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Uncommon occurrence ratios of aflatoxin B₁, B₂, G₁, and G₂ 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 G₁ found routinely at equal or even higher levels than aflatoxin B₁. Aflatoxin B₁ (AFB₁) ratio in a contaminated sample is generally greater than 50 % of total aflatoxin (sum of aflatoxin B₁, B₂, G₁, and G₂). 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 AFG₁ was higher than that of AFB₁. The mean concentration percentages of AFB₁/AFB₂/AFG₁/AFG₂ in reference to total aflatoxins were found to be 47:5:43:5 %, respectively. The AFG₁ and AFB₁ 50/50 trend was observed in maize and groundnuts and was consistent for samples collected in both years. If the AFB₁ measurement was used to check compliance of total aflatoxin regulatory limit set at 10, 20, 100, and 200 µg/kg with an assumption that AFB₁ ≥ 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 B₁ (AFB₁), AFG₁, AFB₂, and AFG₂ (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 AFB₁, AFB₂, AFG₁, and AFG₂. It was established through feeding studies that AFB₁ and AFG₁ (both containing dihydrobisfuran rings) are produced from *O*-methylsterigmatocystin and that AFB₂ and AFG₂ (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 AFB₁ and AFB₂ (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 AFB₁ concentration generally exceeds half of the sum of the aflatoxins and that AFB₂ and AFG₂ occur in the lowest concentrations. In the same regard, several countries have set separate regulatory limits for AFB₁ at half the regulatory limit of the sum of the four aflatoxins (Van Egmond and Jonker 2004). Likewise, analytical methods for quantifying AFB₁ 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 AFG₁-dominant *A. parasiticus* (ratio AFG₁/AFB₁>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 B₁, B₂, G₁, and G₂ and get insights about the types of aflatoxigenic fungi present in Malawi.

Methodology

A meta-analysis was done on aflatoxin (AFB₁, AFB₂, AFG₁, and AFG₂) 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,

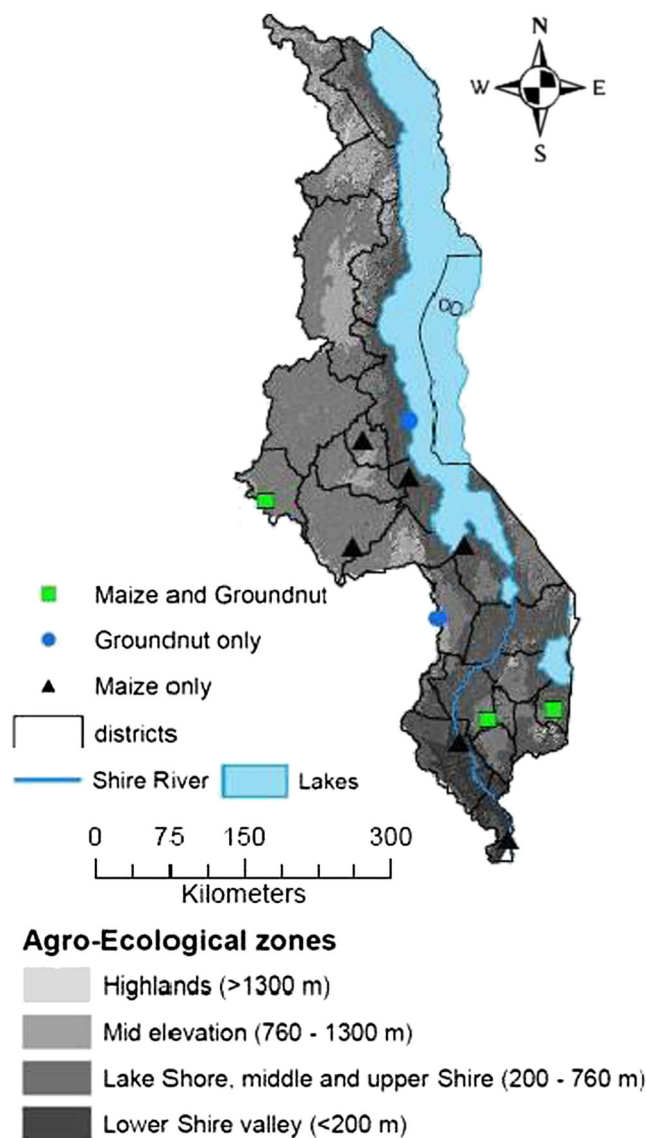


Fig. 1 Sampling sites in different agro-ecological zones of Malawi

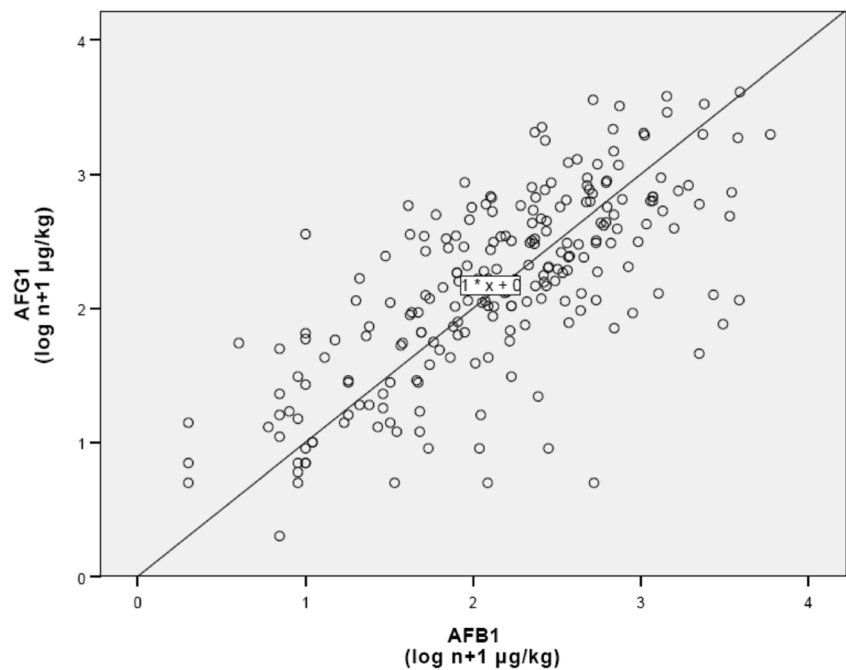
21.6 % from the lower-Shire agro-ecological zone, and 16.9 % from unspecified locations.

Limits of quantification of the analytical results included in this meta-analysis were 0.2 µg/kg for AFB₁ and AFG₁ and 0.1 µg/kg for AFB₂ and AFG₂ (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-

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 %

Fig. 2 Levels of and relationship between AFB₁ and AFG₁ 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 AFG₁>AFB₁ were reanalyzed by LC-MS/MS, and results were comparable.

Aflatoxin data were not normally distributed and were log-transformed for statistical analysis ($\log AFB_1+1$, $\log AFG_1+1$). 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 AFG₁ than AFB₁

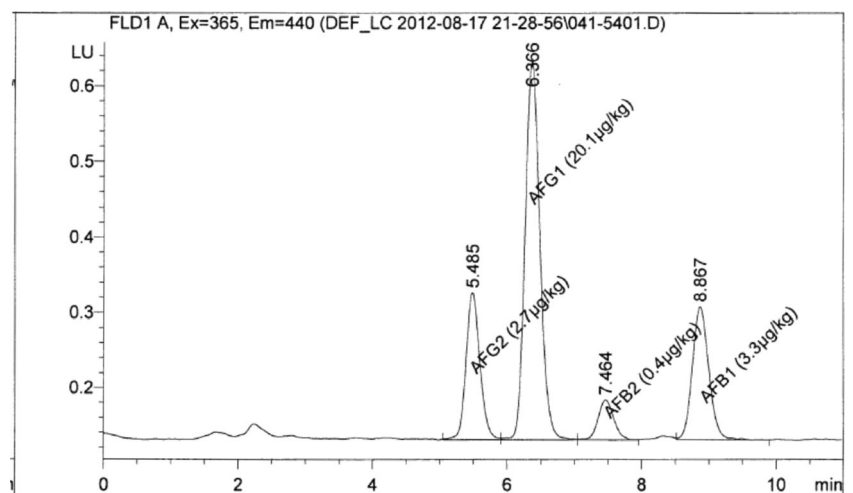
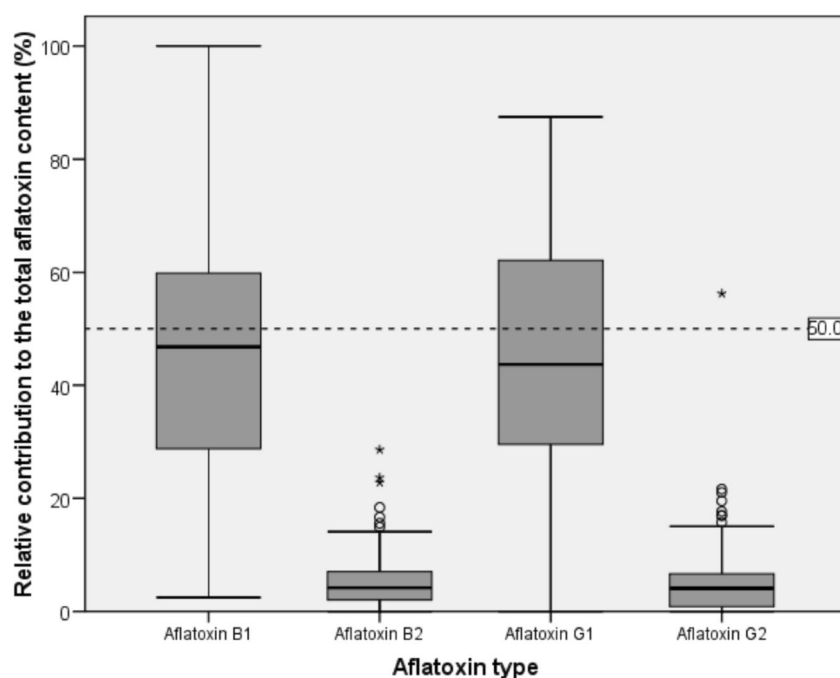


Fig. 4 Relative contribution of aflatoxin B₁, aflatoxin B₂, aflatoxin G₁, and aflatoxin G₂ to the total aflatoxin content in raw maize and groundnut samples from Malawi. The horizontal line within the box represents the median. The bottom and upper ends of the box represent the first and third quartiles, respectively. The bottom and upper whiskers extend from the box to the smallest or largest non-outliers in the dataset (relevant quartile $\pm 1.5 \times$ (interquartile range, IQR)). Circles depict mild outliers ($1.5 \times$ IQR), and asterisks depict extreme outliers ($3 \times$ IQR). Circles depict mild outliers ($1.5 \times$ IQR), and asterisks depict extreme outliers ($3 \times$ IQR). The dotted line represents a 50 % contribution to the total aflatoxin content



As generally expected, all samples that tested positive for aflatoxin contained AFB₁. This AFB₁ co-occurred with AFG₁, AFB₂, and AFG₂ in 95.3, 87.7, and 78.8 % of the samples, respectively (Table 1). With the exception of three samples (1.3 %) where AFG₂ co-occurred only with AFB₁ and AFG₁, in all samples, AFG₂ co-occurred with the three toxins.

The levels of AFB₁, AFB₂, AFG₁, and AFG₂ ranged to 592, 54, 412, and 65 $\mu\text{g}/\text{kg}$, respectively. The mean levels of AFB₁, AFB₂, AFG₁, and AFG₂ levels for the samples were 44.7 ± 79.7 (mean \pm SD), 5.7 ± 8.5 , 42.0 ± 68.0 , and 6.6 ± 11.2 $\mu\text{g}/\text{kg}$. It is worth noting that due to the spread of the aflatoxin data, the mean AFG₁/AFB₁ for all samples (groundnuts and maize) was found to be 2.0 ± 3.2 (mean \pm SD), while the mean AFB₁/AFG₁ was 2.7 ± 8.8 . Interestingly, in 110 samples (47 %), AFG₁ concentration exceeded AFB₁

(Fig. 2) and in 42 % of the samples, AFG₁ contributed to over half of the total aflatoxin concentration. Further, AFB₁ and AFG₁ concentration always exceeded AFB₂ and AFG₂, respectively (Fig. 3). The mean relative percentages to which AFB₁, AFB₂, AFG₁, and AFG₂ 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 B₁, B₂, G₁ and G₂) higher than 10 $\mu\text{g}/\text{kg}$. This value/level corresponds to median limit in food currently established in legislations worldwide (FAO, 2004). e If the AFB₁ measurement was used to check compliance with the COMESA limit with an assumption that AFB₁ ≥ 50 % of the total aflatoxin content, 14 samples with a total aflatoxin > 10 $\mu\text{g}/\text{kg}$ and AFB₁ ≤ 5 $\mu\text{g}/\text{kg}$ would have passed the control. This would represent a 7.7 %

Table 2 Number of samples with total aflatoxin (AFB₁ + AFB₂ + AFG₁ + AFG₂) level greater than the regulatory limit when aflatoxin B₁ 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 AFB ₁ $\leq \frac{1}{2}$ regulatory limit	False-negative rate (%)
10 $\mu\text{g}/\text{kg}$ ^a	182	14	7.7
20 $\mu\text{g}/\text{kg}$ ^b	158	21	13.3
100 $\mu\text{g}/\text{kg}$ ^c	62	15	24.2
200 $\mu\text{g}/\text{kg}$ ^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 AFB₁ measurement; 13.4, 24.2, and 25.5 % false-negative rates would have occurred respectively (Table 2). These results indicate that measurement of AFB₁ 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 AFG₁ > AFB₁ 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 AFB₁ and AFG₁ 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 AFB₁ and AFG₁ 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 AFG₁, 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 AFG₁/AFB₁ 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 AFG₁ do occur in significant proportion comparable to that of AFB₁. Given the great variability of AFG₁/AFB₁ ratios found in the present study, results of the quantification of AFB₁ 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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