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APPLICATION OF QUECHERS-HPLC METHOD FOR THE ANALYSIS OF
AFLATOXINS IN CEREAL-BASED FOODS

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Abstract

This study presents the determination of aflatoxins B1, B2, G1 and G2 in cereal-based food (semolina) using QuEChERS extraction. Placket-Burman design was employed to screen significant factors of QuEChERS, while central composite design (CCD) was used to optimized significant factors. The validation of analytical methodology determined using optimized parameters gave average recoveries at two spiking levels ranging from 98.22 to 104.85 % for semolina samples stored at room temperature and 99.02 to 105.64 % for samples stored in the refrigerator. Linearity was found between 1 – 40 µg/kg with a correlation coefficient greater than 0.99 and the LOD and LOQ ranges from 0.1810 – 1.1769 µg/kg and 0.6033 – 3.9230 µg/kg, respectively. Analysis of samples of two different brands of semolina (Mama Gold and Golden Penny) showed that, samples were contaminated with all the target aflatoxins at both storage conditions. Golden penny contained aflatoxin B1 at a concentration of 6.4565 µg/kg at day 1, while it was found at a concentration of 11.9752 µg/kg when stored for 21 days at room temperature. Aflatoxin B2 was found to increase from 7.5615 µg/kg at day 1 to 9.6744 µg/kg at day 21, while aflatoxin G1 increased from 8.5672 µg/kg to 9.0974 µg/kg and aflatoxin G2 from 4.7278 µg/kg at day 1 to 8.9562 µg/kg at day 21. A similar trend was found for Mama gold, with aflatoxin B1 from 8.4569 µg/kg to 11.9752 µg/kg, aflatoxin B2 from 8.7653 µg/kg to 11.6815 µg/kg, aflatoxin G1 from 9.5621 to 12.0987 µg/kg and G2 6.5682 to 11.7862 µg/kg. It was found that storing the food samples in the refrigerator reduced the growth of aflatoxin, with the reduction observed in the concentration of aflatoxins within days.

Keywords: aflatoxins, cereal-based foods, liquid chromatography, QuEChERS

Introduction

Aflatoxins are a sub-group of mycotoxins produced by filamentous fungi (moulds) which are highly toxic and capable of growing in foodstuffs such as cereals, nuts, spices, fruits, oil seeds or coffee. Temperature, precipitation, relative humidity and

stress conditions in the plants are commonly known factors influencing the production of aflatoxins in foodstuffs (Sirhan *et al.*, 2014). The most commonly used extraction methods applied for the extraction of aflatoxins in food matrices are solvent extraction (Bocacci *et al.*, 2012), solid-phase extraction (SPE), (Chen *et al.*, 2007), supported liquid membrane extraction (SLME) (Megersa *et al.*, 2012), matrix solid-phase dispersion (MSPD) (Abhlilash *et al.*, 2009), solid-phase microextraction (SPME) (Chen *et al.*, 2007).

Aflatoxins (Figure 1), occur naturally when fungi infest food products, aflatoxins such as B1,

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B2, G1, G2, M1 and M2 are usually found in foods, where “B” and “G” refer to the blue and green fluorescent colours produced on thin layer chromatography plates under UV light, while the subscript numbers 1 and 2 indicate major and minor

compounds, respectively. M1 and M2 are the metabolites of B1 and B2 found in human and animal milk. Aflatoxin B1 and B2 are produced by *A. flavus* and *A. parasiticus*. Aflatoxin G1 and G2 are produced by *A. parasiticus* (Chauhan, 2017).

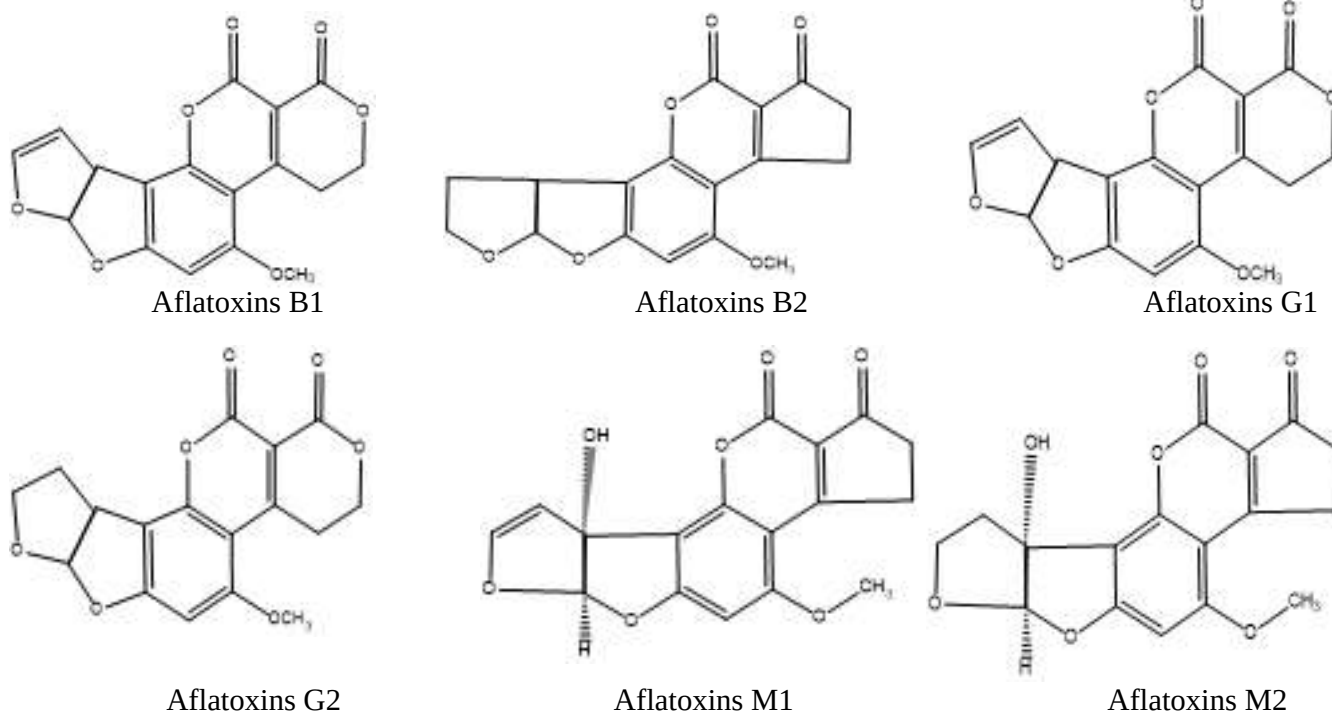


Figure 1: Structure of Aflatoxin (Alsharif *et al.*, 2019)

Health implications of contaminated aflatoxins in humans and animals through consumption, contact or inhalation of foodstuff and feedstuff in both developed and developing countries cannot be underestimated where billions of people are chronically exposed to uncontrolled amounts of aflatoxins leading to aflatoxicosis. Aflatoxins are toxic or fatal to poultry animals (livestock) and are agreed to be carcinogenic to humans (Bursic *et al.*, 2013). Vomiting, abdominal pain, pulmonary oedema, convulsions, coma, death with cerebral edema and enlargement of internal organs such as the liver, kidneys and heart are common symptoms of aflatoxins in humans, while gastrointestinal dysfunction, reduced feed utilization, anaemia, jaundice, liver damage, decrease milk and egg production and immunity suppression is aflatoxicosis characterized in animals (Wacoo *et al.*, 2014).

The role of mathematical and statistical approaches in the optimization of factors like temperature, pH, agitation speed, and liquid volume, among other factors affecting the efficiency of QuEChERS. These factors can be optimized using experimental design, which is

highly significant in the process of large amounts of data collected in a chromatography experiment and to find hidden trends and properties (Abdulrauf *et al.*, 2015).

Multivariate optimization selects all factors and optimises them at the same time and determines the major factors that significantly affect QuEChERS efficiency (Abdulrauf *et al.*, 2014). Plackett-Burman Design (PBD), Box-Behnken Design (BBD) and Central Composite Design are commonly used experimental designs for the screening and optimization of significant factors (Lawal *et al.*, 2018). In this study, the Plackett-Burman design (PBD) was used for the screening of factors, while the central composite design was used for the optimization of significant factor and the optimized parameters were employed for the analysis of aflatoxins in cereal-based foods

Materials and Method

Chemicals and Reagents

Aflatoxin standards were purchased from Sigma Aldrich (St. Louis, MO USA) with purity of 98%, standard stock solutions were prepared in acetonitrile according to the Association of Official Analytical Chemists (AOAC) standard method

(IARC 2002). A working solution containing the aflatoxins standard was prepared daily by diluting

the stock standard solution in acetonitrile to a concentration of 10 µg/ml and stored at 4

Table 1: Levels and factors used in P – B design for QuEChERS – dSPE

S/N	FACTOR	LEVEL	
		LOW (-)	HIGH (+)
1.	Mass of sample	1	5
2.	Mass of MgSO ₄	1	6
3.	Volume of acetonitrile	5	15
4.	Percentage of acetic acid in acetonitrile	0	2
5.	Mass of NaCl	0	2
6.	Centrifugation speed	3000	6000
7.	Centrifugation time	2	6
8.	Centrifugation speed for dSPE	2000	6000
9.	Centrifugation time for dSPE	2	6
10.	Mass of sodium citrate	0	2
11.	Mass of sodium acetate	0	2

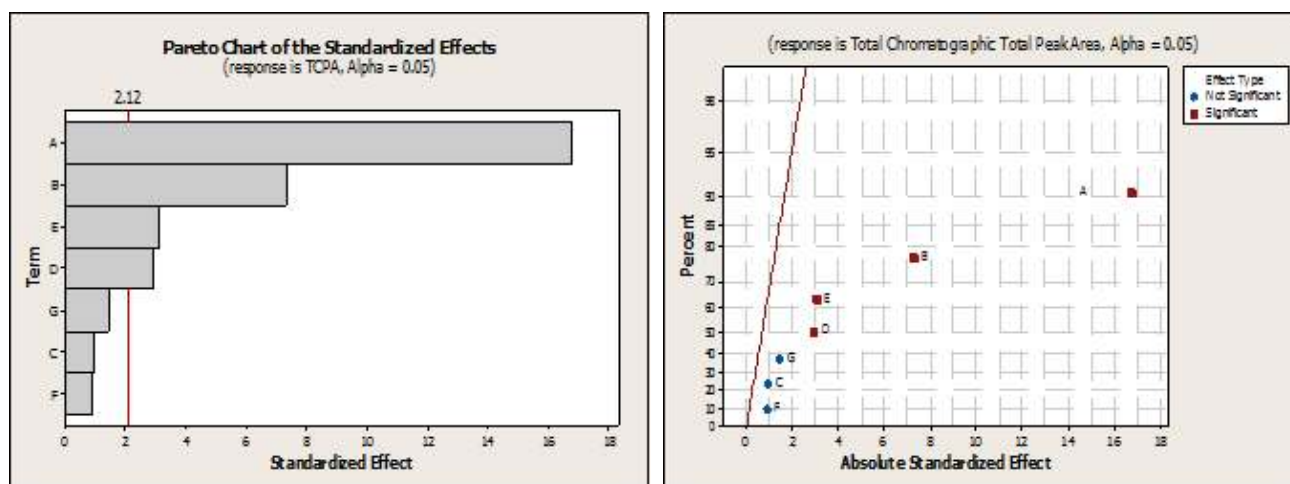
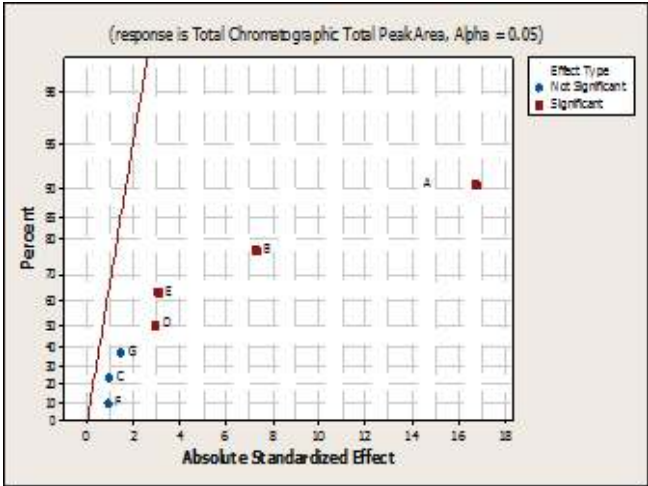
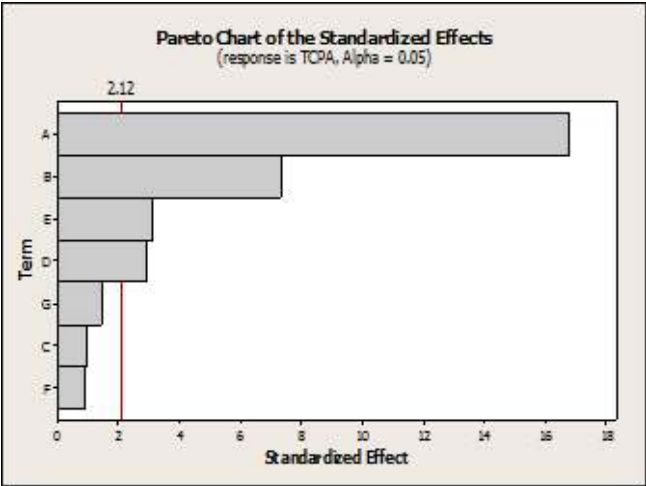


Figure 1: (a) Pareto chart (b) Standardized effect of P-B design showing significant factors

Table 2: Design Matrix for Plackett- Burman design (PBD)

StdOrder	RunOrder	A	B	C	D	E	F	G	H	J	K	L
8	1	1	1	10	2	2	3000	6	6000	2	2	0
4	2	5	1	10	2	0	6000	2	3000	2	2	2
3	3	1	6	10	0	2	3000	2	3000	6	2	2
1	4	5	1	10	0	0	3000	6	6000	6	0	2
2	5	5	6	5	2	0	3000	2	6000	6	2	0
9	6	1	1	5	2	2	6000	2	6000	6	0	2
10	7	5	1	5	0	2	6000	6	3000	6	2	0
12	8	1	1	5	0	0	3000	2	3000	2	0	0
6	9	5	6	10	0	2	6000	2	6000	2	0	0
11	10	1	6	5	0	0	6000	6	6000	2	2	2
5	11	5	6	5	2	2	3000	6	3000	2	0	2
7	12	1	6	10	2	0	6000	6	3000	6	0	0

N.B.: A, mass of sample; B, mass of MgSO₄; C, volume of acetonitrile; D, percentage of acetic acid in acetonitrile; E, mass of NaCl; F, centrifugation speed; G, centrifugation time; H, centrifugation speed for dSPE; J, centrifugation time for dSPE; K, mass of sodium citrate; L, mass of sodium acetate



The RSM screening indicated that some factors were significant to extraction efficiency as illustrated by the Pareto chart (Figure. 1). The Pareto chart of standardized effects is an illustration of horizontal bars for the screened factors and the red vertical line across the bars indicates the level of significant difference (Lawal *et al.*, 2020). The factors found to be significant are mass of sample, mass of MgSO_4 , percentage of acetic acid in acetonitrile, mass of NaCl , and they were further optimized. Therefore, the factors that were not significant (mass of sample, centrifugation speed and mass of salt) were fixed according to the optimal values estimated.

Central Composite Design

The screening experiment obtained by the use of Plackett- Burman design indicates that volume of Acetonitrile, centrifugation speed, centrifugation time, mass of sodium citrate, mass of sodium acetate do not affect extraction efficiency to a significant extent. Therefore, they were fixed according to the optimal value estimated using univariate approach. The mass of sample, mass of MgSO_4 , percentage of acetic acid in acetonitrile and mass of NaCl which are significant variables were further optimized by the second-order central composite design (CCD) utilizing a response surface methodology (RSM). (Tables 3 & 4) These factors increase the extraction efficiency of the QuEChERS technique (Curbelo *et al.*, 2012).

Table 3: The factors of QuEChERS -dSPE Methods

S/N	Factors	Levels	
		Low	High
A	Mass of sample	2	5
B	Mass of MgSO_4	1.5	6
D	Percentage of acetic acid in acetonitrile	0	2
E	Mass of NaCl	0	2

Table 4: Central composite design matrix^a

Std order	Run order	PtType	Blocks	A	B	D	E	Yield
5	1	1	1	2	2.25	1.5	0.875	150
9	2	1	1	2	2.25	0.5	1.625	161
1	3	1	1	2	2.25	0.5	0.875	149
8	4	1	1	4	4.75	1.5	0.875	142
10	5	1	1	4	2.25	0.5	1.625	147
18	6	-1	1	5	3.50	1.0	1.250	165
24	7	-1	1	3	3.50	1.0	2.000	150
29	8	0	1	3	3.50	1.0	1.250	147
27	9	0	1	3	3.50	1.0	1.250	147
3	10	1	1	2	4.75	0.5	0.875	155
26	11	0	1	3	3.50	1.0	1.250	156
4	12	1	1	4	4.75	0.5	0.875	145
21	13	-1	1	3	3.50	0.0	1.250	154
20	14	-1	1	3	6.00	1.0	1.250	161
22	15	-1	1	3	3.50	2.0	1.250	163
16	16	1	1	4	4.75	1.5	1.625	139
15	17	1	1	2	4.75	1.5	1.625	137
7	18	1	1	2	4.75	1.5	0.875	150
19	19	-1	1	3	1.00	1.0	1.250	147
2	20	1	1	4	3.50	0.5	0.875	156
30	21	0	1	3	3.50	1.0	1.250	140
12	22	1	1	4	4.75	0.5	1.625	165
13	23	1	1	2	2.25	1.5	1.625	161
23	24	-1	1	3	3.50	1.0	1.250	159
25	25	0	1	3	3.50	1.0	1.250	147
6	26	1	1	4	2.25	1.5	0.875	175
28	27	0	1	3	3.50	1.0	1.250	156
11	28	1	1	2	4.75	0.5	1.625	160
14	29	1	1	4	2.25	1.5	1.625	149
17	30	-1	1	1	3.50	1.0	1.250	153
31	31	0	1	3	3.50	1.5	1.250	151

^aGenerated using Minitab Statistical Software version 17

Method Validation

Figures of merit of analytical methods is used to confirm the performance of developed method and to determine its suitability for the intended use. The developed method was validated by estimating the linearity, recoveries, relative standard deviation (RSD) limit of quantitation and limit of detection according to EU official guideline (SANTE, 2021). The intra-day precision ($n = 3$) was estimated by performing three extractions in a single day, and inter-day precision ($n = 9$) was estimated based on three extractions per day for 3 days, while the accuracy was reported in terms of the average

recoveries of the spiked sample at three different concentration levels. The limits of detection and quantification values were estimated experimentally at a signal-to-noise ratio of 3 and 10 respectively using the standard deviation of the y-intercept of the regression line of the calibration curve. The linearity was estimated using set of calibration curves prepared with concentrations ranging from 4 to 40 $\mu\text{g/kg}$. As shown in Table 5, the calibration curves were linear over the tested concentration range and the correlation coefficients (r^2) were greater than 0.99. Tables 6 shows the precisions and accuracies (relative recoveries) of

the developed method in semolina sample.

The LOD ranges from 0.1810 to 1.1769 µg/kg, while the LOQ ranges from 0.6033 to 3.9230 µg/kg (Table 5). The average recovery ranges from 98.22 – 105.64 % with intra-day and inter-day RSD of 1.00 – 5.13 % and 1.34 – 9.56 %, respectively. The

figures of merit observed in this study is better or aligns with the study of Choochuay et al. (2018); Gotah et al. (2018). Overall, the use of experimental design ensured efficient optimization of the QuEChERS method, leading to improved recovery, reduced variability, and enhanced sensitivity in aflatoxin determination.

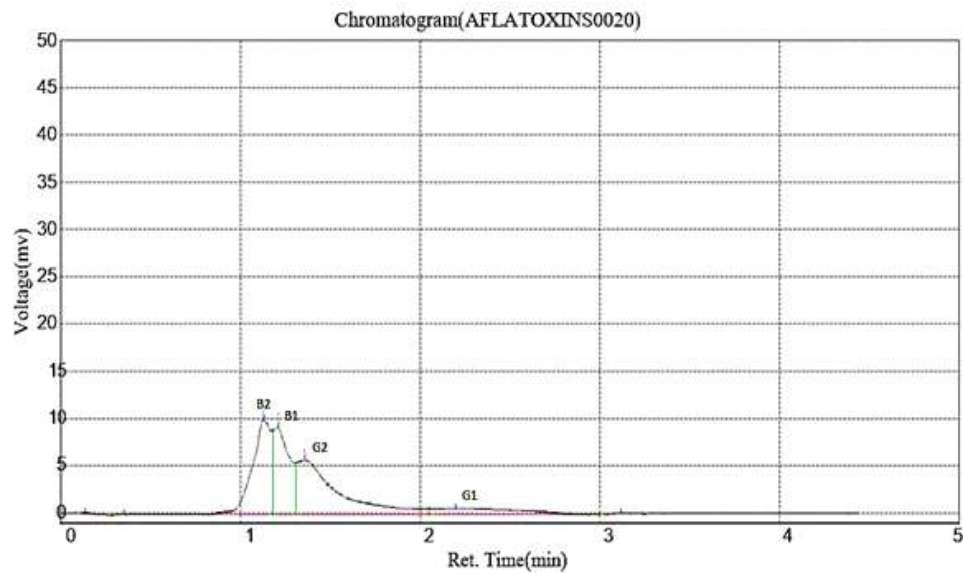


Table 5: Calibration data (4-40 µg/kg), LOD and LOQ of aflatoxins

Analytes	Linearity range µg/kg	Calibration Equation	R ²	LOD (µg/kg)	LOQ (µg/kg)
B1	1.0-4	Y=1802.8+824.16	0.9994	0.1975	0.6583
B2	1.0-30.0	Y=1736.7x+176.03	0.9998	1.1769	3.9230
G1	1.0-40.0	Y=1463.3x+724.09	0.9993	0.1810	0.6033
G2	1.0-30.0	Y=1311.5x+551.41	0.9992	0.3575	1.1917

Table 6: Recovery, inter-and inter-day precision

Aflatoxins	Spike (µg/kg)	RTMM			RFMM			RTGP			RFGP		
		Mean Recovery (%)	Inter RSD (%)	Intra RSD (%)	Mean Recovery (%)	Inter RSD (%)	Intra RSD (%)	Mean Recovery (%)	Inter RSD (%)	Intra RSD (%)	Mean Recovery (%)	Inter RSD (%)	Intra RSD (%)
B1	5	102.54	9.56	2.00	101.14	7.30	2.18	99.49	7.54	1.13	99.92	5.49	1.14
	10	98.22	6.34	3.41	105.64	4.67	5.13	98.78	7.09	1.10	99.08	4.89	1.09
B2	0.5	102.78	5.01	1.00	101.13	4.72	1.00	99.93	1.34	1.10	99.97	1.36	1.23
	1.0	102.65	5.02	1.02	100.78	3.96	1.00	100.20	1.42	1.10	98.98	1.52	1.30
G1	5	104.85	6.12	1.25	101.00	6.52	1.47	101.63	5.44	1.19	101.32	9.40	2.52
	10	103.86	5.45	1.23	101.09	6.42	1.34	101.56	4.89	1.07	100.56	8.97	2.34
G2	0.5	101.08	8.03	2.85	99.68	7.78	1.56	101.03	5.31	1.21	101.04	5.43	2.45
	1.0	101.09	7.89	2.74	99.08	7.72	1.45	101.40	5.35	1.23	100.86	5.34	2.64

Table 7: Analysis of Real Sample for Aflatoxin Content ($\mu\text{g/kg}$)

Analytes	Day 1				Day 7				Day 14				Day 21			
	RTMM	RFMM	RTGP	RFGP	RTMM	RFMM	RTGP	RFGP	RTMM	RFMM	RTGP	RFGP	RTMM	RFMM	RTGP	RFGP
B1	6.4565	5.6231	8.4569	7.9761	8.4561	4.3421	8.9562	6.4561	9.0321	3.9562	9.7654	5.3421	9.8798	2.9673	11.9752	3.6721
B2	7.5615	6.7490	8.7653	7.8921	8.9877	5.3410	8.0123	5.9873	9.0721	4.9862	9.6824	5.0045	9.6744	1.9865	ND	2.9875
G1	8.7358	6.3428	9.5621	8.5672	7.9877	4.9876	9.6754	7.1320	8.9654	4.0032	10.0032	4.9842	9.0974	1.9641	12.0987	1.9864
G2	4.7278	7.0891	6.5682	6.7241	7.7621	3.9843	9.9895	7.1450	7.9895	4.1242	10.1132	5.9832	8.9562	2.5632	11.7862	3.0078

Analysis of Real Samples

The developed method was used for the analysis of real food samples and the results indicated that there were distinct differences concentration of aflatoxins in food stored at room temperature and those stored in the refrigerator (Table 7). The concentration found at both storage conditions were within the European Unions recommended level of aflatoxins. However, the concentration of aflatoxins in the sample stored in the refrigerator were found to be lower than those stored at room temperature, which is in agreement with the result of Omotayo *et al.*, (2019) who determined the storage effects on the concentration of aflatoxins in ginger. The high concentration of aflatoxins in samples stored at room temperature could also result of exposure of the samples to atmospheric air which contains several contaminants. Storage facilities and unhygienic storage of food samples in shops or houses where environmental factors (temperature, humidity and moisture) are not controlled might enhance the growth of fungi and production of mycotoxins (Tia *et al.*, 2016). The effects of duration of storage (1, 7, 14 and 21 days) at room temperature, showed that the concentration of aflatoxins B1, B2, G1 and G2 in Mama gold stored at room temperature (RTMM) increased with days of storage, while it decreases with number of days of storage in Mama Gold stored in the refrigerator (RFMM). Similar effect was found in Golden penny stored at room temperature (RTGP) and the Golden penny stored in the refrigerator (RFGP). In the first day, the concentration of aflatoxins B1, B2, G1 and G2 in RTMM was found to be 6.4565, 7.5615, 8.7358m and 4.7278 $\mu\text{g/kg}$ respectively and were respectively found to 5.6231, 6.7490, 6.3428 and 7.0891 $\mu\text{g/kg}$ in RFMM. After 21 days of storage at different conditions, the concentrations were found to be 9.8798, 9.6744, 9.0974 and 8.9562 $\mu\text{g/kg}$ of aflatoxins B1, B2, G1 and G2 respectively in RTMM, and was found to be 2.9673, 1.9865, 1.9641 and 2.5632 $\mu\text{g/kg}$ of aflatoxins B1, B2, G1 and G2 respectively. Similar trend was observed in golden penny, which were found to contain aflatoxins B1, B2, G1 and G2 at concentration of

8.4569, 8.7653, 9.5621 and 6.7241 $\mu\text{g/kg}$ respectively at day 1 for golden penny stored at room temperature, which increased to 11.9752 $\mu\text{g/kg}$ for B1, 12.0987 $\mu\text{g/kg}$ for G1 and 11.7862 $\mu\text{g/kg}$ for G2 after 21 days, while aflatoxin B2 was not detected at day 21. On the other hand aflatoxin content of the golden penny stored in the refrigerator was found to decrease with days, containing 8.4569, 8.7653, 9.5621 and 6.5682 $\mu\text{g/kg}$ of aflatoxins B1, B2, G1 and G2 respectively at day 1, but after 21 days of storage, the aflatoxin content was found to decrease to 3.6721 for aflatoxin B1, 2.9875 $\mu\text{g/kg}$ for aflatoxin B2, 1.9864 $\mu\text{g/kg}$ for aflatoxin G1 and 3.0078 $\mu\text{g/kg}$ for aflatoxin G2. The Mama gold semolina was found to contain the highest concentration of all the aflatoxins investigated in this study compared to Golden penny.

The concentration of aflatoxins detected in the samples were found to be higher than the maximum of 4 $\mu\text{g/kg}$ according to the European Union (EU) regulation (European Commission, 2023), which showed a potential health risk, but was found to be lower than the US Food and Drug Administration's limit of 20 $\mu\text{g/kg}$ for total aflatoxin. The increase in aflatoxin levels during storage especially at room temperature, indicates the importance of proper storage conditions and timely consumption to minimize exposure. Refrigeration was also found to reduce aflatoxin formation, but did not completely prevent the development and growth of aflatoxins, suggesting that long term storage under refrigerated conditions may still pose a health risk to consumers.

Conclusion

A rapid, effective, simple and quick method based on DSPE clean up coupled with HPLC was used to identify AFB1, AFB2, AFG1 and AFG2 in semolina in this study. The developed method was successfully used to analyse two different brands of semolina (mama gold and golden penny) collected at different market places in Ilorin. Thus, satisfactory linearity, precision and accuracy were obtained. The four aflatoxins were separated and no interference peaks were observed. The concentration of aflatoxins in samples kept at two different conditions (room temperature and

refrigerator) was determined and it was observed that aflatoxins had ability to thrive better at room temperature than in the refrigerator due to the high temperature that enabled the fungi to grow.

Plackett –Burman design and central composite design were developed to screen the factors affecting extraction efficiency using RSM analysis. The four significant variables (mass of sample, mass of MgSO_4 , percentage of acetic acid in acetonitrile, mass of NaCl) determined by employing Plackett-Burman design experiment was found to have linear effects on determination of aflatoxin at 0.05 level of significant (Kong *et al.*, 2012).

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