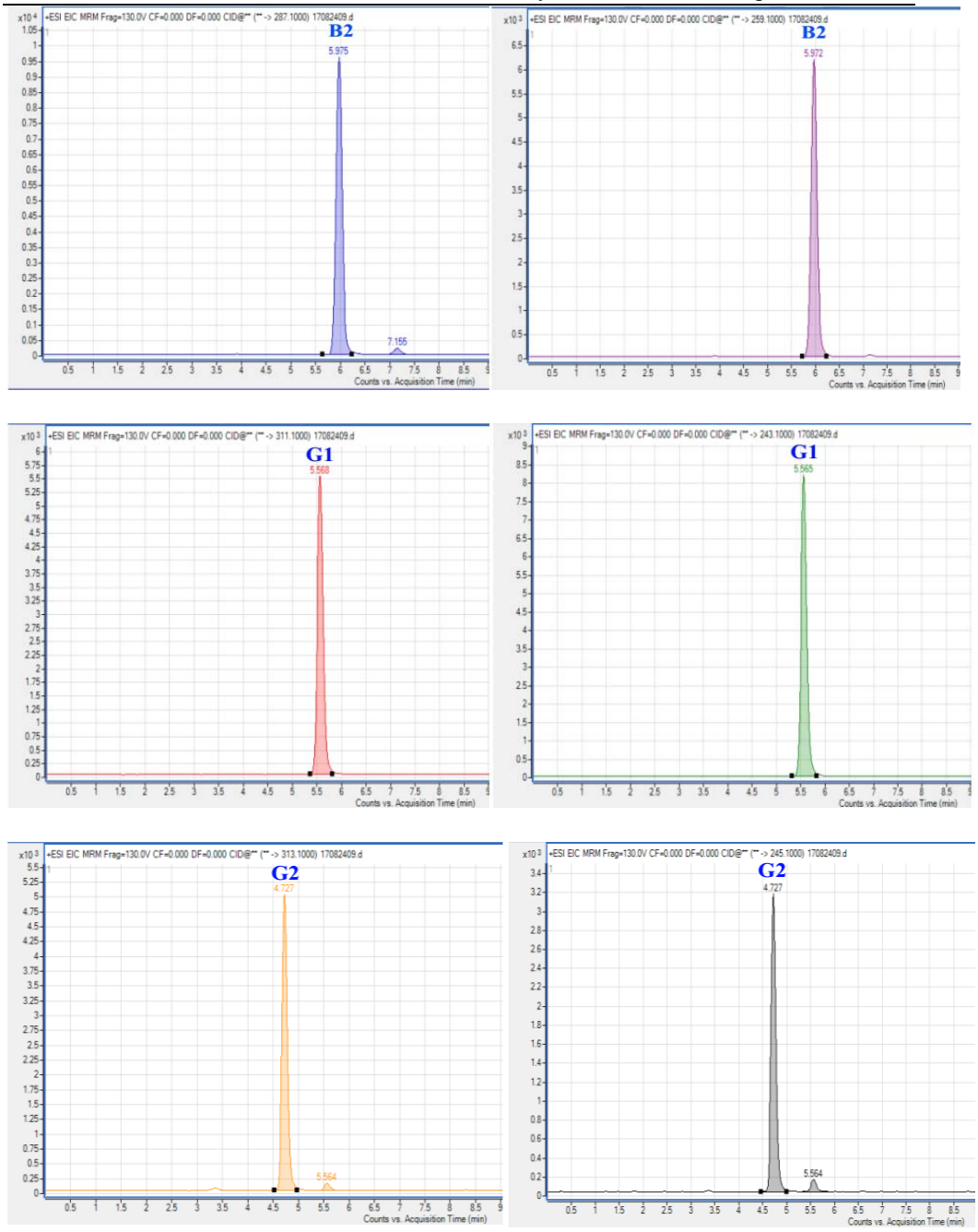
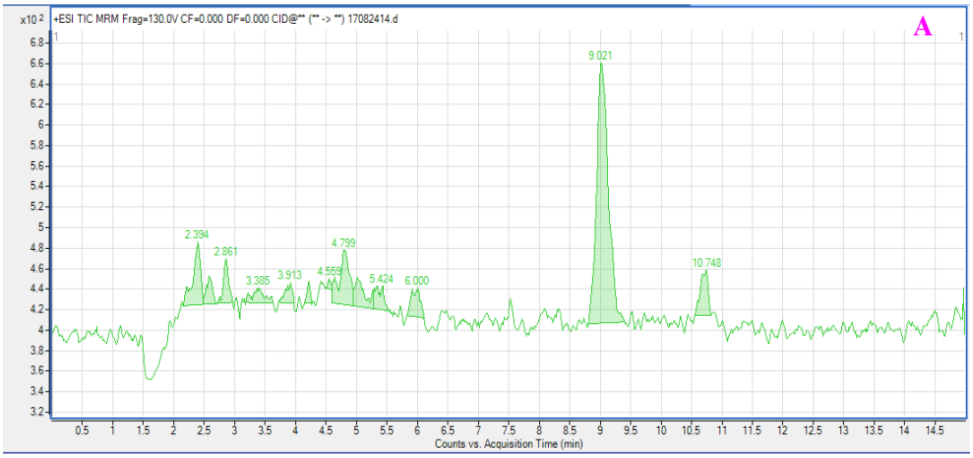


Figure 4. LC-MS/MS chromatogram of standard aflatoxin (B1, B2, G1 and G2) showing molecular ion fragments of primary product ions (left side) and secondary product ion (right side).

Levels of aflatoxin in wheat

The concentration of each aflatoxin was found by using the peak-area integration of chromatogram of wheat sample. The LC-MS/MS chromatogram, counts versus acquisition time of wheat samples from Arada and Azezo market places shows that, as can be seen in Figure 5, there are no large chromatographic peaks observed in both samples within the range of retention time of standard aflatoxins. So, this indicates that, as there is no aflatoxin B1, B2, G1 and G2 detected above the limit of detection in both wheat samples. However at retention time of 9 min, one great chromatographic peak was observed, which was out of the range of retention time of the standard aflatoxin. The retention time of this chromatographic peak was far from the standard aflatoxin, so this compound doesn't belong to aflatoxin (B1, B2, G1 and G2).



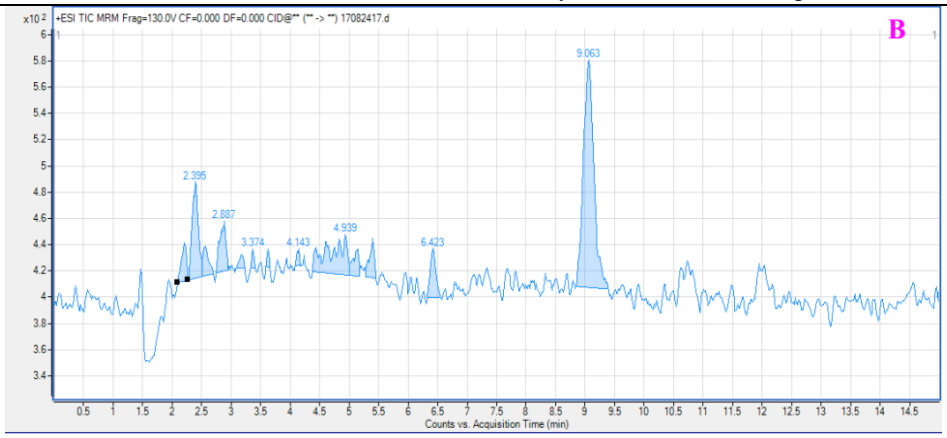


Figure 5. LC-MS/MS chromatogram for wheat samples of Arada (A) and Azezo (B).

Aflatoxins in samples can be identified qualitatively by comparing their chromatogram and retention time with the standard aflatoxins. As can be seen in Figure 5, the LC-MS/MS chromatograms for both wheat samples were almost the same. There is no large chromatographic peak observed in sample chromatograms, as we compare with standard aflatoxin chromatograms with suspected range of aflatoxins retention time.

Recovery

Recovery describes the efficiency of separating analyte from the sample. It is expressed as the percentage of analyte experimentally determined after fortification of sample material at a known concentration and should be assessed over concentrations which cover the analytical range of the method. The recovery study was also used to estimate repeatability (precision), and four replicates (n=4) were carried out for each sample within the same day (intra-day precision). To determine the percentage recovery of the method, both the two wheat samples were spiked with 2 µg/kg of aflatoxin standard. Then after, the aflatoxins extracted with the

same procedure as were done in non-spiked samples, and the analysis was followed.

$$Recovery\ (\%) = \frac{S - U}{C}\ x\ 100$$

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 of standard aflatoxin added.

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

Aflatoxins	Added aflatoxin level in µg/kg	Measured aflatoxin level in µg/kg ± SD	%RSD	Average recovery (%)
B1	2.00	1.4530 ± 0.053	3.65	72.65
B2	2.00	1.4160 ± 0.077	5.44	70.80
G1	2.00	1.5446 ± 0.075	4.86	77.23
G2	2.00	1.4926 ± 0.097	6.50	74.63

The average percentage recoveries of the methods were 72.65, 70.80, 77.23 and 74.63 %, for aflatoxin (B1, B2, G1 and G2) respectively (Table 2). Percent relative standards deviation (%RSD) values were obtained from the analysis of each sample four times (n =4) in the same day. The %RSD was ranged between 3.65 and 6.5. These values indicate that good precision of the analytical method used.

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.[22]

The LC-MS/MS chromatograms of wheat samples spiked with standard aflatoxins were compared with the chromatographic peaks,

retention time and chromatogram of aflatoxin standards. As can be seen in Figure 6 (spiked wheat sample chromatograms), clear chromatographic peaks at almost the same retention time with the standard aflatoxins were observed. Aflatoxin spiked wheat sample chromatograms were almost the same as the chromatograms of standard aflatoxins. The observation of clear chromatographic peaks on chromatogram of spiked wheat samples are an evidence for efficiency of our method.

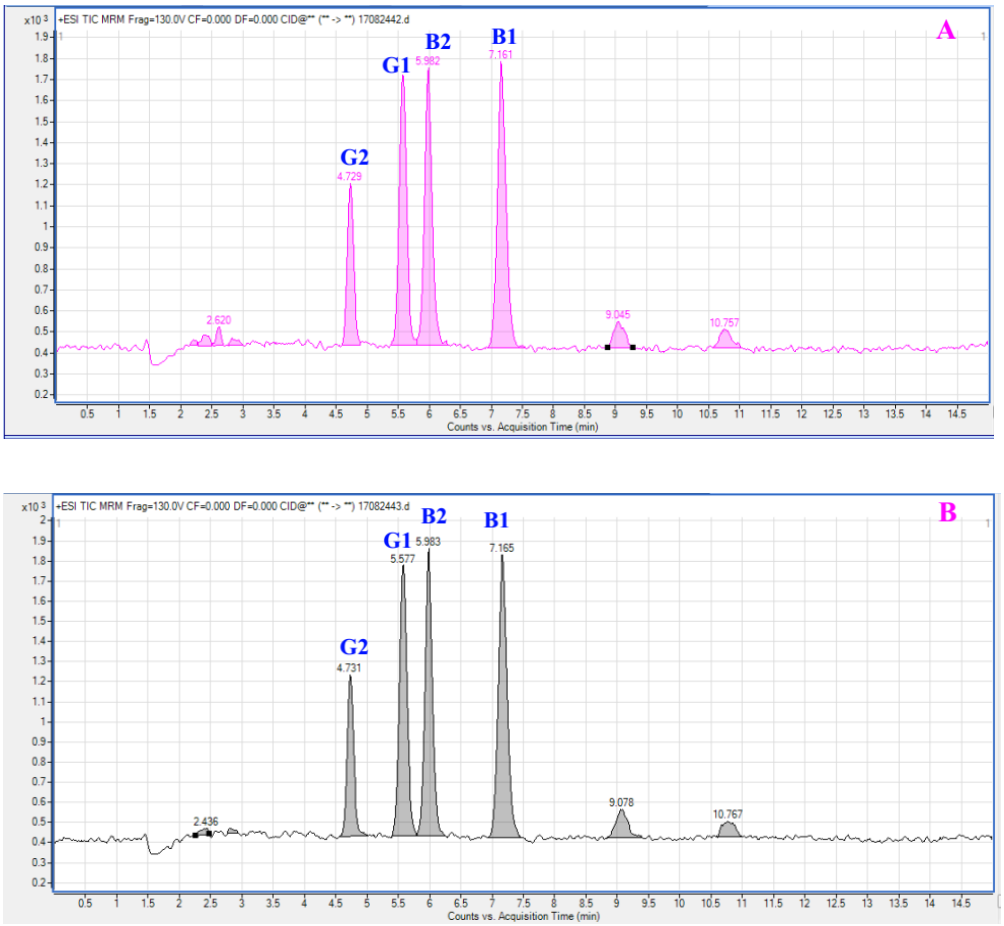


Figure 6. LC-MS/MS chromatogram of 2 µg/kg aflatoxin spiked wheat sample from Arada (A) and Azezo (B).

Comparison of current study results with reported works

When comparing this research finding with other similar studies conducted in different countries on wheat sample, almost all studies conducted in wheat samples depicts that as the wheat samples were contaminated with aflatoxins. In most of the studies the contamination was above maximum residue limits stipulated by European commission and in some studies the contamination was below the maximum residue limits, however in the current study none of the four investigated aflatoxins (B1, B2, G1 and G2) was detected (below limit of detection). For example according to Habtamu and Kelbessa, 2001, investigation on a survey of aflatoxin contamination in Ethiopia, 30 wheat samples were analyzed by using TLC plate. The mean concentrations of aflatoxin B1 in positive samples were above maximum residue limit of the European commission and varied from 19 to 42 µg/kg.[23] The remaining aflatoxins were not found. Again in Ethiopia 120 wheat samples were analyzed by using HPLC-FLD and 4.2 % samples were AFB1 positives and the mean concentration varied from 1.7 to 12.3 µg/kg.[24] In Morocco, wheat flour samples have been analyzed using HPLC-FLD. 17.6 % of wheat flour samples were contaminated with aflatoxins. Aflatoxin B1 levels in wheat flour ranged between 0.03 and 0.15 µg/kg.[25] In Turkey, study was conducted to determine AFB1, AFB2, AFG1 and AFG2 levels in 41 wheat samples by using HPLC-FLD. The concentrations of total aflatoxins in the wheat samples were determined to be ranging from 0.01 to 0.643 µg/kg. 59 % of the samples were found to be positive for total aflatoxins. The recoveries were 70.9, 84.4, 72.8 and 70.0 % for AFB1, AFB2, AFG1 and AFG2 respectively.[26] The recovery result of this article is comparable with the current study. In Iran 100 wheat flour samples were collected from 25 wheat

flour silos. The level of aflatoxins has been determined using HPLC. The mean concentration of AFB1, AFB2, AFG1 and AFG2 levels in all wheat flour samples were 0.53, 0.31, 0.55 and 0.6 μg/kg, respectively.[27]

Experimental

Sample collection and the study area

The samples were collected from commercially available wheat grains of two market places of Gondar town (namely Arada and Azezo). These samples were collected from different wheat retailers randomly. The two samples were composite of eight samples from Arada market retailers and a composite of seven samples from Azezo market retailers. The whole experiment was done in the laboratory of Ethiopian Conformity Assessment Enterprises aflatoxin analysis room, which is found around Megenagna, Addis Ababa, Ethiopia.

Apparatus and chemicals

The apparatuses and instruments used for the experiment were grinding device (Kohinoor), amber glass bottle, 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 (2 mL), filtration apparatus, vortex (Karl Hecht KG D97647 sand heim), auto mechanical shaker, centrifuge, rotatory evaporator, ultrasonic bath, eclipsed plus C-18 column (4.6×15mm), 3.5 μm) and triple quadrupled 6460 model LC-MS/MS (Agilent technologies, USA).

The chemicals, aflatoxin standard (B1, B2, G1 and G2) were HPLC grade and purity ≥ 99.9 %, purchased from Sigma Aldrich (USA); acetone, acetonitrile, methanol, and formic acid were HPLC grade and purity

> 99 %, purchased from Sigma Aldrich (USA). The chemicals were used without any further purification. De-ionized water (conductivity < 0.06 μS/cm, HPLC-grade) was obtained from distilled water passed through a MilliQ water purification system (Millipore LTD, Bedford, MA, USA) and used in all experiments.

Preparation of working standard solutions

Preparation of working standard solution was carried out by accurately weighing 10 mg of each aflatoxin (B1, B2, G1 and G2) and transferred into four different 100 mL volumetric flasks and dissolved using mobile phase for the preparation of 100 ppm individual aflatoxins. With serious dilution of 100 ppm; various concentrations (for instance, intermediate solution of 10 ppm and 1 ppm) of individual standard aflatoxins were prepared. From intermediate of 1 ppm individual standard aflatoxin solutions, mixed standard aflatoxins of 50 ng/mL solution was prepared and finally 0.1, 0.5, 1, 2, 5, 8, 10 and 15 ng/mL were prepared from 50 ng/mL using a dilution law, by mobile phase without formic acid for calibration curve.

Sample preparation and extraction of aflatoxin

Before analysis, the collected wheat samples were mixed for homogenization and then, ground with grinding device. The grinder was cleaned by acetone before and after grinding in order to prevent cross-contamination of aflatoxin. Five g of each wheat flour sample was prepared for sample extraction.

The extraction solvents were acetonitrile and de-ionized water. 15 mL of ACN:H_2O (84:16) was added in to a 5 g wheat sample containing 50 mL centrifuge tube, next blend in vortex mixer at 800 rpm for 30 s to

facilitate the dissolution, and then shacked for one hour on auto mechanical shaker. Then centrifugation is followed, centrifugations were performed for 5 min at 3000 rpm and then quantitatively the supernatant transferred to 250 mL round bottom flask. 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 rotary evaporator at (40 ^{0}C, 772 mbar) and then reconstituted with 10 mL of mobile phase (60 % H_2O : 25 % ACN : 15 % MeOH and 1 % formic acid) followed by sonication for 30 s. Finally, the solution was filtrated using 0.45 μm and 0.2μm syringe filter paper consecutively and then the filtrate was transferred into an auto sampler vial for LC-MS/MS analysis.

The chromatographic conditions during the analysis

Agilent technologies of liquid chromatography series coupled to a 6460 model triple quadrupole mass spectrometer with electrospray source operating in the positive ionization mode (ESI) was applied using multiple-reaction monitoring (MRM) software features. The electrospray ionization settings were gas temperature, 325 °C, gas flow, 10 L/min; nebulizer gas, 50 psi; sheath gas temperature, 350 °C; sheath gas flow, 11.01 L/min; column temperature was 35 ^{0}C. The LC-MS/MS parameters for analytes were cell accelerator voltage (7 V), ionization mode (+ve), fragmentor (130 V). Nitrogen and argon gaseous were used as a nebulizer and collision gas respectively. Mass hunter data acquisition qualitative and quantitative analysis software were used for the data acquisition and data processing for all the analyses, and origin 8 software application also used for quantitative analysis. The chromatographic separation was performed on a reversed phase eclipsed plus C-18 (4.6×15 mm), 3.5 μm particle size) column, by injecting the sample extract. The column was eluted using a gradient flow

(0.55 mL/min) of the mobile phases (59.9 % H_2O, 25 % ACN, 15 % MeOH and 0.1 % formic acid) and the injection volume was 10 µL for LC-MS/MS analysis. Two transition ions were monitored to identify targeted analytes. For quantitative and confirmation purposes, the two most abundant product ions per analyte were chosen. The most intense transition ion was used for quantification, while the second most intense ion was used as a qualifier for confirmation. Using precursor and product ions in mass spectral analysis, all four aflatoxins were detected and confirmed in a sensitive manner.

Preparation of the mobile phase

The mobile phase was prepared by mixing de-ionized water, acetonitrile, methanol and formic acid in v/v % of 59.9: 25: 15: 0.1, respectively. Then the mixture sonicated for 30 s, finally, the mixture was filtered using 0.45 µm filter paper. Similarly, equal volumes of mixture were prepared without formic acid for standard solution preparations, which were used for calibration curve. The formic acid in the mobile phase enhances ionization (gives clear chromatographic peaks).

Conclusion

Liquid-liquid extraction technique was used for extraction of aflatoxins. The mixture of acetonitrile and water were used as extraction solvent, whereas the mixture of water, acetonitrile, methanol and formic acid used as mobile phase. LC-MS/MS method working in multiple reactions monitoring mode was employed for simultaneous analysis of aflatoxin B1, B2, G1 and G2. From the applied method, clear chromatograms for method blank and good chromatographic peaks for standard aflatoxins were observed. So these chromatograms demonstrated that the procedure was suitable for the determination of aflatoxins in the

sample. From the analyzed working standard solution, an excellent coefficient of regression (R^2) were obtained, this is considered as an evidence for linear relationship between the concentration and its response. The levels of aflatoxins in this study were found below the limit of detection. Recoveries were acceptable according to AOAC International guidelines for method validation.

References

1. Perugini, L. D. Genetic characterization of wheat germplasm with Resistance to Fusarium head blight (FHB) and powdery mildew; Doctoral thesis, *North Carolina State University*, **2007**.
2. Wasmi, A. F., Salim, H. A., Subba, R. K. and Abed, M. S. Effect of two bio-agents against root knot nematode meloidogyne graminicola in wheat. *Eur. J. Acad. Res.* **2014**, *2*, 4553-4561.
3. FAO (Food and Agriculture Organization). FAO agricultural database. Rome, Italy, **2004**. www.fao.org/statistics/en/.
4. AACCSA (Addis Ababa Chamber of Commerce and Sectoral Associations). Value chain study on wheat industry in Ethiopia, *Afro universal consult and general trading P.L.C. Addis Ababa, Ethiopia*, **2017**. *www.addischamber.com/wp.../2017/.../Value-Chain study on Wheat-Industry-in-Ethiopia pdf.*
5. Volkova, T. Mycotoxins in brewing grain raw material (barley, malt) in Russia. *J. of Food Scie. and Eng.* **2013**, *3*, 496-502.
6. Bakirdere, S., Bora, S., Bakirdere, E. G., Aydin, F., Arslan, Y., Komesli, O. T., Aydin, I. and Yildirim, E. Aflatoxin species: their health effects and determination methods in different foodstuffs. *Central Eur. J. Chem.* **2012**, *10*, 675-685.
7. Filazi, A. and Sireli, U. T. Occurrence of aflatoxins in food. In: aflatoxins-recent advances and future. (Edited by Mehdi R.), In Tech Janeza Trdine Rijeka, Croatia **2013**, 143-170.
8. Omar, H. E. M. Mycotoxins-induced oxidative stress and disease. In: mycotoxin and food safety in developing countries (Edited by Makun, H. A.). In Tech Janeza Trdine Rijeka, Croatia **2013**, 63-92.

9. Dhanasekaran, D., Shanmugapriya, S., Thajuddin, N. and Panneerselvam, A. Aflatoxins and aflatoxicosis in human and animals. In: aflatoxin-biochemistry and molecular biology. (Edited by Gonzalez, G. R.), In Tech Janeza Trdine Rijeka, Croatia **2011**, 221-264.

10. IARC (International Agency for Research on Cancer). Ochratoxin A. monographs on the evaluation of carcinogenic risks to humans, some naturally occurring substances. *Food items and constituents, heterocyclic aromatic amines and mycotoxins,* **1993**, *56*, 489-521.

11. Creppy, E. E. Update of survey, regulation and toxic effects of mycotoxins in Europe. *Toxicol. Lett.* **2002,** *127*, 19-28.

12. EC (European Commission). Commission regulation no 165/2010 of February 26 amending regulation (EC) no 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards aflatoxins. *OJEU* **2010**, *38*, 244-248.

13. Ok, H. E., Tian, F., Hong, E. Y., Paek, O., Kim, S. H., Kim, D. and Chun, H. S. Harmonized Collaborative Validation of Aflatoxins and Sterigmatocystin in White Rice and Sorghum by Liquid Chromatography Coupled to Tandem Mass Spectrometry. *Toxins* **2016**, *8*, 1-13.

14. Addisu, A. Comparative study of aflatoxins level between traditional and industrial barley malt in Ethiopia. Master thesis, *Addis Ababa University,* **2017**.

15. Geremew, T., Tilahun, B., Ashagrie, Z. and Habtamu, F. Study on Aspergillus species and aflatoxin levels in sorghum (Sorghum bicolor L.) stored for different period and storage system in Kewet districts, Northern Shewa, Ethiopia. *J. Food Sci. Nutr.* **2016**, *2*, 1-8.

16. Dereje, A., Teare, M. and Skinnes, H. Natural occurrence of toxigenic fungi species and aflatoxin in freshly harvested groundnut kernels in Tigray, northern Ethiopia. *J. Dry Lands* **2012**, *5*, 377-384.

17. Assaye, M. A., Gemeda N. and Weledesemayat G. T. Aspergillus species and Aflatoxin Contamination of Pre and Post-Harvest Maize Grain in West Gojam, Ethiopia. *J. Food Sci. Nutr.* **2016**, *2*, 1-7.

18. Tefera, A. K, Determination of Aflatoxins in Maize (Zea Mays L.) Samples from Dera and Fogera Districts of South Gondar Using LC-MS/MS, Master Thesis, *University of Gondar,* **2017**.

19. Ghanghro, A. B., Channa, M. J., Sheikh, S. A., Nizamani, S. M. and Ghanghro, I. H. Assessment of aflatoxin level in stored wheat of godowns of Hyderabad division and decontamination by UV radiation. *Intern. J. Biosci.* **2016**, *8*, 8-16.

20. Sanagi, M. M., Ling, S. L., Nasir, Z., Hermawan, D., Wan Ibrahim, W. A. and Naim, A. A. Comparison of signal-to-noise, blank determination, and linear regression methods for the estimation of detection and quantification limits for volatile organic compounds by gas chromatography. *J. AOAC Int.* **2009**, *92*, 1833-1838.

21. Marijani, E., Charo-Karisa, H., Kigadye, E. and Okoth, S., 2020, "Occurrence and exposure assessment of aflatoxin B1 in Omena (Rastrineobola argentea) from Kenya," *J. Food Qual.* **2020**, *2020*, 1-7.

22. Hong, L. S., Yusof, N. I. M. and Ling, H. M. Determination of aflatoxins B1 and B2 in peanuts and corn based products. *Sains Malays.* **2010**, *39*, 731-735.

23. Habtamu, F., and Kelbessa, U. Survey of aflatoxin contamination in Ethiopia. *Ethio. J. Health Sci.* **2001**, *11*, 17-25.

24. Amare, A., Fehrmann H., Lepschy, J., Beck, R. and Dawit, A. Natural occurrence of mycotoxins in staple cereals from Ethiopia. *Mycopathologia* **2006**, *162*, 57-63.

25. Zinedine, A., Juan C, Soriano J., Molto J., Idrissi, L. and Manes, J. Limited survey for the occurrence of aflatoxins in cereals and poultry feeds from Rabat, Morocco. *Intern. J. Food Microbiol.* **2007***, 115*, 124-127.

26. Giray, B., Girgin, G., Engin, A. B., Aydin, S. and Sahin, G. Aflatoxin levels in wheat samples consumed in some regions of Turkey. *Food Control* **2007***, 18*, 23-29.

27. Kebria, F. G., Joshaghani, H., Taheri, N. S., Semnani, S., Aarabi, M., Salamat, F. and Roshandel, G. Aflatoxin contamination of wheat flour and the risk of esophageal cancer in a high risk area in Iran. *Cancer Epidemiol.* **2013**, *37*, 290-293.