![White Rose university consortium logo - Universities of Leeds, Sheffield & York]

eprints@whiterose.ac.uk
https://eprints.whiterose.ac.uk/

1 **Determination of multi-mycotoxin occurrence in maize based porridges from selected regions**

2 **of Tanzania by liquid chromatography tandem mass spectrometry (LC-MS/MS), a longitudinal**

3 **study**

4 Patrick A. Geary[1], Gaoyun Chen[1], Martin E. Kimanya[2], Candida P. Shirima[3], Michalina Oplatowska-

5 Stachowiak[1], Christopher T. Elliott[1], Michael N. Routledge[4], Yun Yun Gong[1*]

6 [1.] Institute for Global Food Security, Queen's University Belfast, United Kingdom

7 [2.] The Nelson Mandela Institution of Science and Technology, School of Life Sciences and

8 Bioengineering, Arusha, Tanzania

9 [3.] Tanzania Food and Drugs Authority, Dar es Salaam, Tanzania

10 [4.] Division of Epidemiology, Leeds Institute of Genetics, Health and Therapeutics, University of

11 Leeds, United Kingdom

12 **Abstract**

13 Residents of certain areas of Tanzania are exposed to mycotoxins through the consumption of

14 contaminated maize based foods. In this study, 101 maize based porridge samples were collected from

15 villages of Nyabula, Kikelelwa and Kigwa located in different agro-ecological zones of Tanzania. The

16 samples were collected at three time points (time point 1, during maize harvest; time point 2, 6 months

17 after harvest; time point 3, 12 months after harvest) over a 1-year period. Ultra-performance liquid

18 chromatography tandem mass spectrometry (UPLC-MS/MS) was used to detect and quantify 9

19 mycotoxins: aflatoxin B_1 (AFB$_1$), aflatoxin B_2 (AFB$_2$), aflatoxin G_1 (AFG$_1$), aflatoxin G_2 (AFG$_2$), fumonisin

20 B_1 (FB$_1$), fumonisin B_2 (FB$_2$), deoxynivalenol (DON), ochratoxin A (OTA) and zearaleneone (ZEN) in

21 the samples following a QuEChERS extraction method. Eighty two percent of samples were co-

22 contaminated with more than one group of mycotoxins. Fumonisins (FB$_1$+FB$_2$) had the highest

23 percentage occurrence in all 101 samples (100%) whereas OTA had the lowest (5%). For all three

24 villages the mean concentration of FB$_1$ was lowest in samples taken from time point 2. Conversely, In

25 Kigwa village there was a distinct trend that AFB$_1$ mean concentration was highest in samples taken

26 from time point 2. DON concentration did not differ greatly between time points but the percentage

27 occurrence varied between villages, most notably in Kigwa where 0% of samples tested positive. ZEN

28 occurrence and mean concentration was highest in Kikelelwa. The results suggest that mycotoxin

29 contamination in maize can vary based on season and agro-ecological zones. The high occurrence of

30 multiple mycotoxins found in maize porridge, a common weaning food in Tanzania, presents a potential

31 increase in the risk of exposure and significant health implications in children.

32 **1. Introduction**

33 Mycotoxins are naturally occurring toxic secondary metabolites produced by filamentous fungi which

34 can contaminate many kinds of agricultural products. Toxigenic fungi are capable of growing under a

35 wide range of atmospheric conditions depending on the species and they can contaminate crops during

36 pre-harvest, immediate post-harvest, storage, transport and processing (Bennett & Klich, 2003).

37 Mycotoxins have been shown to contaminate a wide range of agricultural products including: cereals,

38 nuts, fruit, spices and wine (Abia et al., 2013; Serra, Braga, & Venâncio, 2005; Van de Perre et al.,

39 2014; Yogendrarajah, Van Poucke, De Meulenaer, & De Saeger, 2013). In the case of aflatoxins, they

40 have also been detected in milk produced by cows that have consumed contaminated feed (Huang et

41 al., 2013). Due to the ubiquitous presence of mycotoxins in both food and feed supply chains, and their

42 association with various toxicological risks in both humans and animals, they have become a major

43 economic and health concern.

44 More particularly, aflatoxin B_1 (AFB$_1$), aflatoxin B_2 (AFB$_2$), aflatoxin G_1 (AFG$_1$), aflatoxin G_2 (AFG$_2$),

45 fumonisin B_1 (FB$_1$), fumonisin B_2 (FB$_2$), deoxynivalenol (DON), ochratoxin A (OTA) and zearaleneone

46 (ZEN) have all been recognised global health, agriculture and trade concerns due to the high

47 occurrence and associated health impacts of these mycotoxins that has been found around the world.

48 However, due to socio-economic and environmental factors, developing countries tend to be more

49 severely affected by the threat of mycotoxins, especially aflatoxins, than developed countries (Wild &

50 Gong, 2010; Williams et al., 2004).

51 The growing recognition of the threat of mycotoxins has stimulated scientific research to better

52 understand how exposure and toxicity impact human health. The toxicity varies among different types

53 of mycotoxins but many have shown the capacity to be acutely toxic, carcinogenic, mutagenic and

54 immunosuppressive (Bakırdere et al., 2012). The most fatal human aflatoxicosis outbreak occurred in

55   Kenya, 2004, with 317 recorded cases of acute hepatitis and 125 deaths (Nyikal et al., 2004). Public

56   health officials discovered that this was linked to the consumption of AFB_1-contaminated maize and a

57   case control study found that maize in case households had higher concentrations of aflatoxins

58   compared to that of the maize from control households (Azziz-Baumgartner et al., 2005). The

59   International Agency for Research on Cancer (IARC) classifies naturally occurring mixtures of aflatoxins

60   (AFB_1+AFB_2+AFG_1+AFG_2) as Group 1 carcinogens (International Agency for Research on Cancer,

61   2002). Previous studies have also provided evidence that aflatoxins may cause immune suppression

62   as a result of decreased protein synthesis, changes in enzymatic activity and changes in metabolism

63   or cell cycles (Jiang et al., 2005; Jiang et al., 2008). A study in West African children reported a strong

64   inverse correlation between the exposure of aflatoxin and body height increase (Gong et al., 2004).

65   Fumonisins, a group of mycotoxins produced by fungal species belonging to the Fusarium genus, have

66   also been shown to have detrimental health effects. Fumonisins are widely distributed around the world

67   and have been classified as possible carcinogens (Joint FAO/WHO Expert Committee on Food

68   Additives, 2011). A study carried out in South Africa was able to demonstrate a positive correlation

69   between fumonisin exposure and high incidences of human oesophageal cancer (Marasas, 2001).

70   Fumonisin exposure has also been linked with increased occurrences of neural tube defects (Missmer

71   et al., 2006). DON, another mycotoxin produced by fungal species within the Fusarium genus, has not

72   yet been associated with any long-term health impacts in humans but animals with low dose chronic

73   exposure to DON have shown that decreased growth and feed intake (Forsell, Witt, Tai, Jensen, &

74   Pestka, 1986; Rotter, Thompson, Lessard, Trenholm, & Tryphonas, 1994). OTA, produced by

75   Aspergillus ochraceus, is a common mycotoxin and a possible human carcinogen. Studies in animals

76   have shown that it has the potential to be carcinogenic, immunosuppressive and neurotoxic (Álavarez,

77   Gil, Ezpeleta, García-Jalón, & López de Cerain, 2004; Lioi, Santoro, Barbieri, Salzano, & Ursini, 2004;

78   Schaaf et al., 2002). ZEN is produced by several fungi in the Fusarium genus including: F. culmorum,

79   F. graminearum and F. crookwellense. It has estrogenic effects in pigs (Jiang et al., 2011) and

80   suggested that it can trigger central precocious pubertal in human females (Massart, Meucci, Saggese,

81   & Soldani, 2007).

82   Due to the toxic effect of mycotoxins in humans and animals it is important to develop analytical methods

83   to detect them in food in order to facilitate their control and regulation. Liquid chromatography tandem

84   mass spectrometry (LC-MS/MS) is an effective method of detection for mycotoxin analysis. In recent

85   years, studies have demonstrated LC-MS/MS methods capable of sub ppb detection for multiple

86   mycotoxins in maize (Frenich, Vidal, Romero-Gonzalez, & Aguilera-Luiz, 2009; Liao et al., 2013;

87   Malachová, Sulyok, Beltrán, Berthiller, & Krska, 2014; Zachariasova et al., 2014). Analytical methods

88   for multiple mycotoxins should be selective for their target analytes, sensitive enough to detect toxins

89   at relatively low concentrations and efficient to ensure rapid and reliable analysis.

90   Recently a mycotoxin study carried out in Tanzania examined the extent of dietary exposure of AFB_1,

91   FB_1 and DON through the quantification of their respective biomarkers in serum and urine (Shirima et

92   al., 2013; Srey, Kimanya, Routledge, Shirima, & Gong, 2014). The study found that young children in

93   Tanzania are chronically exposed to AFB_1, FB_1 and DON through their diet. Urinary FB_1 was found to

94   be negatively associated with length for age Z-scores whilst the negative association between AF-Alb

95   and child growth did not reach statistical significance. In a recent study in Tanzania, maize kernels were

96   sampled from three districts and multi-mycotoxins were measured by LC-MS method (Kamala et al.,

97   2015). The study reported high occurrence of AFB_1 (50%) and FB_1 (73%). The food cooking process is

98   known to have varying impact on mycotoxin levels, therefore measuring the levels of mycotoxins in

99   cooked food can provide more close estimates of exposure than in maize flour.

100   This paper utilises a recently developed multi-mycotoxin detection method to determine the extent of

101   multi-mycotoxin contamination in the maize porridge, in order to build upon mycotoxin occurrence and

102   exposure data from previous studies; and to compare with the exposure biomarker data where possible.

103   **2. Materials and Methods**

104

105   2.1 Reagents and chemicals

106   Acetonitrile (LC-MS grade), ammonium hydroxide (≥25% in water), dimethyl sulfoxide (≥99.9%), formic

107   acid (≥98%), magnesium sulfate (anhydrous, ≥99.5%), methanol (LC-MS grade), mycotoxin standards

108   (AFB_1, AFB_2, AFG_1, AFG_2, FB_1, FB_2, DON, ZEN and OTA), sodium chloride (≥99.0%) and Whatman®

109   Puradisc 4 syringe filters (0.2 μm, PTFE) were all acquired from Sigma-Aldrich (Poole, United Kingdom).

110   Each mycotoxin standard was separately dissolved in acetonitrile (0.2 mg/ml solution) and stored at -

111   20^oC. Bondesil C_{18} was acquired from Agilent Technologies (Waldbronn, Germany).

112   2.2 Study design and sampling

113   Cooked maize porridge samples were collected from households across three rural villages in

114   Tanzania: Nyabula (Iringa region), Kikelelwa (Kilimanjaro region) and Kigwa (Tabora region), which are

115   from different agro-ecological zones. The samples were collected at three time points over the period

116   of a year: Time point 1 (June/July 2010, a maize harvesting season), time point 2 (January 2011, six

117   months after maize was harvested) and time point 3 (June/July 2011, another maize harvesting season

118   12 months after time point 1). The cooked porridge samples were dried after collection. A total of 101

119   samples; 10 samples from each village at time points 1 and 2, 14 samples from Nyabula and Kikelelwa

120   villages at time point 3 and 13 samples from Kigwa village at time point 3 were randomly selected. The

121   samples were oven dried and kept frozen at -80°C until extraction for UPLC-MS/MS analysis. A blank

122   maize flour sample was cooked into a porridge using the same recipe as the other Tanzanian porridge

123   samples, oven dried and subsequently stored at -80°C until LC-MS/MS extraction for UPLC-MS/MS

124   analysis.

125   2.3 Extraction procedure and UPLC-MS/MS analysis

126   A previously developed multi-mycotoxin UPLC-MS/MS method was adopted for the study.

127   (Oplatowska-Stachowiak et al., 2015). Nine mycotoxins of interest were quantified: AFB_1, AFB_2, AFG_1,

128   AFG_2, FB_1, FB_2, DON, ZEN and OTA. Briefly, the LC-MS/MS method was developed on a Waters

129   Acquity UPLC coupled to a Xevo TQ-S triple quadrupole mass spectrometer. Sample extraction method

130   was based on QuEChERS method (Lacina et al., 2012). The quantification was achieved by

131   interpolation from a standard curve prepared by spiking the blank matrix samples at 7 different levels

132   with a mixture of mycotoxins before extraction. Calibrant solutions for matrix-matched calibration curves

133   were prepared in blank matrix before extraction. Limits of detection (LOD) and quantification (LOQ)

134   (S/N≥10) were previously determined in maize as: AFB_1 LOD: 0.05 ng/g, LOQ: 0.125 ng/g; AFB_2/AFG_1

135   LOD: 0.125 ng/g, LOQ: 0.25 ng/g; AFG_2 LOD: 0.25 ng/g, LOQ: 0.5 ng/g; DON LOD: 5 ng/g, LOQ: 12.5

136   ng/g; OTA LOD: 0.625 ng/g, LOQ: 1.25 ng/g; ZEN LOD: 2.5 ng/g, LOQ: 5 ng/g. Limits of detection and

137   quantification were determined in wheat as 0.5 ng/g and 1.0 ng/g respectively for FB_1 and 0.2ng/g and

138   0.5ng/g respectively for FB_2.

139   2.4 Statistical analysis

140   Data was analysed using IBM SPSS Statistics for Windows, Software Version 21.0 (IBM Corp., 2012).

141   Kruskal-Wallis tests were carried out to test for variance between villages within time points and

142 variance between time points within villages where applicable. A p value of <0.05 was considered

143 statistically significant for this test and only positive samples >LOD were used. Spearman's rank tests

144 were carried out to test for correlation between the concentration of AFB_1, FB_1 and DON. A p value of

145 <0.05 was considered statistically significant for this test.

146 **3. Results**

147 Descriptive statistics for all 101 maize porridge samples are displayed in Table 1a and 1b. Both FB_1

148 and FB_2 were detected in all 101 samples. The mean ratio of FB_1:FB_2 for Nyabula, Kikelelwa and Kigwa

149 was 60:40, 60:40 and 64:36, respectively, with a mean ratio of 61:39 for all samples. Median FB_1

150 concentration was significantly higher in Kikelelwa (290.18 ng/g) and Kigwa (383.54 ng/g) than in

151 Nyabula 60.14 ng/g) (p = 0.000). FB_1 concentration was lowest during time point 2 (6 months after

152 harvest) in every village. Eleven percent of the 101 samples analysed showed fumonisin contamination

153 levels greater than the maximum tolerable limit for total fumonisin in maize based foods intended for

154 adult human consumption (FB_1+FB_2 ≥1000 ng/g) set by the European Commission (EC) (European

155 Commission, 2006). Fifty-seven percent of samples also exceeded the EC limit for fumonisins in food

156 products intended for infant consumption (FB_1+FB_2 ≥200 ng/g). At least one type of aflatoxin was

157 detected in 50% of all samples. The mean ratios of AFB_1:AFB_2 and AFG_1:AFG_2 for all samples were

158 both 90:10. The data from Kigwa village was chosen for statistical analysis due to the high (94%)

159 occurrence of aflatoxins in comparison to Kikelelwa (27%) and Nyabula (24%).

160 **Table 1a**

161 Levels of mycotoxin contamination in different villages/regions and time points in Tanzania

Mycotoxin	Village/ Region	Time point	Positive Samples (%)	Mean (ng/g)	Median (ng/g)	Range (ng/g)
Fumonisin B$_1$	Nyabula/ Iringa	1	10 (100)	158.06	109.64	41.70 - 375.64
		2	10 (100)	44.96	46.54	13.01 - 65.53
		3	14 (100)	87.18	60.14	12.87 - 421.36
		All	34 (100)	95.61	60.14	12.87 - 421.36
	Kikelelwa/ Kilimanjaro	1	10 (100)	555.74	393.41	218.19 - 1308.43
		2	10 (100)	159.38	82.10	12.65 - 564.10
		3	14 (100)	438.14	311.63	36.02 - 1850.01
		All	34 (100)	390.74	290.18	12.65 - 1850.01
	Kigwa/ Tabora	1	10 (100)	533.98	489.07	36.26 - 1206.09
		2	10 (100)	299.26	309.83	71.46 - 554.57
		3	13 (100)	394.84	328.50	57.08 - 1141.40
		All	33 (100)	408.04	383.54	36.26 - 1206.09
Fumonisin B$_2$	Nyabula/ Iringa	1	10 (100)	110.42	80.56	27.67 - 261.89
		2	10 (100)	28.21	28.37	9.50 - 38.06
		3	14 (100)	59.35	41.84	7.25 - 282.23
		All	34 (100)	65.21	37.41	7.25 - 282.23
	Kikelelwa/ Kilimanjaro	1	10 (100)	354.47	273.02	152.88 - 867.24
		2	10 (100)	108.95	52.79	10.25 - 414.68
		3	14 (100)	290.03	191.24	26.18 - 1286.93
		All	34 (100)	255.72	191.24	10.25 - 1286.93
	Kigwa/ Tabora	1	10 (100)	298.68	255.38	23.57 - 790.05
		2	10 (100)	177.14	183.44	33.98 - 313.68
		3	13 (100)	240.68	203.59	29.71 - 699.85
		All	33 (100)	239.00	213.09	23.57 - 790.05
Aflatoxin B$_1$	Nyabula/ Iringa	1	4 (40)	7.16	0.45	0.15 - 27.60
		2	2 (20)	7.43	7.43	7.15, 7.70
		3	4 (29)	0.34	0.33	0.20 - 0.50
		All	8 (24)	4.49	0.43	0.15 - 27.60
	Kikelelwa/ Kilimanjaro	1	1 (10)	34.50	34.50	34.50
		2	0 (0)	-	-	-
		3	8 (57)	2.19	0.48	0.20 - 13.05
		All	9 (27)	5.78	0.65	0.20 - 34.50
	Kigwa/ Tabora	1	10 (100)	4.05	1.15	0.40 - 13.55
		2	10 (100)	10.21	5.95	0.55 - 25.80
		3	11 (85)	0.67	0.68	0.20 - 1.55
		All	31 (94)	4.73	0.95	0.20 - 25.80

162

163 **Table 1b**

164 Levels of mycotoxin contamination in different villages and time points in Tanzania

Mycotoxin	Village/ Region	Time point	Positive Samples (%)	Mean (ng/g)	Median (ng/g)	Range (ng/g)
Total Aflatoxins $(B_1+B_2+G_1+G_2)$	Nyabula/ Iringa	1	4 (40)	8.26	0.80	0.15 - 31.30
		2	2 (20)	14.48	14.48	14.05,14.90
		3	4 (29)	0.69	0.60	0.20 - 1.35
		All	8 (24)	6.48	0.93	0.15 - 31.3
	Kikelelwa/ Kilimanjaro	1	1 (10)	39.70	39.70	39.70
		2	0 (0)	-	-	-
		3	8 (57)	3.23	1.10	0.25 - 14.9
		All	9 (27)	7.39	1.10	0.25 - 39.7
	Kigwa/ Tabora	1	10 (100)	6.27	1.65	0.40 - 22.7
		2	10 (100)	14.91	9.58	0.55 - 43.65
		3	11 (85)	1.03	1.10	0.20 - 2.40
		All	31 (94)	7.03	1.60	0.20 - 43.65
Deoxynivalenol	Nyabula/ Iringa	1	1 (10)	4.65	4.65	4.65
		2	4 (40)	127.43	101.15	14.45 - 29.30
		3	10 (71)	177.72	149.85	13.35 - 421.60
		All	15 (44)	136.25	97.70	4.65 - 421.60
	Kikelelwa/ Kilimanjaro	1	10 (100)	102.46	95.35	28.70 - 245.65
		2	9 (90)	115.14	88.85	29.70 - 306.80
		3	10 (71)	116.31	43.80	14.60 - 410.90
		All	29 (85)	111.17	88.85	14.6 - 410.90
	Kigwa/ Tabora	1	0 (0)	-	-	-
		2	0 (0)	-	-	-
		3	0 (0)	-	-	-
		All	0 (0)	-	-	-
Zearalenone	Nyabula/ Iringa	1	0 (0)	-	-	-
		2	2 (20)	12.75	12.75	12.60, 12.90
		3	1 (7)	10.20	10.20	10.20
		All	3 (9)	11.90	12.60	10.20 - 12.90
	Kikelelwa/ Kilimanjaro	1	10 (100)	86.40	59.90	21.70 - 269.90
		2	10 (100)	47.37	52.88	11.25 - 72.10
		3	7 (50)	48.60	38.30	12.20 - 109.25
		All	27 (79)	62.14	46.80	11.25 - 269.90
	Kigwa/ Tabora	1	0 (0)	-	-	-
		2	0 (0)	-	-	-
		3	1 (7)	18.10	18.10	18.10
		All	1 (3)	18.10	18.10	18.10

165

166   **Table 2.** Co-occurrence of mycotoxins in Tanzanian maize porridge across all villages

Village/ Region	Time point	Frequency of mycotoxins co-occurrence in same sample (%)*			
		1 or more mycotoxins	2 or more mycotoxins	3 or more mycotoxins	4 or more mycotoxins
Nyabula/ Iringa	1	100	40	10	0
	2	100	50	20	20
	3	100	86	29	0
	All	100	62	21	6
Kikelelwa/ Kilimanjaro	1	100	100	100	30
	2	100	100	90	10
	3	100	79	57	29
	All	100	91	79	24
Kigwa/ Tabora	1	100	90	10	0
	2	100	100	0	0
	3	100	92	8	0
	All	100	94	6	0
All regions	All time points	100	82	36	10

167   *Mycotoxins were organised into 5 groups for this co-occurrence analysis: 1. Total aflatoxins: $AFB_1+AFB_2+AFG_1+AFG_2$; 2. Total
168   fumonisins: FB_1+FB_2; 3. DON; 4. ZEN; 5. OTA.

169   The highest concentration of AFB_1 in a single sample was found in Kikelelwa village at 34.50ng/g but

170   Kigwa and Nyabula village had more samples with aflatoxins detectable, and also had higher

171   concentrations of aflatoxins; both villages had higher concentrations aflatoxins at time point 2 (during

172   storage) than any other. The Tanzania regulatory limits for AFB_1 concentration ($AFB_1 \geq 5$ ng/g) and total

173   aflatoxins concentrations (total aflatoxins ≥ 10 ng/g) in maize, were exceeded in 14% and 12% of

174   samples, respectively. DON was detected in 44% of all samples. Nyabula samples showed a

175   successive increase in DON concentration between time points whereas Kikelelwa samples showed

176   little difference. DON was not detected in any samples from Kigwa village. None of the analysed

177   samples showed concentrations exceeded the EC limit for DON in maize-based foods intended for

178   human consumption (DON ≥ 750 ng/g); however, 6% of samples were above the limit for infant food

179   (DON ≥ 200 ng/g). ZEN was detected in 31% of all samples. ZEN concentration was highest in all three

180   time points for Kikelelwa (mean: 62.14 ng/g; occurrence: 79%) however there was no significant

181   difference between any of them (p = 0.335). Samples from Nyabula and Kigwa had a noticeably lower

182   occurrence of ZEN (9% and 3% respectively). Only one sample exceeded the EC limit for ZEN in maize-

183   based foods intended for human consumption (ZEN ≥ 200 ng/g). However, 23% of samples exceeded

184   the limit for infant food (ZEN ≥ 20 ng/g). OTA was detected in 5% of all samples (mean: 3.80 ng/g,

185   median: 3.30 ng/g). Each sample that had detectable OTA exceeded the EC limit in maize products

186   intended for both human adult and infant consumption (OTA ≥3.0 ng/g and >0.5 ng/g, respectively).

187   Overall, total aflatoxin concentration and total fumonisins were found to have a positive correlation by

188   Spearman's rank correlation (correlation coefficient: 0.254, p = 0.011), while total aflatoxin was found

189   to be negatively correlated to DON (correlation coefficient: -0.407, p = 0.000). Total fumonisin and DON

190   showed no statistically significant correlation (p = 0.919). ZEN showed no statistically significant

191   correlation with any of the other mycotoxins (p > 0.05). Further data on co-occurrence can be seen in

192   Table 2.

193   **4. DISCUSSION**

194   The LC-MS/MS analysis showed that the maize porridge samples collected from the three Tanzanian

195   villages were subject to contamination of multiple mycotoxins. All three Tanzanian villages showed high

196   occurrences of fumonisins (detected in 100% of samples) for all 101 samples. In all villages a lower

197   fumonisin concentration was seen in samples taken at time point 2, 6 months after harvest, compared

198   to samples taken at time point 1, during harvest. The reason for this difference is not immediately

199   apparent. It is possible that as household food supplies from subsistence farming begin to dwindle

200   during the dry season, and residents may be buying maize from other less contaminated areas. It has

201   been suggested that certain strains of bacteria can be effective in reducing fumonisin levels in maize

202   and that lactic acid, commonly produced by anaerobic bacteria, has a protective role against the growth

203   of Fusarium species in stored maize (Benedetti, Nazzi, Locci, & Firrao, 2006). Potentially, the fungal

204   species in the maize has been gradually degraded during storage by a bacterial agent. When all three

205   time points were taken into account, Nyabula had the lowest FB_1 mean concentration, followed by

206   Kikelelwa. It is possible that the climate or seasonal weather variation in Nyabula may have had an

207   influence in preventing the Fusarium growth in the maize. In terms of maize total aflatoxin concentration,

208   Kigwa had the most positive samples for total aflatoxins, followed by Kikelelwa and Nyabula. Unlike

209   fumonisins, maize aflatoxin concentration was highest in time point 2 compared to time points 1 and 3.

210   This difference in concentrations between the two mycotoxins would suggest that the storage conditions

211   are favoured by fungi that produce aflatoxins and field or harvest conditions at harvest are favoured by

212   fungi that produce fumonisins. This also suggests that the overall climate and weather conditions of

213   Tanzania are favourable for both aflatoxins and fumonisins.

214   It was found that Kikelelwa samples showed the highest frequency of DON in maize followed by

215   Nyabula whilst DON was not detected in any samples from Kigwa. Kikelelwa has a temperature climate

216   which is known to favour growth of DON producing fungi. Unlike aflatoxins and fumonisins, DON

217   concentration between time points was not found to be statistically different. ZEN concentration showed

218   a similar contamination pattern to DON; it was highest in Kikelelwa, followed by Nyabula and Kigwa,

219   where it was only detected in <1% of samples. ZEN occurrence was highest in Kikelelwa village and

220   lowest in Kigwa. The difference in ZEN concentration between time points was not found to be

221   significant in any villages. OTA was the least prevalent of nine mycotoxins analysed. The low frequency

222   meant that it was not possible to ascertain any discernable pattern in the contamination either between

223   regions or time points.

224

225   There were several cases of multiple mycotoxins co-contamination in the analysed maize porridge

226   samples. Mycotoxins were collated into five groups for co-contamination analysis: 1. Total aflatoxins:

227    $AFB_1 + AFB_2 + AFG_1 + AFG_2$; 2. Total fumonisins: $FB_1 + FB_2$; 3.DON; 4. ZEN and 5. OTA. It was found

228   that 82% of all samples contained two or more groups of mycotoxins, 36% contained three or more and

229   10% contained four or more. Samples taken from Kikelelwa contained the greatest proportion of co-

230   contaminated samples with 79% containing three or more groups of mycotoxins. This is much greater

231   in comparison to Nyabula and Kigwa where 21% and 6% of samples were co-contaminated with three

232   or more groups of mycotoxins respectively. Certain mycotoxins also showed patterns of co-

233   contamination in terms of their ratios to one another. The $FB_1:FB_2$ ratio of 60:40 stayed largely consistent

234   for samples across all regions and time points. $AFB_1:AFB_2$ and $AFG_1:AFG_2$ both showed 90:10 ratios.

235   Total aflatoxins and fumonisins showed statistically significant correlations with each other. Aflatoxins

236   and fumonisins were found to have a positive correlation ($r = 0.254$, $p = 0.011$) despite showing different

237   general contamination patterns between time points. This suggests that, even though aflatoxin and

238   fumonisins contamination may differ between seasons, the climate of Tanzania still promotes the growth

239   of certain Aspergillus and Fusarium fungal species. Aflatoxins and DON were found to have a moderate

240   negative correlation. This is most evident in Kigwa village which showed the highest aflatoxin

241   contamination frequency but the lowest for DON. These two correlations highlight the need to further

242   investigate and understand the nature of mycotoxin co-contamination.

243   Many of the samples exceeded EC regulatory limits for maize in both adult and infant food. Tanzania

244   has official regulations for AFB_1 and total aflatoxins in maize but not for the other previously mentioned

245   mycotoxins. Regulatory limits taken from European Commission Regulation No. 1881/2006, as

246   amended, were used as a guideline for the other mycotoxins involved this study. Total aflatoxins and

247   fumonisins were found to exceed the limits in 12% and 11% of samples, respectively. DON and ZEN

248   were the two mycotoxins with the lowest number of samples over the limit at 0% and 1%, respectively.

249   OTA was unique in that it was detected in only five samples but each of those exceeded the regulatory

250   limit. The maize porridge was also compared against EC regulatory limits for food intended for infant

251   consumption which are much lower than their adult counterparts. 50%, 57%, 6% and 23% of samples

252   were over the infant limit for total aflatoxins, total fumonisins, DON and ZEN, respectively based on EC

253   limits. These results show that the mycotoxins in these maize porridge samples are not just a health

254   risk to infants and young children but also a serious risk to adults. Combine this with the fact that many

255   of these samples are co-contaminated with multiple toxins and there is a very real possibility that a large

256   number of these children are consuming food over the recommended limits of several different

257   mycotoxins.

258

259   One of the applications of multi-mycotoxin food analysis such as this is to utilise the data in ongoing

260   biomarker discovery and validation. The data in this study has been compared to biomarker data for

261   samples taken from these villages at the same time points, to determine whether FB_1, AFB_1 and DON

262   contamination in food are consistent with urinary FB_1 (uFB_1), aflatoxin albumin adduct (AF-Alb) and

263   urinary DON (uDON) (Shirima et al., 2013; Srey et al., 2014). Generally speaking, uDON concentration

264   in each Tanzanian village reflected the DON concentrations measured in maize food. The Kigwa maize

265   samples showed no trace of DON and this was reflected in the uDON biomarker levels being the lowest

266   out of the three villages. Table 1b shows the Kikelelwa median DON concentration was similar between

267   time points 1 and 2 with a much lower level at time point 3, which is mirrored in the estimated DON

268   intake from the biomarker study. The Nyabula median DON showed a constant rise from time point 1

269   through to 3 which is the same pattern as reported in the biomarker study (Srey et al. 2014).

270   The comparison of AFB_1 in maize porridge and AF-Alb biomarker in blood was not entirely consistent.

271   The increase in the AFB_1 median concentration from time point 1 to 2 in Kigwa village was in a good

272   agreement with a similar increase in AF-Alb from the same time points. However the maize and AF-

273   Alb data for time point 3 do not agree with each other – a decrease was observed in maize AFB_1

274   whereas AF-Alb stayed the same as time point 2 (Shirima et al., 2013). A possible explanation for this

275   is the increased consumption due to child growth and maize availability at harvest. Also the children

276   may have still been consuming the same AFB_1 contaminated maize from time point 2 which may still

277   affect the AF-Alb biomarker measurements.

278   The FB_1 found in this study was also compared against that of uFB_1 biomarker obtained from the same

279   village and time points. Nyabula village showed the lowest FB_1 contamination in maize but it was

280   Kikelelwa which showed the lowest FB_1 detected in urine. The lower exposure in Kikelelwa is

281   presumably due to lower FB_1 intake of children due to lower maize consumption but high other food

282   types than in the other two villages. It was observed that time point 2 consistently had the lowest FB_1

283   contamination in maize and this is in consistent with $uFB1$ levels.

284   This study was compared against myctoxin occurrence data in maize from Cameroon (Abia et al.,

285   2013), Nigeria (Ezekiel et al., 2014), Malawi (Matumba, Sulyok, Monjerezi, Biswick, & Krska, 2014), two

286   South African studies (Shephard et al., 2013; van der Westhuizen et al., 2010) and another Tanzanian

287   study with uncooked maize samples taken from the same Kikelelwa village at the same season

288   (Kimanya et al., 2014). All data sets showed high frequencies of FB_1 in maize for their respective areas.

289   The FB_1 median for Kikelelwa village determined from this study (290.18 ng/g) was similar to the FB_1

290   median from the Tanzanian study carried out in the same village (329 ng/g) (Kimanya et al., 2014). Both

291   Cameroon and Nigerian maize FB_1 levels were in a similar range as the levels in Kikelelwa and Kigwa

292   village. The two studies from South Africa and the one from Malawi all showed much greater FB_1 levels

293   compared to Tanzania. This comparison shows that FB_1 is heavily prevalent throughout Africa but the

294   levels of contamination not only vary between different countries but also within different areas of the

295   same country. The distribution of AFB_1 was mixed with the majority of areas showing detected

296   frequencies of ≤50%. The two studies featuring maize analysis from Kikelelwa village both showed

297   good agreement with each other. They both gave similar frequencies (29% and 24%, respectively) and

298   medians (0.65 ng/g and 1.27 ng/g, respectively). In comparison to Kigwa village (mean: 0.68 ng/g),

299   Cameroon and Nigeria showed relatively higher means (3.5 ng/g and 2.5 ng/g, respectively). The

300   distribution of DON between countries varied largely amongst countries especially if high DON

301   concentrations in Malawi Highlands (mean: 600 ng/g; max: 2328 ng/g) and compared against the lower

302   ones in Kikelelwa village, Tanzania (mean: 111.17 ng/g max: 410.90 ng/g). The frequency of ZEN was

303   relatively high for Kikelelwa of Tanzania when compared against Cameroon and South Africa. Nyabula

304   and Kigwa of Tanzania showed lower ZEN concentrations comparable to Malawi and Nigeria. The

305   limitation of comparing data between different countries is that mycotoxin contamination levels can vary

306   between seasons and years, so conclusions about relative frequencies of contamination between

307   different regions or countries cannot be drawn without multiple sampling over a period of time.

308   **5. CONCLUSIONS**

309   The UPLC-MS/MS method was successfully used to detect and quantify the nine mycotoxins of interest

310   from the Tanzanian maize porridge samples. All three Tanzanian villages had a considerable mycotoxin

311   contamination as 82% of samples contained two or more groups of mycotoxins. Fumonisins were by

312   far the most prevalent mycotoxins as they were detected in all samples. FB_1 and FB_2 contamination

313   was found to have a seasonal pattern, being lower following a period of storage. AFB_1 was detected

314   primarily in Kigwa, with higher concentrations following storage. DON showed large regional variance,

315   with complete absence from Kigwa village which matched up well the biomarker data in the same

316   village. The data obtained for AFB_1, FB_1 and DON was comparable with exposure findings based on

317   their corresponding biomarkers. Finally, the co-contamination data showed that both Tanzania adults

318   and children from these villages are at risk from multiple mycotoxins in maize. It is not known to what

319   extent co-contamination with multiple mycotoxins may contribute to health effects. This study highlights

320   the need to understand the extent of mycotoxin co-contamination so that proper contamination control

321   can be implemented in these vulnerable areas.

326   References

327   Abia, W. A., Warth, B., Sulyok, M., Krska, R., Tchana, N. A., Njobeh, P. B., . . . Moundipa, P. F.

328   (2013). Determination of multi-mycotoxin occurrence in cereals, nuts and their products in

329   cameroon by liquid chromatography tandem mass spectrometry (LC-MS/MS). Food Control,

330   31(2), 438-453.

331   Álavarez, L., Gil, A. G., Ezpeleta, O., García-Jalón, J. A., & López de Cerain, A. (2004). Immunotoxic

332   effects of ochratoxin A in wistar rats after oral administration. Food and Chemical Toxicology,

333   42(5), 825-834.

334   Azziz-Baumgartner, E., Lindblade, K., Gieseker, K., Rogers, H. S., Kieszak, S., Njapau, H., . . .

335   Aflatoxin, I. G. (2005). Case-control study of an acute aflatoxicosis outbreak, kenya, 2004.

336   Environmental Health Perspectives, 113(12), 1779-1783.

337   Bakırdere, S., Bora, S., Bakırdere, E. G., Aydın, F., Arslan, Y., Komesli, O. T., . . . Yıldırım, E. (2012).

338   Aflatoxin species: Their health effects and determination methods in different foodstuffs. Central

339   European Journal of Chemistry, 10(3), 675-685.

340   Benedetti, R., Nazzi, F., Locci, R., & Firrao, G. (2006). Degradation of fumonisin B1 by a bacterial

341   strain isolated from soil. Biodegradation, 17(1), 31-38.

342   Bennett, J. W., & Klich, M. (2003). Mycotoxins. Clinical Microbiology Reviews, 16(3), 497-516.

343   European Commission. (2006). Commission regulation (EC) no 1881/2006 of 19 december 2006

344   setting maximum levels for certain contaminants in foodstuffs. Official Journal, L364, 5-24.

345   Ezekiel, C. N., Warth, B., Ogara, I. M., Abia, W. A., Ezekiel, V. C., Atehnkeng, J., . . . Bandyopadhyay,

346   R. (2014). Mycotoxin exposure in rural residents in northern nigeria: A pilot study using multi-

347   urinary biomarkers. Environmental International, 66, 138-145.

348   Forsell, J. H., Witt, M. F., Tai, J. H., Jensen, R., & Pestka, J. J. (1986). Effects of 8-week exposure of

349   the B6C3F1 mouse to dietary deoxynivalenol (vomitoxin) and zearalenone. Food & Chemical

350   Toxicology, 24(3), 213-219.

351   Frenich, A. G., Vidal, J. L. M., Romero-Gonzalez, R., & Aguilera-Luiz, M. M. (2009). Simple and high-

352   throughput method for the multimycotoxin analysis in cereals and related foods by ultra-high

353   performance liquid chromatography/tandem mass spectrometry. Food Chemistry, 117(4), 705-

354   712.

355 Gong, Y. Y., Hounsa, A., Egal, S., Turner, P. C., Sutcliffe, A. E., Hall, A. J., . . . Wild, C. P. (2004).

356   Postweaning exposure to aflatoxin results in impaired child growth: A longitudinal study in benin,

357   west africa. Environmental Health Perspectives, 112(13), 1334-1338.

358 Huang, L. C., Zheng, N., Zheng, B. Q., Wen, F., Cheng, J. B., Han, R. W., . . . Wang, J. Q. (2013).

359   Simultaneous determination of aflatoxin M1, ochratoxin A, zearalenone and α-zearalenol in milk

360   by UHPLC-MS/MS. Food Chemistry, 146, 242-249.

361 International Agency for Research on Cancer. (2002). IARC monographs on the evaluation of

362   carcinogenic risks to humans; some mycotoxins, lyons france

363 Jiang, S. Z., Yang, Z. B., Yang, W. R., Gao, J., Liu, F. X., Broomhead, J., & Chi, F. (2011). Effects of

364   purified zearalenone on growth performance, organ size, serum metabolites, and oxidative

365   stress in postweaning gilts. Journal of Animal Science, 89, 3008-3015.

366 Jiang, Y., Jolly, P. E., Ellis, W. O., Wang, J. S., Phillips, T. D., & Williams, J. H. (2005). Aflatoxin B1

367   albumin adduct levels and cellular immune status in ghanaians. International Immunology, 17(6),

368   807-814.

369 Jiang, Y., Jolly, P. E., Preko, P., Wang, J. S., Ellis, W. O., Phillips, T. D., & Williams, J. H. (2008).

370   Aflatoxin-related immune dysfunction in health and in human immunodeficiency virus disease.

371   Clinical & Developmental Immunology, 2008, 1-12.

372 Joint FAO/WHO Expert Committee on Food Additives. (2011). Evaluation of certain food additivies

373   and contaminants, seventy-fourth report of the joint FAO/WHO expert committee on food

374   additives

375 Kamala, A., Ortiz, J., Kimanya, M., Haesaert, G., Donoso, S., Tiisekwa, B., & De Meulenaer, B.

376   (2015). Multiple mycotoxin co-occurrence in maize grown in three agro-ecological zones of

377   tanzania. Food Control, 54, 208-215.

378   Kimanya, M. E., Shirima, C. P., Magoha, H., Shewiyo, D. H., De Meulenaer, B., Kolsteren, P., &

379   Gong, Y. Y. (2014). Co-exposures of aflatoxins with deoxynivalenol and fumonisins from maize

380   based complementary foods in rombo, northern tanzania. Food Control, 41, 76-81.

381   Lacina, O., Zachariasova, M., Urbanova, J., Vaclavikova, M., Cajka, T., & Hajslova, J. (2012). Critical

382   assessment of extraction methods for the simultaneous determination of pesticide residues and

383   mycotoxins in fruits, cereals, spices and oil seeds employing ultra-high performance liquid

384   chromatography–tandem mass spectrometry. Journal of Chromatography A, 1262, 8-18.

385   Liao, C. D., Wong, J. W., Zhang, K., Hayward, D. G., Lee, N. S., & Trucksess, M. W. (2013). Multi-

386   mycotoxin analysis of finished grain and nut products using high-performance liquid

387   Chromatography–Triple-quadrupole mass spectrometry. Journal of Agricultural and Food

388   Chemistry, 61(20), 4771-4782.

389   Lioi, M. B., Santoro, A., Barbieri, R., Salzano, S., & Ursini, M. V. (2004). Ochratoxin A and

390   zearalenone: A comparative study on genotoxiceffects and cell death induced in bovine

391   lymphocytes. Mutation Research, 557(1), 19-27.

392   Malachová, A., Sulyok, M., Beltrán, E., Berthiller, F., & Krska, R. (2014). Optimization and validation

393   of a quantitative liquid chromatography–tandem mass spectrometric method covering 295

394   bacterial and fungal metabolites including all regulated mycotoxins in four model food matrices.

395   Journal of Chromatography A, 1362, 145-156.

396   Marasas, W. F. O. (2001). Discovery and occurrence of the fumonisins: A historical perspective.

397   Environmental Health Perspectives, 109(2), 239-243.

398   Massart, F., Meucci, V., Saggese, G., & Soldani, G. (2007). High growth rate of girls with precocious

399   puberty exposed to estrogenic mycotoxins. The Journal of Pediatrics, 152(5), 690-695.

400   Matumba, L., Sulyok, M., Monjerezi, M., Biswick, T., & Krska, R. (2014). Fungal metabolites diversity

401   in maize and associated human dietary exposures relate to micro-climatic patterns in malawi.

402   Journal of Agricultural Food and Chemistry, 8(34), 269-282.

403 Missmer, S. A., Suarez, L., Felkner, M., Wang, E., Merrill, A. H., Rothman, K. J., & Hendricks, K. A.

404 (2006). Exposure to fumonisins and the occurrence of neural tube defects along the texas-

405 mexico border. Environmental Health Perspectives, 114(2), 237-241.

406 Nyikal, J., Misore, A., Nzioka, C., Njuguna, C., Muchiri, E., Njau, J., . . . Bowen, A. (2004). Outbreak of

407 aflatoxin poisoning - eastern and central provinces, kenya, january-july 2004 78. Morbidity and

408 Mortality Weekly Report, 53(34), 790-793.

409 Oplatowska-Stachowiak, M., Haughey, S. A., Chevallier, O. P., Galvin-King, P., Campbell, K.,

410 Magowan, E., . . . Elliott, C.,T. (2015). Determination of the mycotoxin content in distiller's dried

411 grain with solubles using a multianalyte UHPLC-MS/MS method. Journal of Agricultural and

412 Food Chemistry, 63(43), 9441-9451.

413 Rotter, B. A., Thompson, B. K., Lessard, M., Trenholm, H. L., & Tryphonas, H. (1994). Influence of

414 low-level exposure to fusarium mycotoxins on selected immunological and hematological

415 parameters in young swine. Fundamental and Applied Toxicology, 23(1), 117-124.

416 Schaaf, G. J., Nijmeijer, S. M., Maas, R. F. M., Roestenberg, P., de Groene, E. M., & Fink-Gremmels,

417 J. (2002). The role of oxidative stress in the ochratoxin A-mediated toxicity in proximal tubular

418 cells. Biochimica Et Biophysica Acta (BBA) - Molecular Basis of Disease, 1588, 149-158.

419 Serra, R., Braga, A., & Venâncio, A. (2005). Mycotoxin-producing and other fungi isolated from

420 grapes for wine production, with particular emphasis on ochratoxin A. Research in Microbiology,

421 156(4), 515-521.

422 Shephard, G. S., Burger, H. M., Gambacorta, L., Gong, Y. Y., Krska, R., Rheeder, J. P., . . . van der

423 Westhuizen, L. (2013). Multiple mycotoxin exposure determined by urinary biomarkers in rural

424 subsistence farmers in the former transkei, south africa. Food and Chemical Toxicology, 62, 217-

425 225.

426 Shirima, C. P., Kimanya, M. E., Kinabo, J. L., Routledge, M. N., Srey, C., Wild, C. P., & Gong, Y. Y.

427 (2013). Dietary exposure to aflatoxin and fumonisin among tanzanian children as determined

428 using biomarkers of exposure. Molecular Nutrition & Food Research, 57(10), 1874-1881.

429   Srey, C., Kimanya, M. E., Routledge, M. N., Shirima, C. P., & Gong, Y. Y. (2014). Deoxynivalenol

430   exposure assessment in young children in tanzania. Molecular Nutrition & Food Research, 58(7),

431   1574-1580.

432   Van de Perre, E., Deschuyffeleer, N., Jacxsens, L., Vekeman, F., Van Der Hauwaert, W., Asam, S., . .

433   . De Meulenaer, B. (2014). Screening of moulds and mycotoxins in tomatoes, bell peppers,

434   onions, soft red fruits and derived tomato products. Food Control, 37, 165-170.

435   van der Westhuizen, L., Shephard, G. S., Rheeder, J. P., Burger, H. M., Gelderblom, W. C., Wild, C.

436   P., & Gong, Y. Y. (2010). Simple intervention method to reduce fumonisin exposure in a

437   subsistence maize-farming community in south africa. Food Additives & Contaminants: Part A,

438   27(11), 1582-1588.

439   Wild, C. P., & Gong, Y. Y. (2010). Mycotoxins and human disease: A largely ignored global health

440   issue. Carcinogenesis, 31(1), 71-82.

441   Williams, J. H., Phillips, T. D., Jolly, P. E., Stiles, J. K., Jolly, C. M., & Aggarwal, D. (2004). Human

442   aflatoxicosis in developing countries: A review of toxicology, exposure, potential health

443   consequences, and interventions. The American Journal of Clinical Nutrition, 80(5), 1106-1122.

444   Yogendrarajah, P., Van Poucke, C., De Meulenaer, B., & De Saeger, S. (2013). Development and

445   validation of a QuEChERS based liquid chromatography tandem mass spectrometry method for

446   the determination of multiple mycotoxins in spices. Journal of Chromatography A, 1297, 1-11.

447   Zachariasova, M., Dzuman, Z., Veprikova, Z., Hajkova, K., Jiru, M., Vaclavikova, M., . . . Hajslova, J.

448   (2014). Occurrence of multiple mycotoxins in european feedingstuffs, assessment of dietary

449   intake by farm animals. Animal Feed Science and Technology, 193, 124-140.

450