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Geary, P. A., Chen, G., Kimanya, M. E., Shirima, C. P., Oplatowska-Stachowiak, M., Elliott, C. T., Routledge, M. N., & Gong, Y. (2016). Determination of multi-mycotoxin occurrence in maize based porridges from selected regions of Tanzania by liquid chromatography tandem mass spectrometry (LC-MS/MS), a longitudinal study. *Food Control*, 68, 337-343. <https://doi.org/10.1016/j.foodcont.2016.04.018>

**Published in:**  
Food Control

**Document Version:**  
Peer reviewed version

**Queen's University Belfast - Research Portal:**  
[Link to publication record in Queen's University Belfast Research Portal](#)

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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**

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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<sub>1</sub> (AFB<sub>1</sub>), aflatoxin B<sub>2</sub> (AFB<sub>2</sub>), aflatoxin G<sub>1</sub> (AFG<sub>1</sub>), aflatoxin G<sub>2</sub> (AFG<sub>2</sub>), fumonisin  
 20 B<sub>1</sub> (FB<sub>1</sub>), fumonisin B<sub>2</sub> (FB<sub>2</sub>), deoxynivalenol (DON), ochratoxin A (OTA) and zearalenone (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<sub>1</sub>+FB<sub>2</sub>) 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<sub>1</sub> was lowest in samples taken from time point 2. Conversely, In  
 25 Kigwa village there was a distinct trend that AFB<sub>1</sub> mean concentration was highest in samples taken

Abbreviations: AFB<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub>, AFG<sub>2</sub>, aflatoxin B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, G<sub>2</sub>; DON, deoxynivalenol; FB<sub>1</sub>, FB<sub>2</sub>, fumonisin B<sub>1</sub>, B<sub>2</sub>; IARC, International Agency for Research on Cancer; LOD, LOQ, limit of detection, quantification; OTA, ochratoxin A; QuEChERS, Quick, Easy, Cheap, Effective, Rugged, Safe; UPLC-MS/MS, ultra-performance liquid chromatography tandem mass spectrometry; ZEN, zearalenone.

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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<sub>1</sub> (AFB<sub>1</sub>), aflatoxin B<sub>2</sub> (AFB<sub>2</sub>), aflatoxin G<sub>1</sub> (AFG<sub>1</sub>), aflatoxin G<sub>2</sub> (AFG<sub>2</sub>),  
45 fumonisin B<sub>1</sub> (FB<sub>1</sub>), fumonisin B<sub>2</sub> (FB<sub>2</sub>), 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 (Bakirdere 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<sub>1</sub>-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<sub>1</sub>+AFB<sub>2</sub>+AFG<sub>1</sub>+AFG<sub>2</sub>) 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 Fumonisin, a group of mycotoxins produced by fungal species belonging to the *Fusarium* genus, have  
66 also been shown to have detrimental health effects. Fumonisin 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<sub>1</sub>,  
91 FB<sub>1</sub> 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<sub>1</sub>, FB<sub>1</sub> and DON through their diet. Urinary FB<sub>1</sub> 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<sub>1</sub> (50%) and FB<sub>1</sub> (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<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub>, AFG<sub>2</sub>, FB<sub>1</sub>, FB<sub>2</sub>, 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°C. Bondesil C<sub>18</sub> 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<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub>,  
128 AFG<sub>2</sub>, FB<sub>1</sub>, FB<sub>2</sub>, 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<sub>1</sub> LOD: 0.05 ng/g, LOQ: 0.125 ng/g; AFB<sub>2</sub>/AFG<sub>1</sub>  
135 LOD: 0.125 ng/g, LOQ: 0.25 ng/g; AFG<sub>2</sub> 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<sub>1</sub> and 0.2ng/g and  
138 0.5ng/g respectively for FB<sub>2</sub>.

### 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<sub>1</sub>, FB<sub>1</sub> 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<sub>1</sub>  
148 and FB<sub>2</sub> were detected in all 101 samples. The mean ratio of FB<sub>1</sub>:FB<sub>2</sub> 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<sub>1</sub>  
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<sub>1</sub> 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<sub>1</sub>+FB<sub>2</sub>  $\geq 1000$  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<sub>1</sub>+FB<sub>2</sub>  $\geq 200$  ng/g). At least one type of aflatoxin was  
157 detected in 50% of all samples. The mean ratios of AFB<sub>1</sub>:AFB<sub>2</sub> and AFG<sub>1</sub>:AFG<sub>2</sub> 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

<b>Mycotoxin</b>	<b>Village/ Region</b>	<b>Time point</b>	<b>Positive Samples (%)</b>	<b>Mean (ng/g)</b>	<b>Median (ng/g)</b>	<b>Range (ng/g)</b>
<b>Fumonisin B<sub>1</sub></b>	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
<b>Fumonisin B<sub>2</sub></b>	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
<b>Aflatoxin B<sub>1</sub></b>	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)
<b>Total Aflatoxins (B<sub>1</sub>+B<sub>2</sub>+G<sub>1</sub>+G<sub>2</sub>)</b>	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
<b>Deoxynivalenol</b>	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)	-	-	-
<b>Zearalenone</b>	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<sub>1</sub>+AFB<sub>2</sub>+AFG<sub>1</sub>+AFG<sub>2</sub>; 2. Total  
168 fumonisins: FB<sub>1</sub>+FB<sub>2</sub>; 3. DON; 4. ZEN; 5. OTA.

169 The highest concentration of AFB<sub>1</sub> 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<sub>1</sub> concentration (AFB<sub>1</sub> ≥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  $\geq 3.0$  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<sub>1</sub> 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<sub>1</sub> 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<sub>1</sub>, AFB<sub>1</sub> and DON  
262 contamination in food are consistent with urinary FB<sub>1</sub> (uFB<sub>1</sub>), 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<sub>1</sub> in maize porridge and AF-Alb biomarker in blood was not entirely consistent.

271 The increase in the AFB<sub>1</sub> 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<sub>1</sub>  
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<sub>1</sub> contaminated maize from time point 2 which may still  
277 affect the AF-Alb biomarker measurements.

278 The FB<sub>1</sub> found in this study was also compared against that of uFB<sub>1</sub> biomarker obtained from the same  
279 village and time points. Nyabula village showed the lowest FB<sub>1</sub> contamination in maize but it was  
280 Kikelelwa which showed the lowest FB<sub>1</sub> detected in urine. The lower exposure in Kikelelwa is  
281 presumably due to lower FB<sub>1</sub> 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<sub>1</sub>  
283 contamination in maize and this is in consistent with uFB<sub>1</sub> levels.

284 This study was compared against mycotoxin 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<sub>1</sub> in maize for their respective areas.  
289 The FB<sub>1</sub> median for Kikelelwa village determined from this study (290.18 ng/g) was similar to the FB<sub>1</sub>  
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<sub>1</sub> 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<sub>1</sub> levels  
293 compared to Tanzania. This comparison shows that FB<sub>1</sub> 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<sub>1</sub> 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. Fumonisin were by  
312 far the most prevalent mycotoxins as they were detected in all samples. FB<sub>1</sub> and FB<sub>2</sub> contamination  
313 was found to have a seasonal pattern, being lower following a period of storage. AFB<sub>1</sub> 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<sub>1</sub>, FB<sub>1</sub> 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.

322 *The authors declare that they have no actual or potential conflicts of interest.*

323 *Acknowledgement: We acknowledge the funding support by the Leverhulme-Royal Society Africa Award and the*  
324 *administrative support of Tanzania Food and Drugs Authority for this study. The authors would like to thank the subjects and*  
325 *field workers from all the areas where the study was conducted.*

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