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ORIGINAL PAPER

Uncommon occurrence ratios of aflatoxin B$_1$, B$_2$, G$_1$, and G$_2$ in maize and groundnuts from Malawi

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Abstract We report an unusual aflatoxin profile in maize and groundnuts from Malawi, with aflatoxin G$_1$ found routinely at equal or even higher levels than aflatoxin B$_1$. Aflatoxin B$_1$ (AFB$_1$) ratio in a contaminated sample is generally greater than 50 % of total aflatoxin (sum of aflatoxin B$_1$, B$_2$, G$_1$, and G$_2$). In Malawi, the aflatoxin occurrence ratios were determined by examining LC-MS/MS and HPLC fluorescence detection (FLD) data of 156 naturally contaminated raw maize and 80 groundnut samples collected in 2011 and 2012. Results showed that natural aflatoxin occurrence ratio differed. In 47 % of the samples, the concentration of AFG$_1$ was higher than that of AFB$_1$. The mean concentration percentages of AFB$_1$/AFB$_2$/AFG$_1$/AFG$_2$ in reference to total aflatoxins were found to be 47:5:43:5 %, respectively. The AFG$_1$ and AFB$_1$ 50/50 trend was observed in maize and groundnuts and was consistent for samples collected in both years. If the AFB$_1$ measurement was used to check compliance of total aflatoxin regulatory limit set at 10, 20, 100, and 200 μg/kg with an assumption that AFB$_1$≥50 % of the total aflatoxin content, 8, 13, 24, and 26 % false negative rates would have occurred respectively. It is therefore important for legislation to consider total aflatoxins rather than AFB1 alone.

Keywords Aflatoxin ratios · Maize · Groundnuts · Malawi

Introduction

Aflatoxins are toxic and carcinogenic polyketide-derived secondary metabolites that are produced mainly by certain strains of the *Aspergillus* genus on a wide range of matrices. Most reports have indicated *Aspergillus flavus* and *Aspergillus parasiticus* as major aflatoxin producers, but discovery of more novel aflatoxin producers continues (Horn 1997; Ito et al. 2001; Peterson et al. 2001; Pildain et al. 2008; Varga et al. 2012). Four major naturally occurring aflatoxins include aflatoxin B$_1$ (AFB$_1$), AFG$_1$, AFB$_2$, and AFG$_2$ (in order of decreasing toxicity) (IARC 1993). *A. flavus* normally produces aflatoxin Bs, while *A. parasiticus* produces both aflatoxin Bs and Gs. Other important species that produce both aflatoxins B and G include *Aspergillus toxicarius*, *Aspergillus nomius*, *Aspergillus bombycis*, *Aspergillus parvisclerotigenus*, *Aspergillus minisclerotigenes*, and *Aspergillus arachidicola* (Varga et al. 2009).

The biosynthetic pathway of aflatoxins has been extensively studied and elucidated (Yabe and Nakajima 2004, 1988, 2003; Yu et al. 2004, 2013) and has been estimated to involve up to 27 enzymatic steps (Ehrlich and Yu 2009). It has been found that the different forms of aflatoxin share common pathways that later branch to form AFB$_1$, AFB$_2$, AFG$_1$, and AFG$_2$. It was established through feeding studies that AFB$_1$ and AFG$_1$ (both containing dihydrobisfuran rings) are produced from *O*-methylsterigmatocystin and that AFB$_2$ and AFG$_2$ (both containing tetrahydrobisfuran rings) are produced from dihydro-*O*-methylsterigmatocystin (Bennett and Goldblatt 1973; Bhatnagar et al. 1987; Yabe et al. 1988).

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Experimental results further demonstrated biosynthetic independence of AFB_1 and AFB_2 (Bhatnagar et al. 1987; Yabe et al. 1988) and AFG1 and AFG2 (Yabe et al. 1999).

Different generalized occurrence ratios of the four aflatoxins have been reported (European Commission EC 2012; Kensler et al. 2011; Van Egmond and Jonker 2004), but all agree that AFB_1 concentration generally exceeds half of the sum of the aflatoxins and that AFB_2 and AFG_2 occur in the lowest concentrations. In the same regard, several countries have set separate regulatory limits for AFB_1 at half the regulatory limit of the sum of the four aflatoxins (Van Egmond and Jonker 2004). Likewise, analytical methods for quantifying AFB_1 alone in various matrices have been developed (Ardic et al. 2008; Lee et al. 2004; Yu et al. 2013).

Experimental findings indicate that the ratio of aflatoxin B and G concentrations is greatly influenced by temperature cycling (Lin et al. 1980; Schmidt-Heydt et al. 2010) and population ratios of fungal strains on given matrices (Wilson and King 1995). Furthermore, gene cluster analysis of AFG_1-dominant *A. parasiticus* (ratio $AFG_1/AFB_1>5$) revealed a history of mutation (Carbone et al. 2007). These findings imply that AFB and AFG concentration ratios could be regionally dependent; however, there are hardly any occurrence data on this aspect. In this regard, the present study was undertaken to investigate the occurrence ratios among aflatoxins B_1, B_2, G_1, and G_2 and get insights about the types of aflatoxigenic fungi present in Malawi.

Methodology

A meta-analysis was done on aflatoxin (AFB_1, AFB_2, AFG_1, and AFG_2) positive results of raw maize and raw groundnut samples from the lake shore, middle, and upper-Shire-, mid-elevation-, lower-Shire agro-ecological zone and unspecified locations within Malawi (Fig. 1). This included analytical results of 80 raw groundnuts and 125 raw maize samples measured by immunoaffinity column cleanup coupled with high-performance liquid chromatography and on-line post-column photochemical derivatization-fluorescence detection (IAC-HPLC-PCD-FLD) and 31 raw maize samples measured by LC-MS/MS. The IAC-HPLC-PCD-FLD and LC-MS/MS methodologies used for aflatoxin analysis were similar to those described by Matumba et al. (2014a) and Malachova et al. (2014), respectively. In all cases, the aflatoxin analysis involved a subsample drawn from milled aggregated sample of at least 1-kg mass. In terms of proportions, the dataset comprised of 39.0 % of samples from the upper-Shire agro-ecological zone, 22.5 % from the mid-elevation agro-ecological zone, 21.6 % from the lower-Shire agro-ecological zone, and 16.9 % from unspecified locations.

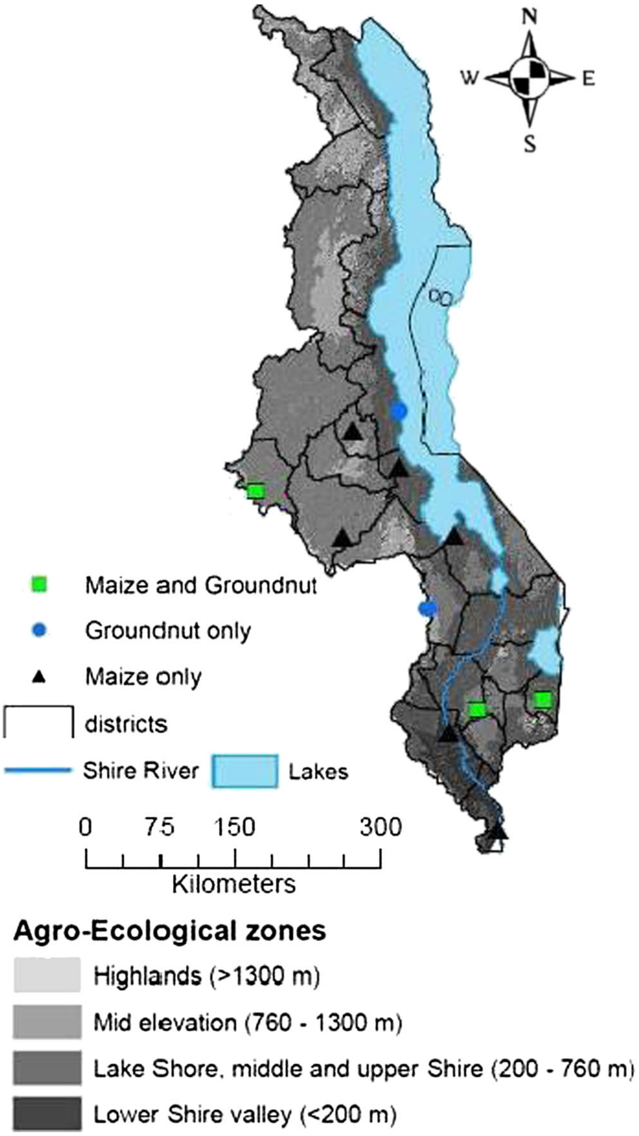

Fig. 1 Sampling sites in different agro-ecological zones of Malawi

Table 1 Co-occurrence matrix for aflatoxin in raw maize and groundnut samples from Malawi

	AFB_1	$AFB_1 + AFB_2$
No AFGs	4.7 %	1.7 %
AFG_1	95.3 %	86.0 %
AFG_2	78.8 %	77.5 %
$AFG_1 + AFG_2$	78.8 %	77.5 %

Limits of quantification of the analytical results included in this meta-analysis were 0.2 μg/kg for AFB_1 and AFG_1 and 0.1 μg/kg for AFB_2 and AFG_2 (IAC-HPLC-PCD-FLD). For the LC-MS/MS method, LOQs for each of the four aflatoxins were 1.3 μg/kg. Quality control in the aflatoxin IAC-HPLC-

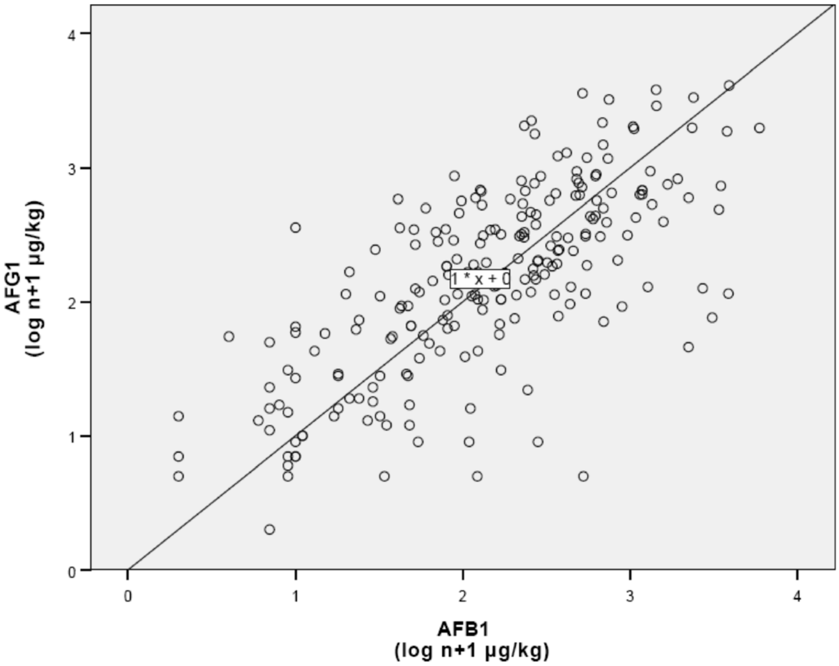

Fig. 2 Levels of and relationship between AFB_1 and AFG_1 in maize and groundnut samples from Malawi (n=236). Linear regression line (Y=1*X+0) indicates equal levels of AFB1 and AFG1. *Points above the regression* line indicate AFG1 > AFB1. The regression line (Y= 1*X+0) is not a fit of the data points but rather a separator of points AFG1 > AFB1 and AFG1 < AFB1

PCD-FLD analyses was achieved by use of naturally contaminated reference materials (Product no.: TR-A100, Batch no.: A-C-268, R-Biopharm AG, Darmstadt, Germany). Further, randomly selected samples previously analyzed by IAC-HPLC-PCD-FLD shown to have contained concentration of AFG_1>AFB_1 were reanalyzed by LC-MS/MS, and results were comparable.

Aflatoxin data were not normally distributed and were log-transformed for statistical analysis (log AFB1+1, log AFG1+1). The difference between means was assessed by analysis of variance (ANOVA) or *t* test. All the analyses were performed using SPSS® (version 16) software (SPSS Inc., Chicago, IL, USA). The level of confidence required for significance was set at $P \leq 0.05$.

Results and discussions

Although a significant proportion of the aflatoxin positive samples included in the study originated from the mid-elevation agro-ecological zone (22.5 %), it is worth noting that this does not reflect the aflatoxin prevalence in the zone as at all times, the majority of the samples that were tested were collected from the mid-elevation which is the main maize and groundnuts-producing area in Malawi. In fact, the aflatoxin problem is more prominent in the lower Shire and the lake shore, middle, and upper-Shire agro-ecological zone than in the mid-elevation and highlands (Matumba et al. 2013; Matumba et al. 2014b).

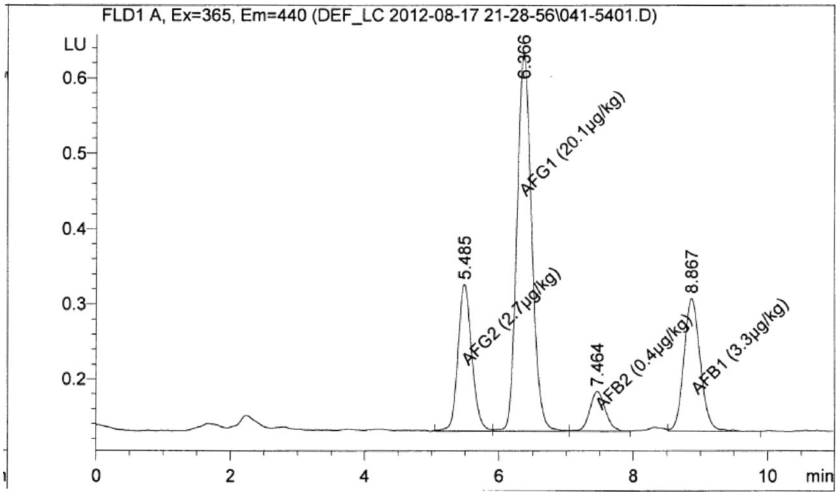

Fig. 3 Example chromatogram (HPLC-FLD) of maize sample extract revealing a much higher concentration of AFG_1 than AFB_1

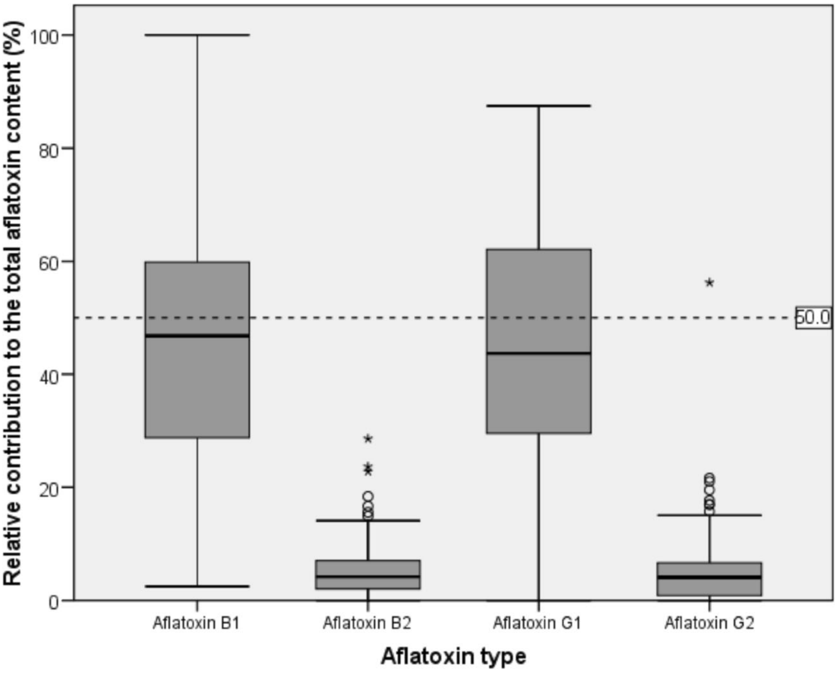

Fig. 4 Relative contribution of aflatoxin B_1, aflatoxin B_2, aflatoxin G_1, and aflatoxin G_2 to the total aflatoxin content in raw maize and groundnut samples from Malawi. The *horizontal line within the box* represents the median. The *bottom and upper ends of the box* represent the first and third quartiles, respectively. The *bottom and upper whiskers* extend from the box to the smallest or largest non-outliers in the dataset (relevant quartile± 1.5*(interquartile range, IQR)). *Circles* depict mild outliers (1.5× IQR), and *asterisks* depict extreme outliers (3×IQR). *Circles* depict mild outliers (1.5×IQR), and *asterisks* depict extreme outliers (3×IQR). The *dotted line* represents a 50 % contribution to the total aflatoxin content

As generally expected, all samples that tested positive for aflatoxin contained AFB_1. This AFB_1 co-occurred with AFG_1, AFB_2, and AFG_2 in 95.3, 87.7, and 78.8 % of the samples, respectively (Table 1). With the exception of three samples (1.3 %) where AFG_2 co-occurred only with AFB_1 and AFG_1, in all samples, AFG_2 co-occurred with the three toxins.

The levels of AFB_1, AFB_2, AFG_1, and AFG_2 ranged to 592, 54, 412, and 65 μg/kg, respectively. The mean levels of AFB_1, AFB_2, AFG_1, and AFG_2 levels for the samples were 44.7±79.7 (mean±SD), 5.7±8.5, 42.0±68.0, and 6.6± 11.2 μg/kg. It is worth noting that due to the spread of the aflatoxin data, the mean AFG_1/AFB_1 for all samples (groundnuts and maize) was found to be 2.0±3.2 (mean±SD), while the mean AFB_1/AFG_1 was 2.7±8.8. Interestingly, in 110 samples (47 %), AFG_1 concentration exceeded AFB_1 (Fig. 2) and in 42 % of the samples, AFG_1 contributed to over half of the total aflatoxin concentration. Further, AFB_1 and AFG_1 concentration always exceeded AFB_2 and AFG_2, respectively (Fig. 3). The mean relative percentages to which AFB_1, AFB_2, AFG_1, and AFG_2 contributed to the total aflatoxin content (100 %) were 47, 5, 43, and 5 %, respectively (Fig. 4).

Out of the 236 aflatoxin positive samples considered in this study, 182 had a total aflatoxin content (aflatoxin B_1, B_2, G_1 and G_2) higher than 10 μg/kg. This value/level corresponds to median limit in food currently established in legislations worldwide (FAO, 2004). e If the AFB_1 measurement was used to check compliance with the COMESA limit with an assumption that $AFB_1 \geq 50$ % of the total aflatoxin content, 14 samples with a total aflatoxin >10 μg/kg and $AFB_1 \leq 5$ μg/kg would have passed the control. This would represent a 7.7 %

Table 2 Number of samples with total aflatoxin ($AFB_1 + AFB_2 + AFG_1 + AFG_2$) level greater than the regulatory limit when aflatoxin B_1 concentration was equal or less than half the regulatory limit and associated false-negative rates

Total aflatoxins regulatory limit ($AFB_1 + AFB_2 + AFG_1 + AFG_2$)	Number of samples with total aflatoxins > regulatory limit	Number of samples with total aflatoxins > regulatory limit and AFB1≤½ regulatory limit	False-negative rate (%)
10 μg/kg[a]	182	14	7.7
20 μg/kg[b]	158	21	13.3
100 μg/kg[c]	62	15	24.2
200 μg/kg[d]	31	8	25.8

[a] Median limit in food currently established in legislations worldwide (FAO, 2004).

[b] Total aflatoxin limit for human consumption enforced by U.S.FDA

[c] Total AF limit for grain intended for breeding livestock enforced by U.S.FDA

[d] Total aflatoxin limit for grain intended for finishing swine of 45.4 kg (100 lb) or greater enforced by U.S.FDA

false-negative rate. Similarly, if the US Food and Drug Administration's (U.S.FDA) limits for human food (20 μg/kg, total aflatoxin), grain intended for breeding livestock (100 μg/kg, total aflatoxin) and grain intended for finishing swine of 45.4 kg (100 lb) or greater (200 μg/kg, total aflatoxin) (FAO 2004) were to be estimated by AFB_1 measurement; 13.4, 24.2, and 25.5 % false-negative rates would have occurred respectively (Table 2). These results indicate that measurement of AFB_1 alone may not satisfactorily be used to control the total aflatoxin concentration in Malawi. In fact, the European Commission (Decision 2002/657/EC) calls for a ≤5 % false-negative rate for a screening technique to be acceptable (European Commission EC 2002). Previously, Matumba et al. (2013) reported to have successfully screened shelled maize using the presence ≥four bright greenish-yellow fluorescence (BGYF) grains per 2.5-kg maize sample as an indicator for total aflatoxin >10 μg/kg with a 4.4 % false-negative rate.

As shown in Table 2, the false-negative rate increased as aflatoxin limits increased from 10 to 200 μg/kg total aflatoxin. This result indicates that $AFG_1 > AFB_1$ phenomenon occurred more frequently at high aflatoxin levels than at low levels (Table 2), which may signify that the aflatoxin-G-dominant producers in Malawi are also high aflatoxin producers. *A. nomius* and *A. parasiticus* are among species that are known to generally produce high amounts of aflatoxins. However, the former is considered to be rare in some geographical regions (Horn et al. 1996; Doster et al. 2009; Tran-Dinh et al. 1999; Horn and Dorner 1998).

The present findings differ with aflatoxin occurrence ratios reported in many surveys conducted in the world where aflatoxin B dominance is observed (Adetunji et al. 2014; Ghiasian et al. 2011; Haryadi and Setiastuty 1994; Oliveira et al. 2009; Younis and Malik 2003). However, similar patterns were reported in nuts of Brazilian origin (Oliveira et al. 2009; Olsen et al. 2008) where the concentration of AFB_1 and AFG_1 was comparable. In particular, Olsen et al. (2008) found the concentration of the AFB1 and AFG1 to be 50/50, and through fungal isolation, they concluded that *A. nomius* was responsible for the aflatoxin contamination of the Brazil nuts. This pattern is also apparent in a publication made on samples from neighboring Mozambique where average AFB_1 and AFG_1 concentrations were comparable (Warth et al. 2012).

Until now, aflatoxigenic fungal strains have not been fully characterized in Malawi. Monyo et al. (2012) only presented characterization information on aflatoxigenic aspergilli by counting colony-forming units on *A. flavus* and *A. parasiticus* agar (AFPA). However, from our data on the high prevalence of AFG_1, we propose that in addition to *A. flavus*, there are other aspergilli stain(s) responsible for the high concentration of the G aflatoxins. It could be further assumed that such strains are distributed across Malawi since no significant mean AFG_1/AFB_1 differences were observed among agro-ecologies of Malawi. One is tempted to speculate that the aflatoxigenic strains may be shared with neighboring Mozambique, hence the similarity of the co-occurrence pattern of the aflatoxin analogs.

In conclusion, the present study has demonstrated that aflatoxin proportions in maize and groundnuts in Malawi generally differ from ratios reported globally. Aflatoxin Gs particularly AFG_1 do occur in significant proportion comparable to that of AFB_1. Given the great variability of AFG_1/AFB_1 ratios found in the present study, results of the quantification of AFB_1 cannot be used to effectively estimate the concentration of total aflatoxins. Knowledge of the aflatoxin proportions and distribution in food may influence the choice of suitable aflatoxin quantitation methods and appropriate regulations in food. Studies are needed in order to characterize aflatoxigenic strains in Malawi.

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Conflict of interest The authors declare that there is no conflict of interest.

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