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The occurrence of mycotoxins in fermented maize products

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The occurrence of mycotoxins in fermented maize dough and Ga Kenkey (an end product of maize dough fermentation) from major processing sites and markets in Accra was investigated. Experiments were then conducted to study the effects of spontaneous fermentation and cooking on aflatoxin and citrinin levels at two processing sites and under laboratory conditions.

Occurrence of aflatoxins and citrinin was widespread with generally high concentrations. Levels as high as 289 $\mu g\ kg^{-1}$ for total aflatoxins and 584 $\mu g\ kg^{-1}$ for citrinin were recorded. Low amounts of ochratoxin A were detected and neither zearalenone nor α-zearalenol were found. Significant increases in aflatoxin levels were observed during the initial stages of fermentation. Furthermore, aflatoxins and citrinin were found to persist throughout the traditional steeping and fermentation processes. Cooking of fermented maize dough for 3 h, as done for Kenkey production, resulted in a 80% reduction in aflatoxins B_1 and G_1 levels, a 35% reduction in aflatoxins B_2 and G_2, and citrinin was no longer detectable.

INTRODUCTION

In Ghana, maize is a dietary staple and contributes the major part of the total calories in the diet of people in the coastal areas (National Food and Nutrition Board, 1962). A large proportion of the maize is consumed in the fermented form produced through traditional spontaneous and uncontrolled fermentations. The fermented dough is used to prepare several foods, the most common being "Kenkey". The preparation of Kenkey involves steeping maize in water for between 24 and 48 h, milling, doughing and fermenting for 72 h. A portion of the fermented dough is then cooked and mixed with fresh fermented dough. The mixture is moulded into balls, wrapped with maize husks and boiled for about 3 h.

Mycological studies of Ghanaian maize have demonstrated the presence of several toxigenic moulds including aflatoxin and citrinin producing species (Jespersen *et al.*, 1994). Aflatoxins which are produced by some strains of *Aspergillus flavus* and *Aspergillus parasiticus* are known to be hepatotoxic, carcinogenic and teratogenic, and a positive correlation has been established between the consumption of aflatoxin contaminated foods and the increased incidence of liver cancer in several South-east Asian and African populations (Shank *et al.*, 1972; Peers & Linsell, 1973; Shank, 1976; WHO, 1979; Groopman *et al.*, 1988). In Ghana no such epidemiological studies have been carried out but it is known that primary liver cancer is one of the commonest cancers with a relative frequency of 14.5% and accounts for 20% of cancers in the male population (Foli & Christian, 1976).

The mycotoxins, citrinin and ochratoxin A, produced by various *Penicillium* spp. and *Aspergillus* spp. particularly *Penicillium citrinum* and *Aspergillus ochraceus*, respectively, have been associated with kidney damage in animals (Krogh, 1987; Richard & Thurston, 1986). Zearalenone produced by some *Fusarium* spp. produced an oestrogenic syndrome in farm animals (Mirocha & Christensen, 1974). No studies have been carried out in Ghana on the extent of maize contamination by mycotoxins though conditions of temperature and relative humidity, coupled with the reliance on sundrying by most small-scale farmers, who, incidentally produce the bulk of the maize in the country, all suggest the possibility of an existing mycotoxin problem.

As indicated earlier, most of the maize produced is consumed in the fermented form. As a result of fermentation, the pH is reduced to about 3.7 and the

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acidified product is cooked for about 3 h for the production of Kenkey. Whilst it is known that studies have been carried out on the effects of several processing methods on aflatoxin levels in various commodities (Samarajeewa *et al.*, 1990), the effect of maize fermentation, low pH, and the combined effect of low pH and heat have not been investigated.

It is the objective of this work to ascertain the levels of aflatoxins, citrinin, ochratoxin A, zearaleone and α-zearalenol in maize dough and Kenkey on sale in some markets and processing sites in Accra, and to investigate changes in the levels of these mycotoxins during fermentation and cooking.

MATERIALS AND METHODS

Samples from production sites and markets

Samples were collected from four production sites and five major markets in Accra. The production sites have a capacity of several tons per week. All samples were promptly analysed for their moisture content (AOAC, 1984) and mycotoxin levels as described below. Mycotoxin determinations in all cases are expressed on a dry weight basis.

Steeping and fermentation trials

A local variety of maize was purchased from a market in Accra. The kernels were well mixed and a portion milled and analysed for moisture and aflatoxin levels. Remaining grains were steeped at 28°C (ambient temperature) in water in the ratio 1:3 (w/v) for 24 h. The steeped grains were then milled in a disc attrition mill. Maize dough of 50% (w/w) moisture content was prepared by kneading the meal with the appropriate amount of water based on the initial meal moisture content. Duplicate samples were placed in containers and left to ferment spontaneously at 28°C for 3 days. Daily samples were taken for moisture, pH and aflatoxin analysis.

Chemicals, reagents and standards

Citrinin, ochratoxin A, zearalenone, α-zearalenol and aflatoxin standards were obtained from Sigma Chemical Co. Ltd (St Louis, MO, USA). Standard stock and HPLC working solutions were prepared by evaporating and dissolving in the appropriate mobile phases. The aflatoxin mobile phase consisted of methanol, acetronitrile and water (10:30:60 v/v) and methanol, acetonitrile, water (20:32:40 v/v) for zearalenone and α-zearalenol. HPLC mobile phase for citrinin consisted of acetonitrile, isopropanol and 0.75 M phosphoric acid (35:10:55 v/v).

Reagents for HPLC separations were of HPLC grade (Merck Chemicals, Darmstadt, Germany). All other chemicals and reagents used were of analytical grade. Distilled and deionized water were used throughout and all mobile phase solutions were filtered through a 0.45 μm Millipore HV disc filter and degassed prior to use through a Millipore filtration unit (Millipore Corp. Bedford, MA, USA).

Extraction and HPLC analysis

Aflatoxins

The extraction procedure was based on that of Pons (1979). Aflatoxins were extracted with methanol followed by precipitation of colour pigments using zinc acetate then extraction into dichloromethane and further clean-up by column chromatography using cellulose and silica gel. Aflatoxins were eluted with dichloromethane:acetone (80:20 v/v) which was evaporated off and the residue quantitatively transferred into 10 ml of HPLC-grade dichloromethane. Five millilitres were evaporated to dryness under a stream of nitrogen and the final residue dissolved in 0.1–1.0 ml HPLC mobile phase and used for HPLC analysis.

The instrument system used for all HPLC analyses was from Waters Associates (Milford, MA, USA) and included a Model 501 Solvent Delivery System fitted with a Rheodyne Model 7125 injector with a 20 μl fixed volume loop, a temperature control system consisting of a temperature control module and two column heaters, a Model 470 Scanning Fluorescence Detector and a Model 746 Data Module.

Identification and quantification of aflatoxins was by reversed-phase liquid chromatography with post-column iodine derivatization. Separation of aflatoxins was carried out on a Spherisorb S5 ODS-1 column of dimensions 25×4.6 mm packed with 5 μm particles (Phase Separations Inc., Norwalk, USA) maintained at 35°C. HPLC mobile phase flow rate was 1.2 ml min^{-1} and post-column iodine derivatizaton of aflatoxins B_1 and G_1 was achieved using saturated iodine solution (Shepherd & Gilbert, 1984) pumped at a flow rate of 0.4 ml min^{-1} using an Eldex precision metering pump (Eldex Laboratories Inc., San Carlos, USA). The derivatization tube consisted of stainless steel tubing (5 m×0.3 mm) maintained at 75°C.

The excitation and emission wavelengths used were 360 and 440 nm, respectively. The aflatoxins were identified by their retention times, and peak areas were used to determine their concentrations in the samples by reference to standard curves obtained by chromatographing pure aflatoxin standard solutions under identical conditions. To compensate for any day-to-day variation, standards were routinely run at the beginning, in-between and after samples had been run.

Citrinin and ochratoxin A

The simultaneous procedure by Lepom (1986) was used. The mycotoxins were extracted into aqueous acidified acetonitrile solution then defatted with iso-octane and purified by several partition steps using chloroform, sodium bicarbonate and after acidification, into chloroform. The extract was rotary evaporated to near dryness

and quantitatively transferred into dichloromethane which was evaporated off and the extract spiked into the HPLC mobile phase consisting of acetonitrile–isopropanol–0.75 M phosphoric acid (35:10:55).

Separation was carried out on a Nova-Pak C_{18} column (3.9×150 mm) (Waters Associates, Milford, USA) maintained at 30°C. The excitation and emission wavelengths used were 360 and 500 nm, respectively. Citrinin and ochratoxin A were identified by their retention times whilst their concentrations in the samples were determined by comparison to standard curves obtained under identical conditions. Using these conditions, retention times for citrinin and ochratoxin A were 3.50 and 6.28 min, respectively.

Zearalenone and α-zearalenol

The extraction procedure was based on that of Bennett *et al.* (1985). The sample was extracted with chloroform from which the mycotoxins were re-extracted into sodium hydroxide. The solution was acidified with citric acid and the mycotoxins extracted into dichloromethane, which was evaporated to dryness and the residue quantitatively transferred into a reactivial with dichloromethane which was evaporated off and the extract dissolved in 0.5 ml mobile phase consisting of methanol–acetonitrile–water (10:16:20) by vortex mixing. The extract was then filtered through a Swinney filter assembly (Millipore Corp. Bedford, MA, USA) and the filtrate subjected to reversed-phase liquid chromatography using fluorescence detection.

The separation and analysis of zealenone and α-zearalenol was carried out on a Nucleosil C_{18} (4.0×200 mm) column (Macherey-Nagel, Dueren, Germany) maintained at 30°C. The detector excitation and emission wavelengths were 236 and 418 nm, respectively. Identification and quantification was by comparison with retention times and peak areas of standards which were analysed under conditions identical to the samples. Retention times for α-zearalenol and zearalenone were 6.78 and 8.49 min, respectively.

Statistical analysis

Data obtained from steeping and fermentation studies on aflatoxins were subjected to analysis of variance (Snedecor & Cochran, 1976). Differences in aflatoxin levels were analysed according to Duncan (1955).

RESULTS AND DISCUSSION

Table 1 shows aflatoxin levels of fermented maize dough samples from two processing sites in Accra over a 1.5-year period. All the samples analysed contained aflatoxins. Levels ranged from 0.7 to 313 μg kg^{-1}. There was no apparent relation between the time of the year and the levels of aflatoxins. However, it is clear (Table 1) that samples from processing site B contained lower aflatoxin levels than those from site A. Variations, therefore, are likely to be determined by the quality of the raw material used and not the time of the year. Generally, all four types of aflatoxins (B_1, B_2, G_1, G_2) were present. In most cases aflatoxin B, was present in the highest ratio.

Table 2 shows the aflatoxin levels of Ga Kenkey samples (the cooked fermented maize) from four processing sites (A, B, C and D) sampled weekly over a 1-month period. Processing sites A and B are the same sites as in Table 1. In agreement with the results for fermented maize (Table 1) samples from site B had lower aflatoxin levels than those from site A. Furthermore, it is seen that the cooking time of 3 h used in the preparation of Kenkey does not appear to destroy the aflatoxins present, as may be expected from the work done by Newberne *et al.* (1966) which showed that the presence of moisture in foods enhances degradation of aflatoxin by hydrolysing the lactone ring of the aflatoxin molecule. Aflatoxin B, in the solid form is known to be stable to dry heat up to its melting point of 260°C (Ciegler & Vesonder, 1983). In the presence of moist heat, however, it undergoes a hydrolytic opening of the lactone ring to form a terminal carboxylic acid which

Table 1. Aflatoxin levels (expressed on dry weight basis) of fermented maize dough from two processing sites A and B in Accra (18-month period)

Collection period (Month)	Site	Aflatoxin type and concentration (μg kg^{-1})				Total aflatoxin (μg kg^{-1})
		B_1	B_2	G_1	G_2	
August	A	267	15.0	6.8	0.4	289
October	A	145	10.0	3.3	0.1	158
February	A	293	15.1	2.1	0.2	313
February	A	13.8	1.0	2.7	0.4	17.9
October	A	140	19.8	101	12.3	273
March	A	63.9	5.6	102	7.8	184
August	B	45.0	2.0	0.6	ND[1]	47.6
October	B	88.0	7.8	5.4	0.4	102
February	B	3.8	0.3	0.8	0.2	5.1
February	B	0.6	0.1	ND	ND	0.7
October	B	212	7.2	12.1	2.2	233
March	B	0.5	0.1	0.4	ND	1.0

[1]ND, None detected. Detection limits, B_1 and B_2 = 0.04 μg kg^{-1}, G_1 and G_2 = 0.06 μg kg^{-1}.

Table 2. Aflatoxin levels (expressed on dry weight basis) of "Ga Kenkey" from four processing sites A, B, C and D in Accra over a 1-month period

Site	Moisture (%)	Aflatoxin type and concentration ($\mu g\ kg^{-1}$)				Total aflatoxin ($\mu g\ kg^{-1}$)
		B_1	B_2	G_1	G_2	
A	70.1	153	43.5	ND[1]	ND	196
A	67.7	57.1	22.6	23.1	5.7	109
A	64.4	39.3	16.9	6.3	1.6	64.1
A	66.7	49.8	8.8	10.4	ND	69.0
B	63.5	4.1	2.7	ND	ND	6.8
B	60.4	4.6	1.5	ND	ND	6.1
B	64.4	7.2	9.4	ND	3.5	20.1
B	61.2	11.1	0.9	1.0	ND	13.0
C	71.4	ND	ND	ND	ND	ND
C	68.6	32.6	7.2	4.0	2.8	46.6
C	67.5	6.1	1.0	9.2	ND	16.3
C	65.3	6.9	1.1	ND	ND	8.0
D	63.3	1.1	0.8	4.0	1.7	7.6
D	64.1	22.9	6.4	30.6	5.5	65.4
D	61.2	26.6	4.2	69.4	6.7	106.9
D	66.5	7.6	0.8	20.4	ND	28.8

[1]ND, for explanation see Table 1.

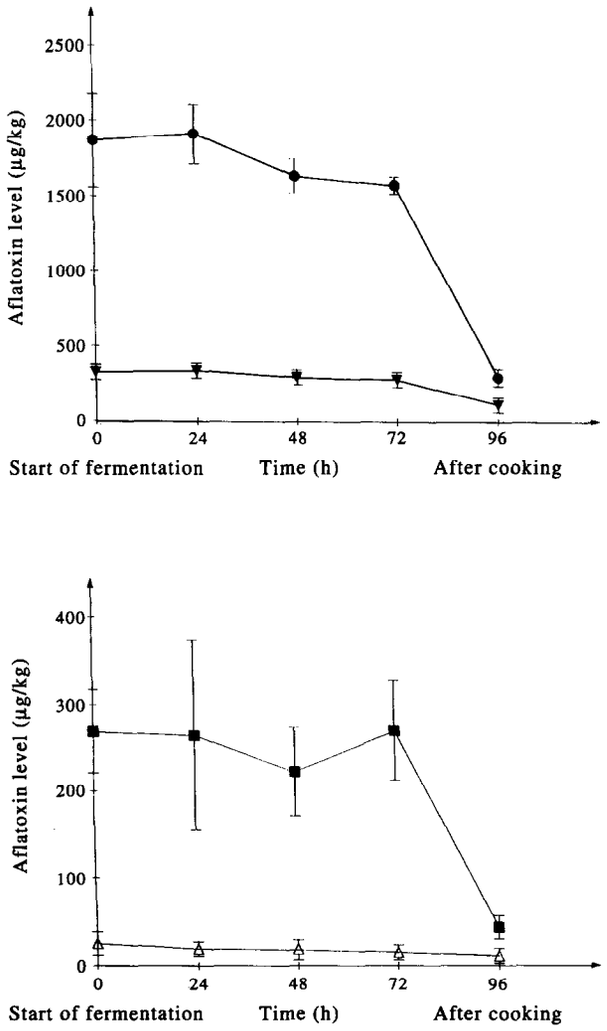

Fig. 1. (a) Changes in aflatoxin B_1 (●) and B_2 (▼) during fermentation and cooking at site A. Average of duplicate samplings. (b) Changes in aflatoxin G_1 (■) and G_2 (△) during fermentation and cooking at site A. Average of duplicate samplings.

then undergoes decarboxylation (Coomes *et al.*, 1966). According to Table 2, moisture content of Ga Kenkey ranged from 60.4 to 71.4%.

Figures 1 and 2 show changes in aflatoxins B_1, B_2, G_1 and G_2 levels during dough fermentation and cooking into Ga Kenkey at production sites A and B. At site A (Fig. 1a and b), fermenting maize dough for 72 h did not appear to reduce aflatoxin levels. However, cooking into Ga Kenkey reduced the levels of aflatoxins B_1 and B_2 by about 80 and 35%, respectively, and aflatoxins G_1 by about 80%, whereas the effect on G_2 appeared to be less pronounced.

At production site B (Fig. 2) where initial aflatoxin levels were low compared to site A, fermentation again did not show any significant effect on aflatoxins. Cooking of Kenkey, however, resulted in a much lower percentage destruction of aflatoxins B_1 and B_2 (38 and 7%, respectively). It was observed that aflatoxins B_2 and G_2 were more heat-stable than B_1 and G_1 during cooking.

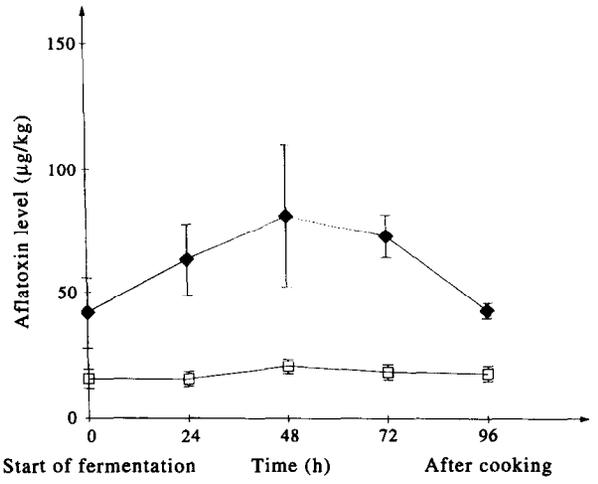

Fig. 2. Changes in aflatoxin B_1 (◆) and B_2 (□) during fermentation and cooking at site B. Average of duplicate samplings.

Similar findings have been reported by Kaminura (1989), but in the absence of water.

Table 3 summarizes results obtained for aflatoxins B_1 and B_2 analyses during fermentation of maize dough in the laboratory. Statistically significant ($P < 0.05$) increases occurred during steeping and the first 48 h of fermentation for aflatoxin B_1, whilst significant increases in aflatoxin B_2 levels were observed during steeping and 24 h of fermentation.

During spontaneous fermentation of maize dough in Ghana, lactic acid bacteria have been found to be the dominating organisms resulting in the production of high levels of lactic acid (Halm *et al.*, 1993). Apart from lactic acid, acetic, butyric and propionic acids are known to be produced and are the main aroma components in fermented maize doughs (Banigo & Muller, 1972; Plahar & Leung, 1982). In this work, there was a reduction of pH from 6.5 in maize kernels to 5.5 in 0-h dough to 4.0, 3.7 and 3.7 after 24, 48 and 72 h fermentation, respectively (data not shown). The effects of acids and alkali on aflatoxins have been discussed by Price & Jorgensen (1985). In the presence of alkali, reduction of aflatoxin occurs with the opening of the lactone ring. In the presence of acid, however, a reformation takes place with the closing of the ring. This suggests that a reduction of pH, such as occurred during maize dough fermentation, could result in reformation of any hydrolysed aflatoxin molecules to give higher aflatoxin levels as observed during the first 48 h of fermentation. No significant increases were found for aflatoxin B_1 after 48 h and this is reflected in the constant pH recorded.

The presence of aflatoxin precursors in the maize kernels and fermenting doughs may also play a role in elevating aflatoxin levels. The biosynthetic pathway for aflatoxin B_1 has been studied by several researchers (Townsend *et al.*, 1982; Bennett & Christensen, 1983; Townsend & Christensen, 1983) and six compounds have been recognized as being intermediate compounds in the biosynthesis of aflatoxin B_1. These are norsolorinic acid, averantin, averufin, versiocoal hemiacetal acetate, versicolorin A and sterigmatocystin. One of these compounds, sterigmatocystin (itself a mycotoxin) can be produced by some *Aspergillus* spp. (Schroeder & Kelton, 1975; Davis, 1981). In this work, the amounts of the individual precursors in the biosynthetic pathway for aflatoxin B_1 were not determined. It is therefore not known if these precursors were originally present in the maize kernels, and the steeping and fermentation procedures only enhanced their conversion to the end product, aflatoxin B_1, which was measured. Further work needs to be carried out to ascertain the levels of these precursors at various stages of the steeping and fermentation procedures. Furthermore, regarding sterigmatocystin, the levels present were not determined, nor were the fungi responsible for its production, namely *Aspergillus versicolor* and *Aspergillus nidulans*, isolated. Work done on fermented maize (Jespersen *et al.*, 1994) showed that, although high numbers of moulds of *Penicillium*, *Aspergillus* and *Fusarium* spp. were detected on maize kernels, a significant reduction in mould numbers was observed during steeping and the early stages of fermentation suggesting that increases in aflatoxin levels could be attributed more to the presence of precursors rather than aflatoxin producing fungi.

It has been established that several substances are able to inhibit or stimulate aflatoxin production, either through their action on growth of *Aspergillus flavus* or *Aspergillus parasiticus*, or by direct action on aflatoxins themselves. These compounds have been reviewed by Zaika & Buchanan (1987). During fermentation, several substances are produced and they include aromatic compounds such as esters, alcohols, aldehydes, lactones and terpenes produced by yeasts (Janssens *et al.*, 1992), and lactic, acetic, butyric and propionic acids produced by bacteria (Banigo & Muller, 1972; Plahar & Leung, 1982). Substances which stimulate growth and/or aflatoxin formation by *A. flavus* and *A. parasiticus* under certain conditions include acetone, ethanol, DL-ethionine, isoprothiolane, nisin, phytate and sodium chloride (Zaika & Buchanan, 1987). Further work needs to be carried out to establish the production of any of these

Table 3. Effect of laboratory fermentation on aflatoxin levels in naturally contaminated maize[1]

Treatment	Aflatoxin type and levels ($\mu g\ kg^{-1}$)[2]	
	B_1	B_2
Maize kernels	69.8 ± 7.5^a	4.5 ± 1.3^a
Kernels steeped 24 h, milled and doughed (0-h dough)	117 ± 10.7^b	11.5 ± 1.4^b
Maize dough (24 h)	206 ± 13.1^c	18.9 ± 2.4^c
Maize dough (48 h)	270 ± 15.9^d	22.2 ± 6.8^c
Maize dough (72 h)	290 ± 6.0^d	25.5 ± 2.7^c

[1]Values are means ± SD of duplicate determinations expressed on dry weight basis.
[2]Means within column sharing the same letter are not significantly different ($P < 0.05$).

Table 4. Changes in citrinin levels during fermentation and cooking into kenkey[1]

Sample	Site A	Site B
Fresh dough[I2]	511	ND[3]
(O h)[II]	273	5
Fermented dough[I]	658	21
(24 h)[II]	526	37
Fermented dough[I]	813	39
(48 h)[II]	1082	43
Fermented dough[I]	227	43
(72 h)[II]	560	55
Kenkey	ND	ND
Cook water	ND	ND

[1]Mean values for two determinations expressed in $\mu g\ kg^{-1}$ on dry weight basis.
[2]I and II refer to two samples from different locations within the same bowl.
[3]ND, None detected (detection limit = 0.1 $\mu g\ kg^{-1}$).

Table 5. Mycotoxin contents of fermented maize dough samples from five markets in Accra

Market code	No. of samples analysed	Mycotoxin range and mean values shown in parentheses ($\mu g\ kg^{-1}$)[1]		
		Total aflatoxins	Citrinin	Ochratoxin A
M	4	2.1–5.5 (3.0)	1.4–552 (145)	ND[2]–2.4 (0.6)
K	4	9.3–249.0 (88.3)	22.6–585 (207)	ND–6.4 (1.6)
L	4	ND–65.9 (23.0)	0.7–110 (34.0)	ND–4.4 (1.1)
P	4	4.1–61.0 (32.6)	10.0–37.4 (18.2)	ND–6.1 (1.6)
S	4	0.6–45.0 (13.7)	3.7–174 (90.8)	ND

[1]Values expressed in $\mu g\ kg^{-1}$ on 50% (w/w) moisture basis.
[2]ND, None detected. Detection limits: citrinin = 0.1 $\mu g\ kg^{-1}$; ochratoxin A = 0.3 $\mu g\ kg^{-1}$; aflatoxin B_1 and B_2 = 0.04 $\mu g\ kg^{-1}$; aflatoxin G_1 and G_2 = 0.06 $\mu g\ kg^{-1}$.

substances during steeping and fermentation of maize and their respective roles in relation to aflatoxins.

Table 4 shows changes in citrinin levels during fermentation and cooking into Ga Kenkey. Fermentation did not appear to affect citrinin levels. Cooking of fermented dough into Kenkey, however, seemed to destroy citrinin in both the Kenkey and its "cook-water". This observation agrees with literature reports on the heat-lability of citrinin (Jackson & Ciegler, 1978; Roberts & Mora, 1978; Nelson *et al.*, 1980). The absence of citrinin after cooking is encouraging; however, further studies are required on the toxicity and occurrence of breakdown or bound products in the light of the high citrinin levels observed in market samples of maize dough as shown in Table 5. All 20 samples from five major markets in Accra were found to be positive for citrinin with generally high levels. Five of the 20 samples contained detectable amounts of ochratoxin A, which is known to co-occur with citrinin. Levels of ochratoxin A were generally low. No zearalenone or α-zearalenol was detected in the maize dough samples. Nineteen of the 20 samples were positive for aflatoxins with aflatoxin B_1 dominating. The high levels of aflatoxins and citrinin in the dough samples confirm earlier work on dominating fungal species in maize for kenkey production (Jespersen *et al.*, 1994). Their work revealed dominating mould genera to be *Penicillium*, *Aspergillus* and *Fusarium* with detailed investigations showing the species with highest frequency to be *Penicillium citrinum*, *Aspergillus flavus* and *Aspergillus parasiticus*, which are responsible for the production of citrinin and aflatoxins B_1, B_2, G_1 and G_2.

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