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Mycological Analysis and Multimycotoxins in Maize from Rural Subsistence Farmers in the Former Transkei, South Africa

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ABSTRACT: Maize harvested in the Centane region of the former Transkei, Eastern Cape Province, South Africa, by subsistence farmers has been shown over many seasons to be contaminated with fumonisin mycotoxins. However, there are limited data on the presence of other mycotoxins. Two multimycotoxin LC-MS/MS methods were applied to good and moldy maize samples, as separated by the farmers themselves from the 2011 harvest. One method involved extract cleanup on multitoxin immunoaffinity columns before LC-MS/MS analysis for aflatoxins, fumonisins, deoxynivalenol (DON), zearalenone (ZEN), and T-2 and HT-2 toxins. The other method was based on a "dilute-and-shoot" approach for the above mycotoxins and a wide range of other fungal secondary metabolites. Both methods showed high incidences of fumonisins B_1 and B_2 (FB_1 and FB_2) in good maize (100% for both by the first method, means were 2083 and 927 μg/kg for the two analogues; 93% for both by the second method, positive means of 2764 and 1050 μg/kg, respectively). All samples of moldy maize were contaminated (mean FB_1 of 27.64 and 35.98 mg/kg, respectively; mean FB_2 of 10.58 and 14.14 mg/kg, respectively). Comparison of the two methods for FB_1 and FB_2 over the entire range of samples gave R^2 values 0.9144 and 0.8859, respectively. Low levels of DON were found by both methods (positive means of 12 and 4.7 μg/kg in good maize, respectively, and of 14 and 5.8 μg/kg in moldy maize, respectively). ZEN was determined with positive means of 108 and 25 μg/kg in good maize, respectively, and of 111 and 135 μg/kg in moldy maize, respectively. No aflatoxins, OTA, or T-2 or HT-2 toxins were detected. A wide range of other *Fusarium*, *Aspergillus*, *Alternaria*, and *Penicillium* mycotoxins and secondary metabolites were determined.

KEYWORDS: *deoxynivalenol, fumonisin, maize, mycotoxin, multimycotoxin, ochratoxin, secondary metabolites, zearalenone*

INTRODUCTION

The Centane region in the southeast of the former Transkei, Eastern Cape Province, South Africa, is characterized by scattered villages in which maize is grown on a subsistence basis in small plots, mostly adjacent to the houses. This maize, which is traditionally sorted by the inhabitants themselves into good and moldy cobs on the basis of visual examination, is entirely consumed locally. The maize placed aside as moldy is frequently used for making local maize beer, a product consumed in large quantities at community parties. This area has also been characterized as a hotspot for esophageal cancer, with incidence rates for males during 1998−2002 of 33 per 100,000.[1] Previous studies on home-grown maize in this region have shown that *Fusarium verticillioides* occurs widely and that, as a consequence, fumonisin (**1**−**3**) levels can be high, especially in the moldy cobs.[2−5] Long-term averages of fumonisin contamination of good home-grown maize used for an exposure assessment were 820 and 321 μg/kg for fumonisins B_1 (FB_1) and B_2 (FB_2), respectively.[3] Maximum levels reported were 7.90 and 3.77 mg/kg for the two analogues, respectively. Fumonisin levels in the moldy maize have been measured as high as 117 and 23 mg/kg for the two fumonisin analogues, respectively.[2] The diet in this area consists of large amounts of maize or maize-based dishes, with mean daily maize consumption estimated to average 400−500 g/person/day, with the high consumers (90th percentile) consuming >700 g/day.[3] Due to this high maize consumption, estimates of mean adult female probable daily intake (PDI) for fumonisins from consumption of home-grown maize in the Centane region were 8.15 and 6.7 μg/kg bw/day, depending on measurement methodology.[3,6] These estimates are well above the group provisional maximum tolerable daily intake (PMTDI) of 2 μg/kg bw/day set by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) for FB_1, FB_2, and FB_3 alone or combined.[7]

Although multiyear studies of fumonisin contamination have been conducted, the presence of other *Fusarium* mycotoxins has been measured in only a limited number of maize samples. Initial research on a pooled moldy maize sample harvested in 1977 and a single moldy sample from 1978, both collected from the southeast Transkei (Butterworth region), showed the

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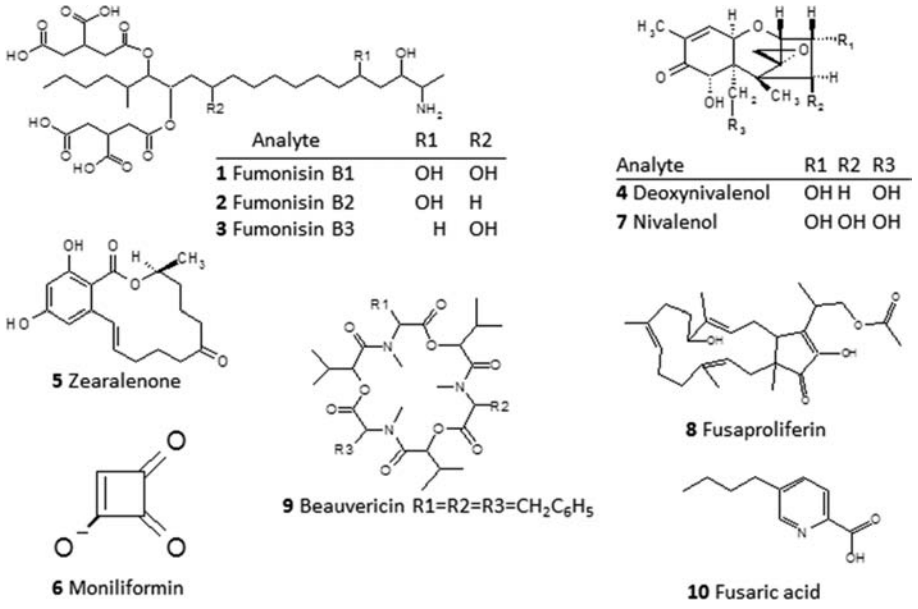

Figure 1. Structures of the most important *Fusarium* mycotoxins quantitated in Transkeian maize.

presence of deoxynivalenol (DON) (**4**; see Figure 1 for structures) at levels of 0.07 and 0.42 mg/kg for the two years, respectively.[8,9] Similarly, zearalenone (ZEN) (**5**) was found at 1.1 and 4.00 mg/kg, respectively, whereas the latter sample also contained moniliformin (MON) (**6**) at 16 mg/kg,[9] and a hand-selected subsample of moldy kernels from this single sample was shown to contain fusarin C (0.28 mg/kg).[10] A larger study on moldy maize collected after the 1985 harvest from 12 households in Centane showed contamination with MON (mean = 0.8 mg/kg), ZEN (mean = 0.4 mg/kg), DON (mean = 0.3 mg/kg), and nivalenol (NIV) (**7**) (mean = 1.8 mg/kg).[11] In all three harvest years, none of the samples showed the presence of T-2 toxin or diacetoxyscirpenol (DAS) (limit of detection (LOD) approximately 0.1 mg/kg). Further information on the range of mycotoxins in Transkeian maize was obtained when 10 moldy maize samples collected during 1997 from the Centane region were analyzed by LC-MS/MS for the *Fusarium* mycotoxins fusaproliferin (FUS) (**8**) and beauvericin (BEA) (**9**).[12] All samples contained BEA (mean = 258 μg/kg), and nine contained FUS (mean of positives = 33 μg/kg).[13] Two of these samples were analyzed for MON by LC-MS/MS and showed low-level contamination (10 and 17 μg/kg).[14]

Recent advances in mycotoxin analysis have led to the development of multimycotoxin LC-MS/MS analytical methods. In general, two different approaches have been taken, namely, one in which the sample extract is purified on a multitoxin immunoaffinity column (IAC) for analysis of the main regulated mycotoxins[15] or a "dilute-and-shoot" method without extract cleanup that allows a wider range of mycotoxins and other secondary metabolites to be determined.[16,17] This latter technique was used to investigate the fumonisin levels in individual maize kernels from batches of good and moldy maize collected in the Centane region.[18] This same survey indicated that individual kernels could contain the fungal secondary metabolites agroclavine, alternariol, altertoxin-I, BEA, calphostin C, chanoclavine, cyclosporins A, C, and H, emodin, equisetin, FUS, fusaric acid (**10**), MON, NIV, regulosin, and tentoxin. The *Streptomyces* bacterial metabolites valinomycin, monactin, and nonactin were also detected. The dilute-and-shoot technique has also recently been applied to determine the contamination levels in a range of African crops (maize, groundnuts, sorghum, millet, rice, sesame seeds, wheat, soy, peanut cake, dried fruits, and certain local processed foods) collected in Burkino Faso, Cameroon, Mozambique, and Nigeria.[19−22] These multimycotoxin methods provide a wider perspective on the mycotoxin contamination of certain crops and are indicative of multitoxin exposure in these populations.

As described above for the Centane region of the former Transkei, there is limited and mostly outdated knowledge about contamination of home-grown maize with *Fusarium* mycotoxins other than fumonisins, as well as on other secondary metabolites. For these reasons, the main aim of this study was to determine a wide range of mycotoxins and fungal secondary metabolites present in a single harvest of the home-grown maize (both good and moldy) collected from households in this rural subsistence farming area and thus reveal the extent of coexposures resulting from home-grown maize consumption by this population with high esophageal cancer incidence. As a minor aim, the use of two different LC-MS/MS methods previously validated[15−17] in the authors' individual laboratories allowed a comparison of results for the principal mycotoxins.

MATERIALS AND METHODS

Sample Collection. Fifty-four samples (approximately 1 kg each) of good home-grown maize were collected from houses in the villages of Gcina, NoBuswana, Qolora-by-the-Sea, and Nontshinga in the Centane region of the former Transkei region, Eastern Cape Province, South Africa, during July 2011. In addition, 38 moldy home-grown maize samples, as separated by the householders themselves, were also collected when available from the same households. At the same time, a cooked maize-based evening meal (porridge or maize kernels) was sampled (approximately 200 g each) as part of a urinary multibiomarker of exposure study (unpublished data). Samples were transported to the PROMEC Unit, Tygerberg, for shelling of cobs (unless kernels had been collected), mycological analysis of kernels, and milling. After mixing, milled subsamples of maize and the food samples were dispatched to the Institute of Sciences of Food Production (ISPA), National Research Council of Italy, Bari, Italy (for LC-MS/MS analysis after IAC cleanup of extracts) and to the University of Natural Resources and Life Sciences, Vienna (BOKU),

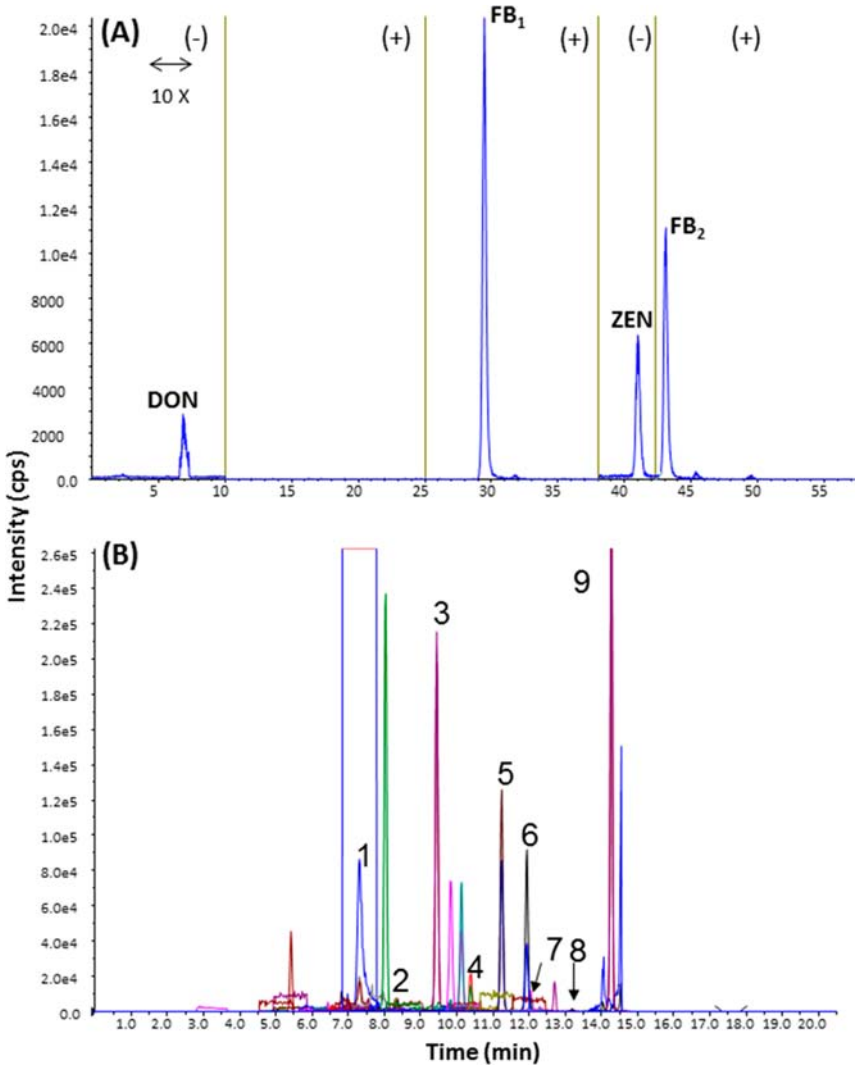

Figure 2. (A) Representative chromatogram of maize analyzed by LC-MS/MS with IAC cleanup, showing DON (10 μg/kg), FB_1 (3111 μg/kg), FB_2 (1509 μg/kg), and ZEN (197 μg/kg); (B) positive ion chromatogram of maize analyzed by "dilute-and-shoot" LC-MS/MS showing (1) fusaric acid (557 μg/kg), (2) diacetoxyscirpenol (6 μg/kg), (3) FB_1 (6845 μg/kg), (4) FB_3 (451 μg/kg), (5) FB_2 (2269 μg/kg), (6) aurofusarin (3878 μg/kg), (7) culmorin (33 μg/kg), (8) fusaproliferin (184 μg/kg), and (9) beauvericin (138 μg/kg). Other metabolites are either too low in response or quantitated in the negative ion mode.

Department for Agrobiotechnology (IFA-Tulln), Center for Analytical Chemistry, Tulln, Austria (for LC-MS/MS analysis by a dilute-and-shoot method). The study was approved by the Ethics Committee of the South African Medical Research Council and informed consent obtained from participants.

Mycological Analysis. A 100 g subsample of kernels from each collected good maize sample was surface disinfected for 1 min in a 3.5% sodium hypochlorite solution and rinsed twice in sterile water. One hundred kernels per sample were plated out (5 kernels per Petri dish) onto 1.5% malt extract agar, containing 150 mg/mL novobiocin. The plates were incubated at 25 °C for 5−7 days. *Fusarium* species were identified according to the method of Nelson et al.[23]

Multimycotoxin Analyses at ISPA. Maize samples were analyzed for FB_1, FB_2, DON, T-2 toxin, HT-2 toxin, ochratoxin A (OTA), ZEN, and aflatoxins AFB_1, AFB_2, AFG_1, and AFG_2 as previously described,[15] with modifications. In particular, the test portion size was increased from 10 to 20 g to reduce subsampling variation; therefore, the volumes of extraction solvents were proportionally increased. The immunoaffinity column was further eluted with 2 mL water after passing 3 mL of methanol according to the manufacturer's instructions. Briefly, the sample (20 g) was extracted twice, first with phosphate-buffered saline (PBS, 100 mL) for 60 min, and then, after centrifugation, 70 mL of PBS extract was collected. Then methanol (70 mL) was added to the remaining solid for the second extraction, centrifugation, and collection of sample extract, which was then diluted 1:10 with PBS. Aliquots of the two extracts were filtered through a glass microfiber filter and separately applied to a Vicam (Watertown, MA, USA) Myco6in1 IAC (equivalent to 1.18 g of the sample), which was washed with water (10 mL) and eluted with methanol (3 mL) and water (2 mL). The eluate was dried at 50 °C under a stream of air and reconstituted in methanol/water (20:80, 200 μL) containing 1 mM ammonium acetate and 0.5% acetic acid. Analysis of 20 μL of purified sample extract was performed on an Agilent (Waldbronn, Germany) 1100 series binary pump and autosampler interfaced with an AB Sciex (Applied Biosystems, Foster City, CA, USA) Q Trap MS/MS system. The column used was a 150 mm × 2 mm i.d., 5 μm, Gemini C18 (Phenomenex, Torrance, CA, USA), which was eluted at 40 °C with a water/methanol gradient, both constituent mobile phases containing 0.5% acetic acid and 1 mM ammonium acetate (Figure 2A). Gradient conditions and MS/MS parameters were as previously described.[15] Calibration was achieved with matrix-matched standards, and results were corrected for method recovery by spiking ground maize samples containing no or low levels of mycotoxins. Samples with FB_1 and FB_2 levels >4000 and >2000 μg/kg, respectively, were reanalyzed as above following appropriate dilution of the crude extracts.

Multimycotoxin Analyses at IFA-Tulln. Maize and maize-based food samples (as sent, undried) were analyzed according to the method previously described[16,17,24] and used for analysis of various African food samples.[19−22] Briefly, maize meal was extracted with acetonitrile/water/acetic acid (79:20:1), and after centrifugation, an aliquot of the extract was diluted with an equal volume of acetonitrile/water/acetic acid (20:79:1) and an aliquot (5 μL) directly injected into the LC-MS/MS consisting of an Agilent 1290 HPLC and an AB Sciex 5500 QTrap MS/MS. Chromatographic separation was performed at 25 °C with two consecutive chromatographic runs, one with positive and the other with negative electrospray ionization (Figure 2B). The column used was a 150 mm × 4.6 mm i.d., 5 μm, Gemini C18 (Phenomenex) eluted with a gradient of methanol/water containing both 5 mM ammonium acetate and 1% acetic acid. Gradient conditions and MS/MS parameters were as previously described.[20] Quantitation was achieved with external calibration, and results were corrected for extraction recovery and matrix effects (apparent recovery) by spiking three different samples. Although previous studies have used the transition m/z 355/265 for DON quantitation due to its low baseline noise,[17] the present study used m/z 355/59 as the former transition was affected by an unidentified interference, which occurred in almost all samples and was rather a broad band indicating a matrix constituent was the cause.

Statistics. Simple linear regression and goodness-of-fit analysis on Stata/IC 11.0 (StataCorp, College Station, TX, USA) was used to determine the relationship between the two different LC-MS/MS methods for FB_1 and FB_2 separately, with the good and moldy maize samples combined. The regression intercept on the axes was set at zero.

RESULTS AND DISCUSSION

The results of mycological analyses of the good maize are presented in Table1. *F. verticillioides*, the primary producer of fumonisins, was present in all samples with a mean incidence of 28.2% of kernels infected. This is within the range (23.1−43.0%) of infection previously recorded in studies of good maize from the Centane region conducted between 1976 and 1989.[2] *Fusarium proliferatum*, also a fumonisin producer, was found in only one sample at 1% incidence, which indicates it is not an important contributor to fumonisin contamination in these samples. *Fusarium subglutinans* and *Fusarium graminearum sensu lato*, which are not fumonisin producers, were identified in 46 samples (overall mean incidence = 4.7%) and 18 samples (overall mean = 1.3%), respectively. The former, together with some *F. verticillioides* strains, is a MON producer, whereas the latter is mainly a trichothecene and ZEN producer. Compared with the harvests investigated between 1976 and 1989, *F. subglutinans* incidence in the present study was similar to the lower levels (3.5 and 5.5%) previously observed, whereas the *F. graminearum* incidence was below the previous lowest level of 2.5%, which accords with the low levels of DON discussed below.[2] *Aspergillus flavus*, a producer of aflatoxins, was found in three samples at 1% incidence in each. This confirms previous unpublished observations of the general absence of *A. flavus* or *Aspergillus parasiticus* in the maize grown in the Centane region.

Table 1. Mycological Analysis of the Good Maize (n = 53) Collected from Participants

	fungal incidence (% positive kernels)	
fungal species	mean ± sd (%)	median
Fusarium verticillioides	28.2 ± 19.1	24
Fusarium proliferatum	0.02 ± 0.14	0
Fusarium subglutinans	4.7 ± 4.6	4
Fusarium graminearum sensu lato	1.3 ± 2.6	0
other *Fusarium*	1.4 ± 2.5	0
Stenocarpella maydis	3.3 ± 4.3	1
Stenocarpella macrospora	0.8 ± 1.9	0
Aspergillus flavus	0.06 ± 0.23	0
other fungi	35.2 ± 11.1	35

The results of LC-MS/MS analysis of the good and moldy maize samples by both analytical techniques are shown in Table 2 for the agriculturally important mycotoxins (FB_1, FB_2, FB_3, DON, ZEN, OTA, and AFB_1) and in Table 3 for the other minor mycotoxins and fungal secondary metabolites determined by the dilute-and-shoot method. With regard to the food analysis, the levels of the agriculturally important mycotoxins have been determined in relation to urinary multibiomarker determinations (unpublished results), whereas Table 4 contains data on the minor mycotoxins found in the food samples by the dilute-and-shoot method. The fumonisin contamination levels measured in this maize harvest are the highest yet recorded for this rural area[2,4,5] in that the mean and maximum levels of FB_1 in positive samples of good maize (by both methods) exceed the mean of 1840 μg/kg and the maximum of 7900 μg/kg determined in the 1989 and 1985 harvests, respectively.[2] Studies on fumonisin levels in this geographic area have shown there is considerable interyear variation, presumably due to differing growing conditions. Similarly, the levels of FB_2 measured in the 2011 harvest exceed previous highest levels, namely, the mean of 610 μg/kg and the maximum of 2250 μg/kg, measured in the 1985 harvest. In general, fumonisin levels in moldy maize are an order of magnitude greater than the corresponding parameter for good maize (Table 2). Mean FB_1 and FB_2 levels are lower than the previous highest recorded levels (1989 harvest with means of 53,740 and 13,680 μg/kg, respectively), except for a marginally higher mean FB_2 (14,140 μg/kg) determined by the dilute-and-shoot method. Despite this, the maxima measured for FB_1 and FB_2 far exceed the previous highest levels determined in the 1989 harvest of 117,520 and 22,960 μg/kg for these two analogues, respectively. The two analytical methods employed in these analyses represent different approaches to multimycotoxin analysis. The wide range of contamination in these maize samples allows the two methods to be compared over an analytical range of nearly 4 orders of magnitude. A highly significant linear relationship ($p < 0.001$) was shown to exist between the two methods for both FB_1 and FB_2 (Figure 3) with R^2 values of 0.9144 and 0.8859, respectively, with the regression being forced through zero. On the basis of this evidence, the dilute-and-shoot method would appear overall to be measuring 12 and 21% higher values than the IAC cleanup method for FB_1 and FB_2, respectively. The difference between the methods could be explained by the variability of the multimycotoxin methodologies currently available as confirmed in a recent proficiency test among 41 laboratories using LC-MS(/MS) in which a robust and reliable method for simultaneous determination of mycotoxins in maize was not identified.[25]

As may be expected from the low incidence of *F. graminearum*, only low levels of DON were found (Table 2). The dilute-and-shoot method quantitated low levels of DON in all samples of good maize with a mean of 4.7 μg/kg and a maximum of 14 μg/kg. Comparable levels of DON were measured with the IAC cleanup method, but the toxin was detected in only 6% of these samples with mean and maximum of 12 and 14 μg/kg, respectively. The higher LOD value (3.6 μg/kg) of the IAC cleanup method as compared to the LOD

Table 2. Contamination Levels of Agriculturally Important Mycotoxins in Good (n = 54) and Moldy (n = 38) Maize from Centane Region, Former Transkei

			good maize				moldy maize			
toxin	recovery (%)	LOD[a] (μg/kg)	% positive	mean[b] ± sd[c] (μg/kg)	median[b] (μg/kg)	range[b] (μg/kg)	% positive	mean[b] ± sd[c] (μg/kg)	median[b] (μg/kg)	range[b] (μg/kg)
IAC Cleanup Method										
FB_1	93	3.9	100	2083 ± 3630	848	56–14990	100	27640 ± 38970	14940	514–190100
FB_2	95	3.7	100	927 ± 1565	299	38–6444	100	10580 ± 13810	5792	222–64840
DON	71	3.6	6	12 ± 2	12	10–14	11	14 ± 8	11	7.5–25
ZEN	60	1.5	32	108 ± 185	29	4.2–675	61	111 ± 167	23	1.6–614
OTA	79	3.6	0				0			
AFB_1	72	1.2	0				0			
Dilute-and-Shoot Method										
FB_1	55	3	93	2764 ± 3584	1405	11–17120	100	35980 ± 41790	18330	927–178800
FB_2	58	1.5	93	1050 ± 1472	429	7.9–7680	100	14140 ± 17030	6810	314–74680
FB_3	66	1	93	192 ± 268	75	0.5–1312	100	2438 ± 2739	1355	90–11280
DON	125	0.8	100	4.7 ± 2.1	4.3	2.2–14	100	5.8 ± 2.6	5.2	1.1–12
ZEN	95	0.3	39	44 ± 88	4.1	0.6–329	74	184 ± 420	11	0.1–1648
OTA	92	0.4	0				0			
AFB_1	33	0.3	0				0			

[a]Limit of detection. [b]Mean/median/range of positive samples. [c]Standard deviation.

value of the dilute-and-shoot method (0.8 μg/kg) would explain the lower percentage of positive samples. Interestingly, the levels measured in moldy maize were no different from those in the good maize. Thus, consumption of moldy maize carries a risk of increased fumonisin exposure, but not of DON. Approximately a third of good maize samples had detectable levels of ZEN, with the IAC method yielding levels (mean = 108 μg/kg, maximum = 675 μg/kg) about 2-fold higher than those of the dilute-and-shoot method (mean = 44 μg/kg, maximum = 329 μg/kg). The 2-fold difference of ZEN levels measured by the two laboratories for good maize (Table 2) could, as for fumonisins, be explained by the variability of current multimycotoxin methodologies.[25] ZEN levels in moldy maize as determined by the IAC method were similar to those of the good maize, whereas levels as determined by dilute-and-shoot method were greater. Both methods indicated that there was a higher incidence of positive samples in the moldy maize as compared to the good maize. A previous study on 12 moldy maize samples from the Centane region found a mean DON level of 300 μg/kg, a mean ZEN level of 400 μg/kg, and a mean *F. graminearum* incidence of 8.0%, all figures being higher than in the current samples.[11] Despite detection limits in the low parts per billion range, none of the good or moldy maize samples showed the presence of OTA or aflatoxins. This is consistent with the general absence of the producing fungi.

The PDI of mycotoxins from consumption of good home-grown maize can be estimated by assuming a daily maize consumption of 500 g and a mean contamination level equal to the higher of the two means produced by the analytical methods. Thus, at a mean total fumonisin (FB_1 + FB_2 + FB_3) level of 4006 μg/kg (as determined by the dilute-and-shoot method) and assuming a mean body weight (bw) of 60 kg, the PDI would be 33.4 μg/kg bw/day, well above the PMTDI of 2 μg/kg bw/day set by the JECFA for FB_1, FB_2, and FB_3 combined.[7] A similar calculation for DON, assuming a mean contamination level of 4.7 μg/kg (mean contamination from IFA-Tulln data on all samples), would result in an estimated PDI of 0.04 μg/kg bw/day, well below the group PMTDI of 1 μg/kg bw/day set by JECFA for DON and its acetylated analogues.[26] For ZEN at a mean contamination of 108 μg/kg (positive samples by IAC method), estimated mean PDI would be 0.9 μg/kg bw/day, well above the PMTDI of 0.5 μg/kg bw/day established by the JECFA, which recommended that the combined intake of ZEN and its metabolites should not exceed this level.[27] However, if the negative samples are included by assigning a level of half the LOD, then this mean PDI drops to 0.3 μg/kg bw/day.

The dilute-and-shoot LC-MS/MS method quantitated another 17 *Fusarium* metabolites, including very low levels of the masked mycotoxins DON-3-glucoside and ZEN-4-sulfate and the ZEN metabolites α- and β-zearalenol. T-2/HT-2 toxins and acetyl-DON analogues were not detected. Fusarin C, previously identified in moldy maize,[10] was not included in the suite of toxins analyzed due to the absence of a standard. The widespread presence of BEA was confirmed (100% in both good and moldy maize) at similar levels in moldy maize (mean = 238 μg/kg) to that previously found[13] (mean = 258 μg/kg), and a lower but persistent contamination was shown in good maize (mean = 19 μg/kg). FUS was quantitated in 31 and 42% of good and moldy samples, respectively, with mean levels of 40 and 98 μg/kg. These mean levels are of similar order to the mean level of 33 μg/kg previously found in 9 of 10 moldy maize samples.[13] Mean MON levels in both good and moldy maize (68 and 180 μg/kg, respectively) were lower than in the previous study[11] in Centane in which a mean of 800 μg/kg was reported. There was widespread occurrence of NIV (81 and 87% in good and moldy maize, respectively), but mean levels (36 and 140 μg/kg, respectively) were well below the levels found by Sydenham[11] of 1800 μg/kg in moldy maize from the 1985 harvest in Centane. Although FB_1, FB_2, and FB_3 have been widely found in Transkeian maize, this is the first report of the occurrence of hydrolyzed FB_1 in untreated maize from Transkei. The hydrolyzed FB_1 was identified by MS/MS transitions 406/370 and 406/388 with an ion ratio of 0.90 occurring at the chromatographic retention time of the standard. Previous applications of this dilute-and-shoot method to maize collected in Burkina Faso and Mozambique have also found low levels of hydrolyzed FB_1.[19] However, levels are

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Table 3. Minor Mycotoxin and Secondary Metabolite Contamination Levels in Good (n = 54) and Moldy (n = 38) Maize from Centane Region, Former Transkei

			good maize (n = 54)				moldy maize (n = 38)			
mycotoxin	recovery (%)	LOD[a] (μg/kg)	% positive	mean[b] ± sd[c] (μg/kg)	median[b] (μg/kg)	range[b] (μg/kg)	% positive	mean[b] ± sd[c] (μg/kg)	median[b] (μg/kg)	range[b] (μg/kg)
***Fusarium* Metabolites**										
apicidin	96	0.1	2	0.02			8	2.0 ± 2.3	1.3	0.08−4.5
aurofusarin	69	1	89	223 ± 575	36	0.2−3878	82	640 ± 1566	107	1.2−8480
beauvericin	69	0.02	100	19 ± 34	6.3	0.2−161	100	238 ± 484	84	2.5−2905
butenolide	82	4	61	38 ± 41	22	1.8−200	66	61 ± 69	29	2.7−311
chlamydosporol	97	0.2								
culmorin	73	0.8	39	17 ± 21	8.4	0.8−88	39	29 ± 27	14	3.2−75
diacetoxyscirpenol	76	0.2	50	1.2 ± 1.2	0.8	0.2−6.4	89	6.1 ± 13	1.7	0.3−60
equisetin	167	0.2	59	25 ± 57	2.3	0.2−242	89	131 ± 245	33	3.1−1134
fusaproliferin	48	1.5	31	40 ± 48	27	1.7−184	42	98 ± 83	71	6.7−263
fusaric acid	89	20	93	152 ± 162	79	20−886	100	1773 ± 1362	1558	101−6666
hydrolyzed FB_1	53	0.1	57	54 ± 153	11	0.1−849	100	631 ± 850	209	6.1−2986
moniliformin	96	0.5	35	68 ± 143	3.4	0.9−480	53	180 ± 330	49	0.9−1323
monoacetoxyscirpenol	78	0.5	28	3.9 ± 4.1	2.8	0.5−18	71	12 ± 11	9.2	1.4−51
nivalenol	100	0.7	81	36 ± 85	15	0.7−557	87	140 ± 250	38	0.6−1203
α-zearalenol	91	0.5	9	2.8 ± 3.5	0.8	0.7−8.8	39	5.1 ± 6.0	1.6	0.3−18
β-zearalenol	83	0.5	24	2.4 ± 3.7	1.2	0.5−15	53	12 ± 19	2.6	0.2−57
Masked *Fusarium* Metabolites										
DON-3-glucoside	100	0.2	6	0.5 ± 0.2	0.4	0.3−0.7	13	1.1 ± 0.6	0.8	0.5−1.9
ZEN-4-sulfate	89	0.03	28	3.6 ± 9.3	0.7	0.1−36	24	16 ± 23	1.7	0.5−67
***Aspergillus* Metabolites**										
cytochalasin E[d]	63	0.5	4	126 ± 177	126	0.9−251	11	6.7 ± 7.6	4.0	1.0−18
3-nitropropionic acid	109	0.2	6	0.5 ± 0.2	0.5	0.2−0.7	11	1.1 ± 0.6	1.1	0.5−1.7
α-ochratoxin A[e]	66	3	0				5	11 ± 8	11	5.3−17
terrein[e]	70	5	6	31 ± 36	15	5.6−72	11	95 ± 128	42	11−287
***Alternaria* Metabolites**										
alternariol methyl ether	90	0.02	59	0.6 ± 1.4	0.1	0.02−7.7	84	2.7 ± 7.7	0.3	0.01−42
macrosporin	96	0.05	15	1.8 ± 1.9	1.2	0.06−6.2	39	8.3 ± 13	3.5	0.2−52
monocerin	66	0.05	28	7.0 ± 12	1.5	0.4−40	45	11 ± 27	1.6	0.3−111
***Penicillium* Metabolites**										
agroclavine	65	0.005	6	1.0 ± 0.8	0.9	0.2−1.8	3	0.4		
andrastatin A			9	*i*			13	*i*		
chanoclavine	75	0.015	56	0.5 ± 0.6	0.2	0.02−3.0	100	3.3 ± 3.5	2.1	0.1−15
chrysophanol	92	0.4	46	3.0 ± 3.2	2.0	0.4−16	39	12 ± 34	2.7	1.2−136
citreoviridin	66	3	7	68 ± 75	41	14−175	5	26 ± 36	26	0.3−51
curvularin	100	0.08	28	21 ± 72	0.6	0.08−282	53	31 ± 79	0.8	0.2−326
cyclopenin	68	0.05	50	7.9 ± 15	0.5	0.09−66	71	38 ± 76	10	0.3−350
cyclopenol	71	0.05	59	98 ± 250	6.3	0.05−1301	76	447 ± 1146	95	0.4−5855
dechlorogriseofulvin	68	0.2	6	2.2 ± 0.4	2.1	1.8−2.5	11	433 ± 860	4.5	0.3−1724
emodin[f]	93	0.02	67	1.0 ± 1.1	0.6	0.02−5.2	100	7.0 ± 21	2.4	0.01−131
griseofulvin	66	0.2	6	1.9 ± 0.9	1.5	1.4−2.9	18	131 ± 339	2.4	0.02−899
mycophenolic acid	78	0.4	81	186 ± 424	43	0.4−2495	95	436 ± 784	95	1.5−3984
penitrem A	87	3	7	8.6 ± 3.0	8.2	5.3−12	16	44 ± 52	30	4.3−145
pestalotin	75	0.2	85	4.4 ± 7.3	2.1	0.2−42	100	28 ± 42	10	0.2−158
roquefortine	78	0.2	50	15 ± 32	2.2	0.2−152	21	15 ± 25	2.2	0.4−67
rugulosin	198	0.4	43	15 ± 21	3.6	0.4−64	66	62 ± 88	19	0.6−303
skyrin	67	0.2	94	6.7 ± 13	1.5	0.2−60	100	131 ± 270	22	0.5−1411
viridicatin	69	0.04	57	4.1 ± 7.2	1.3	0.04−37	71	14 ± 25	7.0	0.02− 107
Other Metabolites										
monactin[g]	43	0.02	11	0.7 ± 0.6	0.5	0.1−1.6	55	2.4 ± 4.2	0.8	0.1−18
nonactin[g]	43	0.02	17	0.08 ± 0.07	0.05	0.02−0.2	45	0.3 ± 0.5	0.1	0.01−2.2
radicicol[h]	109	0.4	2	12			5	11 ± 6.0	11	6.6−15

[a]Limit of detection. [b]Mean/median/range of positive samples. [c]Standard deviation. [d]Also *Alternaria* metabolite. [e]Also *Penicillium* metabolite. [f]Also *Aspergillus* metabolite. [g]Bacterial (*Streptomyces*) metabolite. [h]*Nectria* spp. metabolite. [i]Detected.

relatively low with respect to those of FB_1, and recent data indicate that the hydrolyzed fumonisin moiety has low toxicity in vivo.[28] Other secondary metabolites occurring at levels of note are the red naphthoquinone pigment, aurofusarin, which

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Table 4. Minor Mycotoxin and Secondary Metabolite Contamination Levels in Cooked Maize-Based Food (n = 53) from Participant Households

mycotoxin	recovery (%)	LOD[a] (μg/kg)	% positive	mean[b] ± sd[c] (μg/kg)	median[b] (μg/kg)	range[b] (μg/kg)
***Fusarium* Metabolites**						
beauvericin	104	0.02	100	1.0 ± 2.5	0.1	0.02−16
chlamydosporol	97	0.2	30	3.9 ± 11	0.6	0.4−43
culmorin	73	0.8	36	1.9 ± 2.0	1.3	0.4−7.8
equisetin	200	0.2	70	2.6 ± 8.8	0.4	0.1−52
fusaproliferin	56	1.5	42	835 ± 963	445	75−4267
fusaric acid	89	20	60	91 ± 92	49	22−378
hydrolyzed FB_1	89	0.1	74	3.9 ± 5.2	2.0	0.2−22
moniliformin	89	0.5	32	11 ± 13	7.4	0.7−50
nivalenol	111	0.7	74	20 ± 74	1.6	0.3−438
β-zearalenol	107	0.5	9	1.2 ± 0.5	1.2	0.6−1.9
Masked *Fusarium* Metabolites						
DON-3-glucoside	109	0.2	15	1.2 ± 2.1	0.5	0.2−6.4
ZEN-4-sulfate	98	0.03	21	0.4 ± 0.4	0.2	0.1−1.2
***Aspergillus* Metabolite**						
3-nitropropionic acid	95	0.2	68	13 ± 55	1.6	0.4−328
***Alternaria* Metabolites**						
alternariol methyl ether	98	0.02	32	0.3 ± 0.4	0.1	0.04−1.5
monocerin	98	0.05	25	3.8 ± 11	0.3	0.03−42
***Penicillium* Metabolites**						
andrastatin A			8	[e]		
chanoclavine	84	0.015	70	0.1 ± 0.1	0.05	0.01−0.5
emodin[d]	85	0.02	79	0.6 ± 0.6	0.4	0.1−3.2
mycophenolic acid	104	0.4	51	54 ± 120	7.9	1.1−551
skyrin	66	0.2	45	1.3 ± 2.1	0.8	0.05−11

[a]Limit of detection. [b]Mean/median/range of positive samples. [c]Standard deviation. [d]Also *Aspergillus* metabolite. [e]Detected.

has some antibiotic properties, and fusaric acid, which has been shown to alter brain and pineal neurotransmitters and their metabolites in rats and may contribute to overall toxic effects observed in *Fusarium*-contaminated animal feed.[29] Of the other *Fusarium* metabolites, all of which occurred at low levels, equisetin was also found in the study of single kernels,[18] whereas apicidin, butenolide, culmorin, and di- and mono-acetoxyscirpenol are newly identified in Transkeian maize.

Only four *Aspergillus* metabolites, cytochalasin E, 3-nitropropionic acid, α-OTA, and terrein, were identified in a small number of samples, and of these, the first could have arisen from *Alternaria* species and the last two from *Penicillium* species. Unlike previous studies[20] on maize from Cameroon, no aflatoxin biosynthetic intermediates or metabolites, such as sterigmatocystin, the versicolorins, averufin, or norsolorinic acid, were identified; neither was the *Aspergillus* metabolite kojic acid. These results, together with the absence of aflatoxins, reflect the very low incidence of *Aspergillus* species in the maize from Centane. A small number of *Alternaria* metabolites (alternariol methyl ether, macrosporin, and monocerin) occurred at very low levels in these samples of both good and moldy maize. In contrast, 18 *Penicillium* metabolites, mostly present at low levels, were detected. These are expected to occur during postharvest storage in rural households, in contrast to the metabolites from *Fusarium*, *Aspergillus*, and *Alternaria* species discussed above, which would be expected to be produced preharvest. The bacterial (*Streptomyces*) metabolites, monactin and nonactin, were also identified at very low levels, as they were in the single kernel study.[18]

Many of the minor *Fusarium* metabolites, including the masked mycotoxins, identified in the maize samples were similarly present in the cooked food samples, although generally at lower levels as would be expected due to the high water content of the undried food. However, only a few of the *Aspergillus*, *Alternaria*, and *Penicillium* metabolites were identified in the food samples, possibly due to the mostly low incidence rates in good maize and the low levels at which they are generally present. The fate of these during conventional cooking is also an unknown factor.

The multimycotoxin analysis of good and moldy maize has highlighted the coexposures that occur in rural subsistence farming communities of the Centane region of the former Transkei, as has also been previously highlighted for various food commodities consumed in Burkina Faso, Cameroon, Mozambique, and Nigeria.[19−21] However, apart from the mycotoxins that are recognized to affect human health, the exact implications of these multiple exposures, with respect to both the secondary metabolite properties and the levels at which they are consumed, are not understood. A previous review on toxicological interactions among fungal toxins has indicated that these interactions can be additive, synergistic, less than additive, or antagonistic and can vary with dose, exposure time, animal species involved, and toxicological end point.[30]

In conclusion, this is the first comprehensive application of modern multimycotoxin LC-MS/MS analytical methods to good and moldy maize samples from the high esophageal cancer incidence area of Centane in the former Transkei. Apart from multiyear data on fumonisin contamination, previously published data mostly concerned a few mycotoxins (DON, BEA, FUS, MON, and ZEN) in an extremely limited number of maize samples. The present study confirms and greatly expands these previous data and provides new information on the natural co-occurrence of 50 mycotoxins and fungal secondary metabolites in both good and moldy home-grown maize. The

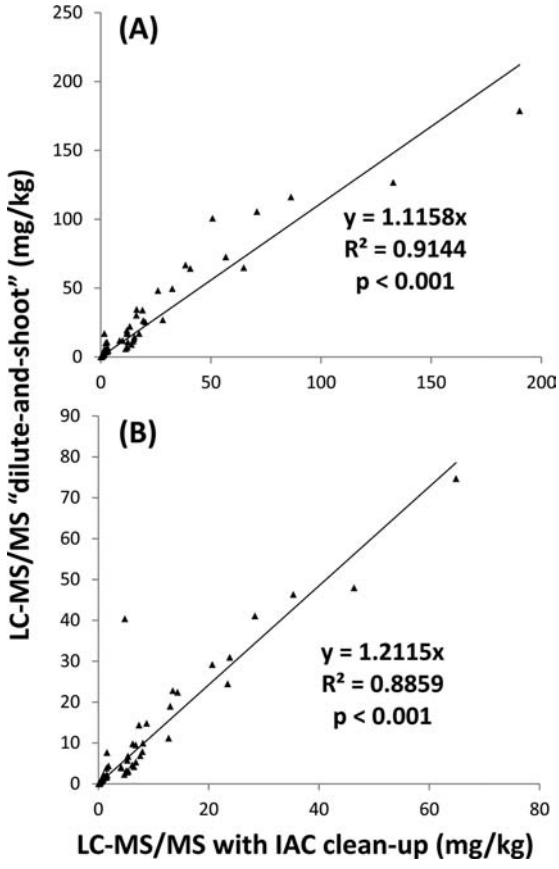

Figure 3. Comparison of combined FB_1 (A) and combined FB_2 (B) results for the good and moldy maize samples obtained by the two different LC-MS/MS methods. The trendline has been forced through zero.

results indicate multiple coexposures to a wide range of mycotoxins from various fungal genera and indicate that apart from high fumonisin exposure, exposure to ZEN could be above the JECFA PMTDI for this myco-estrogen. As more standards become available, such as fusarin C, the range of secondary metabolites will increase and our knowledge of coexposures will become more comprehensive. Exposure to other secondary metabolites quantitated in these samples of good home-grown Transkeian maize is generally low, although the effects of multiple coexposures are as yet unknown, as are the generally higher exposures resulting from the use of moldy maize during the preparation of locally brewed maize beer.

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Notes

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