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Abstract
The bright greenish yellow fluorescence (BGYF) test has been used with varying success in screening for aflatoxins in maize. This test was applied to 180 maize samples collected from different markets within 12 districts of Malawi in order to evaluate its performance against high performance liquid chromatographic analysis. The number of BGYF grains in 2.5 kg unground samples ranged from 0 to 35 and about 49% of all tested samples had aflatoxin ranging from 1 to 382µg/kg. A total of 65(36%) of the examined unground samples showed no BGYF. The European Commission (Decision 2002/657/EC) recommends a false negative rate of less than 5%, for a screening technique to be acceptable. In this study, 4 BGYF grains per 2.5 kg unground maize sample successfully indicated aflatoxin contamination of >10 µg/kg (COMESA maximum tolerable limit), with a 4.4% false negative rate. In this case, the amount of confirmatory analyses would be reduced by 63%, if the BGYF test was employed as a screening method. The screening technique therefore offers a practical screening tool for Malawi and possibly for the sub-Saharan region.

Keywords: Maize, aflatoxin, BGYF, screening, Malawi

1.0 Introduction

Maize is the most important food crop in Sub-Saharan Africa. In Malawi, it contributes significantly to the diet of more than 80% of the population (Pingali, 2001). In this context, contamination of maize with mycotoxins, mainly aflatoxins, is a matter of concern for food quality control (Matumba *et al.*, 2009). Aflatoxins are toxic secondary metabolites produced by several species of filamentous *Aspergillus*, including *A. flavus, A. parasiticus and A. nomius* on a wide variety of food matrices. They are immunosuppressant, mutagenic, teratogenic, carcinogenic and cause growth retardation in humans and animals (IARC, 1993; Khlangwiset *et al.*, 2011; Preisler *et al.*, 2000; Wangikar *et al.*, 2005) and as such, aflatoxins are regulated world-wide (Van Egmond *et al.*, 2007).

The presence of aflatoxins is determined by rapid presumptive and screening tests after which positive results are confirmed using chromatographic techniques such as high-performance liquid chromatography (HPLC) with fluorometric detection, liquid chromatography with mass spectrometry (LC-MS) and tandem mass spectrometry (LC-MS/MS) (Anklam *et al.*, 2002; EC, 2002; Shephard, 2009). However, confirmatory tests are expensive and often unavailable in most developing countries, such as Malawi. The use of screening techniques reduces the number of samples that must be tested using confirmatory methods and thus reduces the cost of analyses. Unfortunately for the developing world, available screening techniques (e.g.

ELISA) are still expensive, not readily available and often require special storage such as refrigeration for antibodies (Goryacheva and Saeger, 2011).

The presence of bright greenish yellow fluorescence (BGYF) is used as a presumptive test (black light test) to identify maize lots that may contain aflatoxins. Basically, the BGYF test involves inspection of samples under a "black" or long-wave ultraviolet light (365 nm) in a dark cabin or room. The presence of BGYF is due to a product of a peroxidase catalyzed reaction of kojic acid (2-hydroxymethyl-5-hydroxy-γ-pyrone). Kojic acid is formed by *A. flavus and A. parasiticus* (Basappa *et al.*, 1970). The BGYF test is fast and requires no reagents and has been used with varying success (Bothast and Hesseltine, 1975; Dickens, 1987; Dickens and Whitaker, 1981; Glória *et al.*, 1998; Maupin *et al.*, 2003; Schmitt and Hurburgh Jr, 1989; Shotwell and Hesseltine, 1981). The differences in the performance of the method could be attributed to variance in *Aspergillus* strains across study ecologies since *Aspergillus* strains have varying kojic acid and aflatoxin production abilities (Basappa *et al.*, 1970).

Nevertheless, the BGYF test is an approved Official Method 45-15.1 of the American Association of Cereal Chemists (AACC, 2000). The BGYF test therefore offers a simple screening tool for the developing world. However evaluation studies need to be carried out before its adaptation. In this context, the objective of this study was to evaluate the BGYF test for screening aflatoxin contamination in maize from Malawi and possibly the sub-Saharan region. Such a tool would be instrumental in dietary aflatoxin risk management.

2.0 Materials and methods

2.1. Maize samples

A total of 180 (2.5kg) samples of shelled white maize were collected in May 2012 from markets in 12 districts of Malawi namely: Karonga, Mzimba, Nkhatabay, Ntchisi, Salima, Lilongwe, Dedza, Mangochi, Blantyre, Phalombe, Mulanje, and Chikhwawa.

2.2. BGYF test

The entire working maize sample (2.5 kg) was examined in a light-tight viewing cabinet fitted with a long wave UV lamp (Luckham, Sussex, UK) at 365nm. The maize was spread at the base (66 cm by 46 cm) of the cabinet in batches in such a way that grains were not lying on each other until the entire working sample was examined. Each maize batch was turned twice to ensure that all sides of the maize grains were viewed. The maize sample was viewed under UV light for an accumulated period of up to 10 minutes. Grains that exhibited BGYF were counted and temporarily stored separately. The remaining sample (excluding the grains that exhibited BGYF) was coarsely broken using a laboratory mill (Christy and Norris Ltd) without a sieve. A sub-sample (1kg) was examined again in order to check for grains that could exhibit BGYF only after breaking. Average particle sizes of the broken maize grains were determined by passing ten 1kg subsamples through 2.0 and 4.0 MM laboratory test sieves. (Endocotts, London, England). The number of BGYF exhibiting particles was estimated and coded as follows: 1 (< 5 particles), 2 (5-10 particles), 3(11-20) particles, 4(21-50 particles), 5(50-10 particles) and 6(>100 particles).

2.3. Aflatoxins analysis by HPLC-FLD

2.3.1. Chemical and reagents

Acetonitrile, methanol and HPLC-grade water were supplied by Merck (Darmstadt, Germany). 5.0 μg/mL total aflatoxins (Aflatoxin B1 (AFB1)/AFB2/AFG1/AFG2, 4/1/4/1) were purchased from Trilogy Analytical Laboratory (Washington, MO, USA). After

reconstitution in 10mL acetonitrile, the stock standard was kept securely at -15 °C, wrapped in aluminium foil to avoid photo-degradation and held for 6 months. Working aflatoxins standard solutions were made by diluting the stock solution in methanol/water (50/50, v/v).

2.3.2. Precautions and safety consideration

Aflatoxins are carcinogenic compounds; consequently, disposable latex gloves were worn at all times during handling of solutions, extracts and samples only in properly ventilated hoods. Aflatoxin residues on laboratory ware, pipette tips and kit components were destroyed using 10% solution of household bleach before discarding. Accidental spills of aflatoxins were swabbed with 5% NaOCl bleach.

2.3.3. Extraction and clean-up

After the BGYF test, the entire sample (BGYF exhibiting grains + the broken grains) was thoroughly mixed, ground using a laboratory blender (Waring Products, New Hartford, Connecticut) and fine-milled using a laboratory mill (Christy and Norris Ltd, Suffolk, UK) to pass a sieve #20. A modified procedure for extraction and clean-up of aflatoxin in maize grains and feed (Neogen Corporation, Scotland) was then used. Briefly, a sub-sample (30.0g) of the finely ground maize was added to 3.0g of NaCl and extracted with 60.0 mL of methanol/water (80/20, v/v) in a covered flask. The suspension was shaken using an orbital shaker (SSL1, Stuart, Bibby Scientific Limited, Staffordshire) set at 220 rpm for 90 minutes. The extract (10.0 mL) was diluted four fold with HPLC grade water and filtered twice (first through a coarse fluted filter, and second through a glass filter) before passing 20.0 mL of the diluent through a neocolumn® aflatoxin (wide bore) column (Neogen Corporation, Scotland UK). The column was then washed with 23.0 mL of water/methanol (75/25, v/v) to remove maize intrinsic compounds and finally the aflatoxins were selectively eluted with 2.0 mL of 100% methanol followed by 2.0 mL of 100% HPLC water. The total volume of the eluent (4 mL) was mixed using a vortex for 30 seconds after which a sub-sample was ready for HPLC analysis.

2.3.4. Aflatoxins determination using HPLC- FLD

Determination of aflatoxins was done using Agilent 1200 Series HPLC System (Agilent, Waldbronn, Germany) consisting of G1322A degasser, a G129A autosampler, a G1330B thermostat, a CY1311A quaternary pump, a G1316A temperature controller and a G1321A fluorescence detector (FLD). Chromatographic separation was achieved using a ZORBAX Eclipse® XDB-C18 column (150mm×4.6mm i.d., particle size 5μm), protected by a C18 security guard cartridge, 4×3 mm i.d. (all supplied by Agilent Technologies). An isocratic mobile phase of water/ methanol/ acetonitrile (55/35/10 v/v/v) was used at a flow rate of 1.0 mL/min. The column oven temperature was maintained at 30°C and the injection volume was 20 and 40 μL for standards and samples, respectively. Post-column derivatization was achieved using a photochemical reactor for enhanced detection (LCTech UVE, Dorfen, Germany). Fluorescence excitation and emission wavelengths were set at 365 and 440 nm, respectively. Retention times of AFG2, AFG1, AFB2 and AFB1 were 5.6, 6.5, 7.7 and 9.1 minutes respectively. Data acquisition and processing was achieved using chromatographic software (ChemStation®).

Aflatoxin determination in samples was based on a six point external standard calibration curve, using a mixture of aflatoxin standards (AFB1 and AFG1, each ranging from 0.75 to 60 ng/mL, and AFB2 and AFG2, ranging from 0.19 to 15 ng/mL). Recovery rates were calculated using maize samples spiked with 25.5 μg/kg of total aflatoxins. The results were corrected by mean recovery rates obtained from the recovery experiments (Table 1).

2.4. Data analysis

For data evaluation, all analytical values less than the limit of detection (LOD) (Table 1) were treated as ½ LOD and values of ½ limit of quantification (LOQ) (Table 1) were assigned to analytical values ≥LOD and <LOQ. Data were evaluated by analysis of variance (ANOVA) and Tukey's HSD test was used to compare the means of aflatoxins across the districts. Since aflatoxin concentration in the samples was not normally distributed, data were log transformed before analyses. Simple linear regression was used to determine the correlation between aflatoxin concentrations and the number of BGYF grains in the samples. Spearman correlation was used to determine the relationship between the number of BGYF grains in unground maize samples and the number of BGYF particles that were seen in coarsely broken samples. The analyses were conducted using SPSS version 15 (SPSS inc., Chicago, IL, USA).

3.0 Results and Discussion

3.1. HPLC-FLD method performance

Mean recoveries of maize samples spiked with AFB1 and AFG1, (each at 10 μg/kg) and AFB2 and AFG2 (each at 2.5 μg/kg) are provided in Table 1. The relative standard deviations (RSD) of the recoveries were generally low (≤ 4.1) for all types of aflatoxins which demonstrated that the method was well under control during the analytical sessions and the values complied with the European Commission Regulation (EC) No 401/2006 (EC, 2006). All calibrations curves had $R^2 \geq 0.9995$. LOQ for individual aflatoxins were all below 1 μg/kg.

3.2 Occurrence of BGYF grains and aflatoxins in analyzed samples

A summary of data on the occurrence of BGYF grains and the total aflatoxins (AFB1+AFB2+AFG1+AFG2) is provided in Table 2. The number of BGYF grains in unground samples ranged from 0 to 35. About 49% of all the tested samples had aflatoxins ranging from 1 μg/kg to 382 μg/kg. Aflatoxins were detected in at least one sample from all the districts except Lilongwe and Ntchisi. District mean total aflatoxins ranged from 0.3 to 156 μg/kg, with significant variance between districts. Samples from Chikhwawa district had significantly higher aflatoxins than the other districts (except Phalombe district). In fact, all samples from Chikhwawa were distinctly contaminated with aflatoxins above LOD, with a lowest value of 2.9 μg/kg. The Common Market for Eastern and Southern Africa (COMESA), of which Malawi is a member, has proposed maximum tolerable limits (MTL) of 10μg/kg for total aflatoxins in unprocessed cereals (COMESA, 2012). Under these regulations, 77% and 78% of the maize samples in Chikhwawa and Phalombe, respectively, were non-compliant. In a separate survey conducted at the end of July 2011, 75% of the samples collected from Nsanje, Chikhwawa's neighboring district aflatoxins exceeded the COMESA limit whereas consistently low aflatoxin levels were detected in samples from Mzimba, Lilongwe and Dedza districts (Matumba *et al.*, manuscript in preparation). However, vendor interviews revealed that 60% of the samples that were collected from Chikhwawa district had been purchased from high and mid altitude districts of Dedza (mean monthly temperature (mean±std.dev): 13.3±4.2˚C (min), 22.5±5.1˚C (max), years 1971-2012) and Lilongwe (mean monthly temperature (mean±std.dev): 14.0±5.3 ˚C (min), 27.1±4.7˚C (max), years 1971-2012) respectively and transported before drying thoroughly. The differences in prevalence rates and concentrations of aflatoxin across districts therefore suggest Chikhwawa district presented a more favourable climatic condition for fungal growth and aflatoxin production before the maize was dried. Indeed Chikhwawa district has a low altitude (< 200m above sea level), with a hot climate (mean monthly temperature (mean±std.dev): 20.0±3.6 ˚C (min); 32.5±3.5 ˚C

(max), years 1971-2012). The climatic conditions are likely to have favoured fungal and aflatoxins contamination (Cotty and Jaime-Garcia, 2007). Moreover, under favourable conditions *Aspergillus* fungi are capable of producing aflatoxin within 24 hours after infestation (Gwinner *et al.*, 1996) and therefore transporting the maize before drying may have increased the risk of aflatoxin contamination.

It is also worth noting that the aflatoxin concentrations reported in this study are higher than those generally reported for Sub Saharan Africa (Bankole *et al.*, 2006). An exception is the very high aflatoxin levels (> 1000 µg/kg) reported for maize samples from Kenya (Lewis *et al.*, 2005; Probst *et al.*, 2007). However, since most of maize production in Malawi is realized in high and mid altitude areas (FEWSNET_Malawi, 2006), the present results indicate the country's potential of producing maize with low aflatoxin contamination.

3.3. Relation between aflatoxin concentration and number of BGYF grains.

Data on the relationship between aflatoxin and BGYF grains in unground samples is provided in Table 3. A total of 65(36%) unground samples showed no BGYF, of which 58 (89%) had aflatoxin contamination of less than 1 µg/kg. Six (6) of the samples that exhibited no BGYF had an aflatoxin contamination between 1 and 10 µg/kg, and one sample contained 11 µg/kg aflatoxin (Table 3).

A total of 89 samples had aflatoxin levels of ≥1µg/kg. If the presence of at least one (1) BGYF exhibiting grain was used as an indicator of aflatoxin contamination of ≥1µg/kg, 115 of 180 samples would have been accepted by the BGYF test. However, 7 of 89 aflatoxin positive samples would have been falsely classified as complying samples, thus representing a 7.8% false negative rate. This screening criterion would therefore not satisfy European Commission Decision 2002/657/EC which calls for a less than 5% false negative rate at the level of interest (EC, 2002). In addition, the criterion could also falsely classified 33 samples with actual concentration of ≤1µg/kg as a positive representing 29 % false positive rate.

A total of 59 samples had aflatoxin levels above COMESA maximum tolerable limit (10 µg/kg). If the presence of at least 4 BGYF grains per 2.5kg sample was used as an indicator of non-compliance with the COMESA standard, a total of 114 samples could have passed the BGYF test. Therefore, 5 samples containing aflatoxin levels of > 10 µg/kg would have falsely been classified as complying, representing 4.4% false negative rate. This would be acceptable according to EU standards. In this case, 12 of 66 samples with actual aflatoxin concentration of ≤10µg/kg could have falsely been classified as positive, representing 18 % false positive rate. This criterion could reduce the number of confirmatory analyses to only 66 (37% of the total number of sample) hence reduce the costs.

A strong significant ($p<0.01$) positive correlation ($R^2 = 0.83$) was observed between the number of BGYF grains in unground maize samples and the number of BGYF particles that were missed during unground examination and were only detected after coarsely grinding the remaining sample (Table 4). Therefore, coarsely grinding the samples and the subsequent BGYF examination was not necessary since the examination of unground maize already gave satisfactory results.

In the present study, a weak correlation ($R^2=0.53$) was obtained between aflatoxin concentration and the number of BGYF exhibiting grains in a 2.5 kg sample (Figure 1). However, a strong correlation between mass fractions of BGYF grains and aflatoxin has been reported for maize samples from eastern North Carolina (Dickens, 1987). The variation in the

findings is attributable to the variance in production ability and pathways of the synthesis of kojic acid and aflatoxins among *Aspergillus* strains (Basappa *et al.*, 1970). It is this variance that necessitates studies on validation of the BGYF test in different ecologies. The present results indicate that BGYF test could not be used to quantitatively estimate aflatoxin concentration but could rather effectively be used to screen samples with > 10 μg/kg aflatoxin content.

4.0 Conclusions

The performance of BGYF screening test for aflatoxin contamination in maize has been evaluated in Malawi. The results show that the presence of at least 4 BGYF grains in maize samples (2.5kg) in Malawi could potentially be used to screen for > 10 μg/kg aflatoxin content maize samples. This screening criterion could reduce the number of samples to be confirmed by reference methods thus reducing the cost of managing aflatoxin in maize in Malawi. However it is worth noting that some aflatoxins may be lost in the process of transferring and re-mixing of maize grains during the BGYF screening and HPLC quantification and thus affecting aflatoxin quantification especially at low concentrations. Despite this limitation, the methodology in its present form still offers a practical screening tool for Malawi and possibly for sub-Saharan region.

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Table 1: Recoveries, Limit of Detection and Limit of Quantification of aflatoxins in spiked Maize samples

Analyte	LOD[a] (µg/kg)	LOQ[a] (µg/kg)	Mean Recovery (RSD)% [b]
AFB_1	0.2	0.7	90(3.5)
AFB_2	0.08	0.3	93(4.1)
AFG_1	0.2	0.7	97(3.7)
AFG_2	0.08	0.3	95(2.8)

[a]LOD limit of detection [S/N = 3/1] and LOQ limit of quantification [S/N = 10/1] both expressed as µg/kg sample

[b]Mean recoveries of five (5) analyses of maize spiked with AFB_1 and AFG_1 (each at 10 µg/kg) and AFB_2 and AFG_2 (each at 2.5 µg/kg)

Table 2: Occurrence of BGYF grains and aflatoxins in maize in some districts of Malawi

District	No. of Samples	No. of BGYF grains in unground samples (2.5 kg)		Total aflatoxin concentration (AFB_1+AFB_2+AFG_1+AFG_2) (µg/kg)[1]		No. of samples above 10 µg/kg[a] (%)
		Range	Mean	Range	Mean	
Karonga	4	0-5	2.5	0.3[*]-107.9	31.6 bc[2]	2(50)
Nkhata-bay	24	0-11	2.0	0.3-108.6	9.4 c	4(16.7)
Mzimba	15	0-3	0.6	0.3-9.6	1.2 c	0
Ntchisi	7	0-2	0.4	0.3-0.3	0.3 c	0
Salima	16	0-15	2.8	0.3-52.2	7.2 c	3(18.8)
Lilongwe	17	0-3	0.5	0.3-0.3	0.3 c	0
Dedza	15	0-9	1.8	0.3-96.6	9.3 c	0
Mangochi	14	0-26	7.1	0.3-381.9	55.5 bc	7(50)
Blantyre	18	0-11	2.9	0.3-76.9	15.4 c	7(38.9)
Mulanje	15	0-9	2.5	0.3-44.5	9.6 c	6(40)
Phalombe	9	2-28	9.9	0.3-318.4	122.2 ab	7(77.8)
Chikhwawa	26	2-35	10.5	2.9-351.1	155.5 a	20(76.9)

[a]Maximum tolerable level for raw maize for EU also proposed by COMESA

[*]Value of 0.3 is the sum of ½ LODs for AFB_1, AFB_2, AFG_1 and AFG_2

[1]Aflatoxin concentration as quantified by the HPLC-FLD

[2] Means in the same column followed by the same letter are not significantly different ($p>.05$) according to Tukey's HSD test or t-test

Table 3: Relation between aflatoxin concentration and number of BGYF grains in unground samples (2.5 kg)

Aflatoxins Concentration (μg/kg)*	Number of BGYF grains							Total No. of samples
	0	1	2	3	4	5-10	>10	
<1	58	19	10	3	0	1	0	91
1≤10	6	3	6	4	2	8	1	30
>10-20	1	0	0	1	6	6	0	14
>20-100	0	0	0	3	2	13	6	24
>100	0	0	0	0	0	10	11	21
Total No. of samples	65	22	16	11	10	38	18	180

Highlighted and bordered with continuous and dotted lines indicate false negative and positive results respectively if presence ≥4 BGYF grains would be used to screen for samples with >10 μg/kg sample

*Aflatoxin concentration as quantified by HPLC-FLD

Table 4: Relation between BGYF grains in unground maize samples (2.5 kg) and BGYF broken particles in coarsely ground samples (1.0 kg) missed during viewing of the unground samples.

Number of BGYF broken particles*	Number of BGYF grains							Total
	0	1	2	3	4	5-10	>10	
<5	63	20	13	10	8	21	4	139
5-10	2	2	3	1	0	10	5	23
11-20	0	0	0	0	2	6	9	17
20-50	0	0	0	0	0	0	1	1
Total	65	22	16	11	10	37	19	180

Particle sizes of the broken grains obtained after coarsely breaking the sample were: 26% (< 2.0 MM), 44% (2.0-4.0 MM), and 30% (>4.0 MM) particles

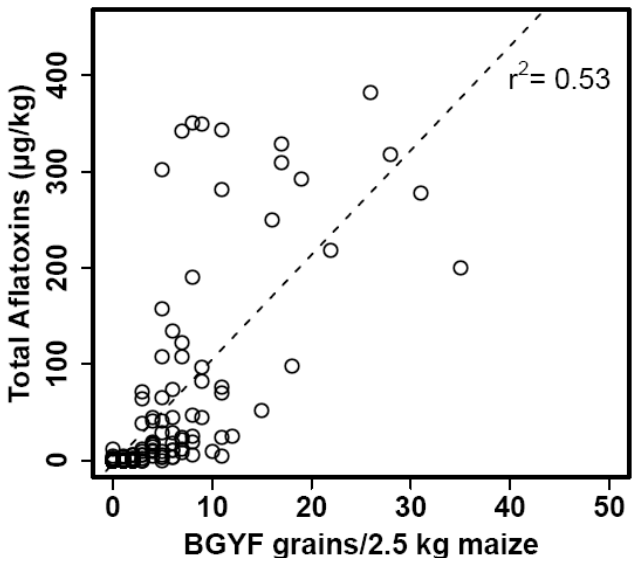

Figure 1: Scatterplot of aflatoxin concentration (µg/kg) as quantified by HPLC-FLD versus the number of BGYF grains in a 2.5 kg sample