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Moulds And Ochratoxin A Associated With Green Coffee (Coffea *Arabica*) Beans Processed By Dry And Wet Methods In Nyeri County.

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Abstract: *Coffee when contaminated with moulds such as Aspergillus and Penicillium species due to poor processing and storage can produce mycotoxins such as Ochratoxin A (OTA) associated to many health hazards to consumers. The aim of this research was to determine the levels of moulds and Ochratoxin A in coffee processed by dry and wet methods. The coffee samples were collected from Estates and Factories located in the main coffee processing zone in Nyeri County, Kenya. The parameters determined in the green beans were; moisture content, levels of moulds and OTA contamination. The moisture content of coffee samples were determined by dry oven method, OTA levels by use of HPLC and moulds enumeration by serial dilution technique. The results showed no significant difference on the level of moisture content in the coffee samples from Estates and Factories. Ochratoxin A was not detected in all coffee samples. Mould counts of coffee samples from Factories and Estates were found to be between* 1×10^3 *CFU/ml to* 6.0×10^1 *CFU/ml. The highest mould contamination was observed in dry processed coffee as compared to wet processed coffee. Although few samples showed mould contamination, there were no OTA detected in the samples.*

Key words: *Factories and estates, Moulds, Ochratoxin A, Processing methods.*

I. Introduction

Coffee plant belongs to the *Rubiaceae* family and genus *coffea* [1]. Coffee cherries are harvested when they are bright red, glossy and firm and processed either by dry, wet or semi-dry process methods. For dry processing, the coffee cherries are spread out in the sun in thin layers of about 5–8 cm thick on drying beds to dry to a moisture content of below 12% [2]. In wet processing, the pulp is mechanically removed from ripe cherries, fermented and washed with plenty of water before drying [3]. Poor processing and handling can lead to mould contamination and eventually production of OTA [4]. Ochratoxin A (OTA) is one of the most widespread mycotoxin that can be found in coffee beans and its beverage [5]. This mycotoxin is produced mainly by two genera of microorganisms, *Penicillium* and *Aspergillus* [6]. Practices such as picking of fallen old cherries from the ground during harvesting , poor washing of coffee parchment, contamination of coffee by drying on the ground or dirty drying surfaces , storing partially dry coffee for long periods or rewetting during drying influences the growth of moulds on coffee which affects the quality and safety of the final product due to production of mycotoxins [7]. The toxin is dangerous and often present noxious effect to eukaryotes, including humans, animals, and plants. It is considered nephrotoxic, cytotoxic, carcinogenic, teratogenic and immunosuppressive [8]. It has been classified by the International Agency for Research on Cancer (IARC) as a possible human carcinogen [9]. The OTA is highly resistant to physical and chemical treatment and once in food it persists during the processing and storage conditions [10]. In Kenya the peak coffee harvesting and processing season coincides with the main rainy and cold seasons. Due to the cold weather conditions coupled with the wet season, coffee takes long period of time to dry and some may reabsorb moisture. These conditions encourage mould growth on the coffee beans which may lead to the production of mycotoxins [11]. There are few reports on the level of mould and OTA contamination of coffee in Kenya. This study aimed at determining the level of mould and OTA contamination in green coffee beans processed by dry and wet methods. The information on the levels of moulds contamination and OTA in green coffee beans processed by dry and wet methods will be useful for extension services geared towards training of farmers and coffee handlers by relevant bodies, on effective ways of handling coffee to avoid mould contamination and OTA occurrence in coffee.

II. Materials And Methods

2.1 Study site and samples preparations

Coffee samples processed by wet and dry methods were drawn from coffee factories and estates located in the main coffee processing zones in Nyeri County, Kenya. Nyeri County was selected as suitable site because of the cool and wet conditions which may favor growth of moulds and OTA production during harvesting and processing of coffee. The County is situated between longitudes 36°38´ East and 37°20´ East and between the equator and latitude 0°38° South. The coffee samples were collected from 5 different factories and 3 estates. From each factory and estate, a sample of 200g of coffee processed by wet and dry method was drawn randomly from different sites of the coffee heaps.

The control samples were prepared by taking a total of 100kg of ripe coffee cherries of mixed variety which were harvested from Dedan Kimathi University farm located in Nyeri County, Kenya. Ripe cherries were harvested by selective method sorted and pulped using a wet pulper machine. Parchment coffee obtained was then fermented for 72 hours in fermentation tanks using natural method of fermentation. It was then washed with plenty of water to remove mucilage and sun dried on raised beds for 12 days to attain a moisture content of approximately 9.0%. Another batch of coffee cherries amounting to 100kg was processed by dry method by exposing to the sun directly for 30days to attain a moisture content of approximately 9.0%. After drying, the samples were packaged in sealed polythene bags and stored at frozen temperatures of -18°C awaiting analysis.

2.2 Moisture content analysis

The moisture content of coffee samples was determined by dry oven method [12] and the results expressed as percentage moisture content.

2.3 Mould enumeration

Moulds were enumerated by the serial dilution method. Samples of green coffee beans weighing 5g were transferred to a bottle containing 1800 ml of saline peptone diluent (0.1% of peptone, 0.5% of NaCl, 0.03% $Na_2H_2PO_4$) and mixed for 15 minutes. Five-fold dilutions were prepared and appropriate dilutions spread on triplicate plates of Potato dextrose agar. The plates were incubated at 28ºC for 5 days after which they were examined for colony growth visually and with the aid of a microscope. The colonies were then counted using a colony counter and expressed as CFU/ml [2].

2.4 Isolation and identification of *Aspergillus* and *Penicillium* species

The suspected colonies of *Aspergillus spp*. and *Penicillium spp*. were isolated by streaking on Malt Extract Agar and incubated at 25°C for 7 days. Morphological analysis of *Aspergillus* and *Penicillium* species was done by microscopic and macroscopic technique. *Aspergillus* species was identified by observing colony characters such as color and texture of the mycelium, characteristics of conidiophores such as shape of conidial heads and color of stipes [13]. *Penicillium* species was identified by examining the texture of the colony, color of conidia, texture and color of mycelium, conidiophores with branching patterns [14].

2.5 Screening of *Aspergillus* and *Penicillium* species for OTA production

Testing for OTA production of isolated *Aspergillus* and *Penicillium* was done by agar plate technique according to the method as described by [15] with some modification. One agar plug from each plate was cut out near the center of the mold colony and removed using a flame sterilized stainless steel scalpel. By means of a syringe, a drop of extraction solution (Chloroform and methanol in the ratio of 1:2) was placed directly on the mycelium. While still wet, the mycelium side of the plug was gently pressed against the application line on a thin-layer chromatography (TLC) plate and then removed immediately. After drying the application spot, the TLC plates (Silica gel on alluminium) were put in the development tank with the eluting solvent (Chloroform, Ethyl acetate and Formic acid in the ratio 6:3:1) and separation allowed until the solvent reached the solvent front. The plate was removed, allowed to dry and then viewed under infrared. The standard was compared with the samples for the presence of OTA.

2.6 Ochratoxin A analysis

Ochratoxin A was analyzed according to the method described by [16]. One hundred milliliters of chloroform and 12.5 ml of phosphoric acid (0.1 M) were added to an Erlenmeyer flasks containing 10g of ground coffee samples. The mixture was magnetically stirred for 30 minutes and filtered through Whatman no.100. The filtrate was transferred into a 500-ml separatory funnel, 50ml of the lower layer transferred to round-bottom flasks and evaporated to dryness on a rotary evaporator. The residue was dissolved in 5 ml of hexane and 5 ml of methanol/water mixture (1:1, v/v), filtered using Whatman paper no. 100 and the filtrate transferred to a 50-ml separatory funnel for separation. The lower layer was collected in a 25-ml Erlenmeyer flasks and the upper phase extracted twice more with 5 ml of the methanol/water mixture (1:1, v/v) used to rinse

the round bottom flask containing the residue. All the lower layers were combined and 5ml of it loaded at a flow rate of 1 ml/min into a C18 SPE Cartridges previously conditioned with 5 ml of methanol and 5 ml of methanol/water mixture (1:1, v/v). The Cartridges were then washed with 5 ml of methanol/water mixture (3:1, v/v) and 3 ml of methanol at a flow rate of 2 ml/min. Ochratoxin A was then eluted with 10ml of methanol/formic acid mixture (98:2, v/v) at a flow rate of 2 ml/min and the solutions evaporated to dryness under nitrogen stream. The residue was dissolved in 500 µl of the mobile phase (Acetronitrile: Glacial acetic acid: Distilled water, 5:1:4, v/v) and 50 µl injected into the HPLC (Knauer, Japan) fitted with Nucleosil RP 18 C_{18} column and a fluorescence detector (FLD).

2.7 Data analysis

The data obtained was analyzed using statistical package for social scientist (SPSS version 20) where variability among the different samples was done using ANOVA. The differences between group means was analyzed using Duncan and statistical significance established at $p \leq 0.05$.

III. Results And Discussion

3.1 Moisture content of coffee samples

The results for percentage moisture content of coffee samples from estates and factories are shown in TABLE 1. The percentage moisture content of all coffee samples ranged between 6.85 ± 0.22 to 9.27 ± 0.54 which were slightly lower than what has been reported by other authors [17] who reported a range of 8.5% - 12% in green coffee beans. This could be attributed to the variations in the prevailing drying conditions, method of drying and the method of monitoring the moisture content level of coffee parchment during drying by different processors. From the study, it was observed that the samples from the different estates and factories showed a significantly lower moisture content level than those of the control samples for both the wet processed and the dry processed samples. Similarly, the wet processed coffee samples from Estate A showed a significantly ($p \leq 0.05$) higher levels of moisture content than those of Estate B and Estate C (TABLE 1).

The wet processed samples from the different Factories also showed significant variations with the coffee samples from Factory D showing a significantly higher level of moisture content than those of the other factories. Considering the dry processed coffee samples, there was no significant variations in the moisture content level for the coffee samples from the different Estates. However coffee samples from different Factories showed significant variations in the moisture content for the samples obtained by the dry method. The different variations observed in the level of moisture content from the different Estates and Factories could be attributed to common factors in processing factories which includes differences in prevailing weather conditions, lack of equipment to monitor the moisture content during drying, differences in storage conditions of dry coffee and the combination of coffee from different farms. Considering the lack of equipment in most of the Estates and Factories, as a common practice, the monitoring of moisture content in the coffee beans during drying is checked manually where the experts takes a dry coffee bean and bites with the teeth. From their experience, the personnel involved can know that a coffee bean is having correct level of moisture or not by the marks of their teeth on the bean tested. For a bean with correct level of moisture content (10-12%) the bean will be quite hard and will crack rather than form dents after biting but for the bean with higher moisture content above 12%, some dents will be seen on the surface of the bean [18]. The storage conditions for the dry coffee may also vary between the different Estates and Factories and this could contribute to the variations in the level of moisture content. In Kenya, the Estates used in the study represent big farms which produce and processed their coffee under one management. The Factories on the other hand represent the small scale farmers which produce their coffee separately and later combine them together under the cooperative management. Hence due to the nature of the different methods of handling coffee by the different Estates and Factories studied, the level of moisture content in the coffee beans they produce could also vary.

Table 1: Moisture content of coffee samples from factories and Estates processed by wet and dry method (%)

	Estates			**Factories**					**Control sample**
	A	B	C	D	E	F	G	H	
WP	8.47± 0.47^{d}	7.51± 0.29^{bc}	7.06± 0.30^{ab}	9.27± 0.54^{e}	8.48± 0.48^{d}	7.18± 0.02^{ab}	7.40± 0.09^{abc}	7.85± 0.34^{c}	9.00± 0.11^{de}
DP	7.43± 0.11^{abc}	7.20± 0.37^{ab}	7.17± 0.32^{ab}	7.16± 0.50^{ab}	7.65± 0.27^{bc}	6.85± 0.22^{a}	-	-	9.05± 0.72^{e}

(WP) Wet processed coffee (DP) Dry processed coffee (-) Sample not available.

Values within a row marked with different letters are significantly different ($p \leq 0.05$).

3.2 Mould content of coffee samples

During different phases of harvesting, processing, transport and storage, coffee may be subjected to microbial contamination and subsequent colonization [2]. In this study, the mould content was not detected in the wet processed samples from Estates but for the dry processed samples only two Estates showed some presence of moulds in the samples. The level of mould content in the dry samples from Estates was 3.13×10^3 and 6.0×10^1 CFU/ml, for Estate A and Estate C, respectively. Considering the Factories, the mould counts for wet processed coffee samples were found to be between 2.0×10^1CFU/ml to 2.5×10^2CFU/ml while that of dry processed coffee samples ranged between 1.46×10^3 CFU/ml to 5.56×10^3CFU/ml (TABLE 2). However the highest mould contamination was found in dry processed coffee as compared to wet processed coffee. The high mould contamination observed in dry processed coffee could be attributed to inefficient drying of the cherries since for dry processed coffee the whole coffee cherries are dried without separation of pulp from the beans. Hence depending on the weather conditions, the coffee cherries may take long to dry. Similarly, it may take longer time to dry the whole coffee cherry as compared to the parchment coffee [19]. The different Estates and Factories used in the study dry their coffee cherries by exposing to direct sun and this may offer them challenges such as poor weather conditions leading to poor drying of coffees.

Generally, coffee samples from Factories had slightly higher level of mould contamination than the samples from Estates. This can be explained by the fact that the Factories are formed by small scale farmers mainly managed by cooperatives which are characterized by inadequate processing facilities than the Estates. Cooperative societies collect coffee from many small scale farmers and due to logistics of collecting coffee and storage, their coffee may stay long in the stores before being processed and marketed. Due to poor storage, the coffee in the stores may develop mould growth especially during wet and dump weather conditions where the coffee could reabsorb moisture [11].

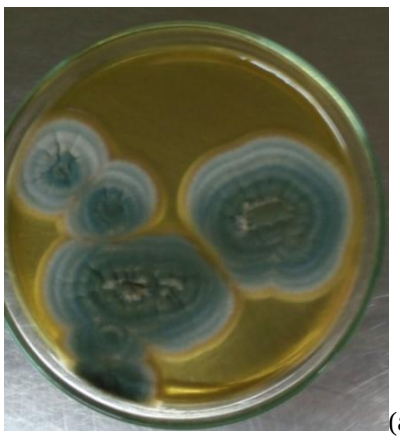
Table 2: Total counts of moulds of coffee beans from Estates and Factories processed by wet and dry methods (CFUs/ml)

Estates				**Factories**					**Control sample**
	A	B	C	D	E	F	G	H	
WP	ND	ND	ND	ND	2.5×10^2	ND	ND	2.0×10^1	ND
DP	3.13×10^3	ND	6.0×10^1	5.56×10^3	2.18×10^3	1.46×10^3	-	-	ND

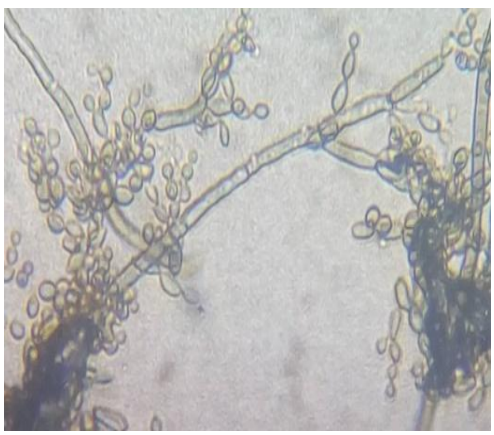
WP– Wet processed coffee DP– Dry processed coffee (-) Sample not available ND- Not detected

3.3 Mould species in coffee samples

The mould species were identified in the coffee samples basing on the characteristics such as appearance of the colonies, color of the mycelium, degree of growth and the microscopic characteristics. The appearance of the moulds species as observed under a microscope (OPTIKA, Italy) are presented in Fig 1 and 2. The two species that were identified in the coffee samples are *Penicillium and Aspergillus* species. The same mould species have also been identified in green coffee bean samples as reported by [20]. Their presence in food affects the quality and safety of the final product due to their ability to produce mycotoxins such as OTA, Aflatoxins B1 and B2 [21]. Literature have shown that *Aspergillus* and *Penicillium* species are among the most important contaminants of coffee beans [20, 22].



(a)



(b)

Figure 1: The photo of *Penicillium* species, (a) Colony on MEA, 25°C (5 days) (b) Conidiophores of *Penicillium* spp (×40)

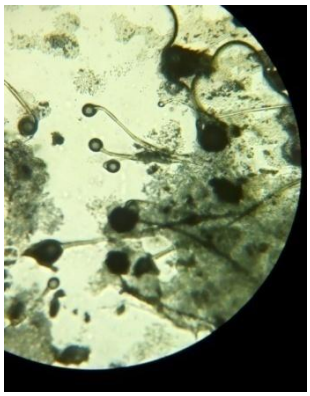
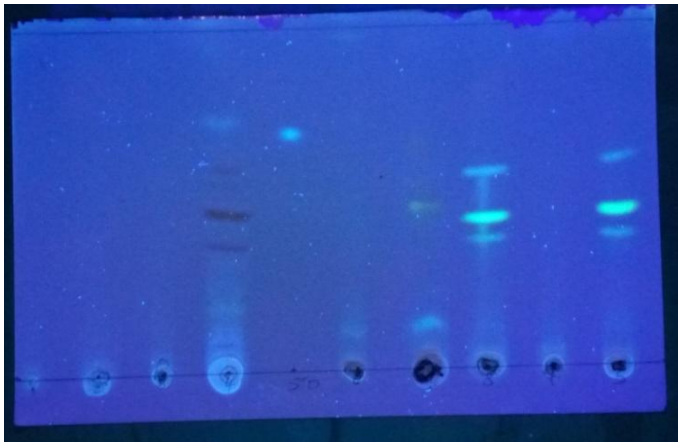

(a)

(b)

Figure 2: The photo of *Aspergillus* species, (a) *Aspergillus* niger group on MEA 25°C (5 days) (b) *Aspergillus* spp (×40)

3.4 Analysis of OTA

Isolates of *Apergillus* and *Penicillium* species identified under a microscope (OPTIKA, Italy) (Fig 1 and 2) were screened for OTA production on Thin Layer Chromatography (TLC) plate. Sample from Estates and Factories with detection of mould contamination were used for OTA analysis. The results showed that the samples analyzed tested negative for the presence of OTA (Fig 3). Hence the dry and wet coffee samples from the Estates and Factories which indicated presence of moulds did not show any OTA contamination in the beans. This could be because the mould content level detected in the samples could have been too low or the coffee beans were not kept long enough to allow any OTA production [23].



1 2 3 4 5 6 7 8 9 10

Figure 3: TLC plate (silica gel on aluminium) representing OTA analysis after development in Chloroform: Ethyl acetate: Formic acid (6:3:1), as visualized under 366 nm UV light.

Key: Tracks 1, 2, 3, 4, 5, 7, 8 and 6, 9 represents pure *Aspergillus* and *Penicillium* spp. isolates cultures respectively. Track 5 represents OTA standards. Estate C(m) 1, Estate C(m) 2, Estate C(m) 3, Factory F(m) 4, Estate C(m) 6, Factory F(m) 7,Estate A(m) 8,and Factory F(m) 9 .The names and the number represent the estates and factories where the isolates were obtained from. (m) –Dry processed coffee.

Ochratoxin A is one of the most dangerous mycotoxin produced by certain filamentous fungi such as *Aspergillus ochraceus, Aspergillus carbonarius* with some isolates of *Aspergillus niger* and *Penicillium verrucosum* [24, 25, 26]. A study carried out by [26] showed that the toxicogenic micro flora that was associated to the coffee beans which was contaminated with OTA mainly comprised of *Aspergillus* species. According to [23] the incidences of OTA in green coffee beans is attributed to the climate, the method used to process the coffee, how long the coffee is stored after processing and transportation as they influence contamination and growth of moulds.

Table 3: Ochratoxin A content of coffee beans from Factories and Estates processed by dry and wet methods (µg/kg)

Estates				**Factories**					**Control sample**
	A	B	C	D	E	F	G	H	
Wet Processed	ND	ND	ND	ND	ND	ND	ND	ND	ND
Dry processed	ND	ND	ND	ND	ND	ND	-	-	ND

ND - Not detected (-) Sample not available

IV. Conclusion

Research findings from this study indicated that there were significant differences in coffee samples in terms of moisture content of wet processed and dry processed coffee from the different Estates and Factories. Regarding mould contamination, dry processed coffee samples showed higher levels of mould contamination as compared to wet processed coffee. Ochratoxin A was not detected in wet and the dry processed coffee samples from the Estates and Factories. The information obtained from this paper is useful for extension services geared towards training of farmers and coffee handlers by relevant bodies, on effective ways of handling coffee to avoid mould contamination and OTA occurrence in coffee.

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