ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES
CENTER FOR FOOD SCIENCE AND NUTRITION

OCCURRENCE OF OCHRATOXIN A IN GREEN COFFEE: IN RELATION TO PROCESSING METHODS IN JIMMA ZONE, OROMIYA REGION OF ETHIOPIA

A thesis submitted to the School of Graduate Studies of Addis Ababa University in partial fulfillment of the requirement for the Degree of Master of Science in Food Science and Nutrition

By: Ambi Bekele Zewdie

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Dedication

This thesis is dedicated to coffee farmers in Jimma zone of Oromiya Region, Ethiopia.

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Abstract

Ochratoxin A (OTA) is one of the important mycotoxins that contaminate a wide range of food commodities. OTA contamination of coffee is a worldwide problem. Coffee is a major Ethiopian export commodity generating about 25% of Ethiopia's total export earnings. OTA is mainly produced in coffee by fungal species of *Aspergillus ochraceus, Aspergillus carbonarius* and *Aspergillus westerdijkiae* (section *Circumdati*). This study aims to assess the levels of OTA in green coffee in relation to drying process in Jimma zone, Oromiya, Ethiopia. A total of 18 coffee samples were collected from three coffee producing weredas of Jimma zone. The samples were comprised of dry and wet processed. Fungi profile was evaluated by direct plating techniques. OTA levels were analyzed using HPLC technique after extraction and filtration using an immunoaffinity column. Results obtained revealed an overall percentage of fungal contamination between 76.67%-100% and 73%-93% in dry and wet processed coffee, respectively. There was no drying process method difference in composition of ochratoxigenic species in dry processed coffee in three sites. Coffee from dry processed samples had a fungal incidence of 29.63% *Aspergillus flavus*, 40.74% *Aspergillus niger*, 30% *Aspergillus ochraceus*, 29.26% *Penicillium species*, 21.11% *Fussarium* species and 29.26 *Rhyzopus* and other unidentified species. While those of wet processed had a fungal incidence of 30.74% *Aspergillus flavus*, 42.59% *Aspergillus niger*, 29.26% *Aspergillus ochraceus*, 26.66% *Penicillium* species, 23.70% *Fussarium* species and 28.52% *Rhyzopus* and other unidentified species. There was no fungal diversity in types of drying process. *Aspergillus niger* was the predominant fungal infection in both process with 42.59% and 40.74% in wet and dry processed coffee respectively. The least fungal occurrence in both processed coffee was *Fussarium* species with 23.7% in wet and 21.11% in dry processed. From a total of 54 isolates (36 *Aspergillus niger* and 18 *Aspergillus ochraceus)*, only 4 isolates (7.14%) were capable of producing ochratoxin A with the range of concentration of 2.04 to3.32µg/kg. From the total of 18 samples analyzed, 44.44% were OTA contaminated with the contamination level ranged between 2.74µg/kg and 6.46µg/kg. OTA levels were mostly below the recommended standards by EU (5µg/kg). There was no difference in OTA contamination between wet and dry processing while the mean value is 2.71µg/kg and 0.97µg/kg in dry and wet processed respectively.

Keywords: *Coffee arabica L, A. niger, A. ochraceus,* Ochratoxin A, Drying method

List of Acronyms and Abbreviation

AOAC: Association of Official Analytical Chemists

BEN: Balkan Endemic Nephropathy

CE: Capillary Electrophoresis

ECX: Ethiopian Commodity Exchange

EFSA: European Food Safety Authority

GAIN: Global Agricultural Information Network

HPLC-FL High Pressure Liquid Chromatography with -Fluorescence Detection

IAC: Immuno Affinity Column

IARC: International Agency for Research on Cancer

ITC: International Trade Center

IUPAC: International Union of Pure and Applied Chemistsry

LC-MS: Liquid Chromatography Mass Spectroscopy

OP-OT: Open ring Ochratoxin

OTA: Ochratoxin A

OTB: Ochratoxin B

OTC: Ochratoxin C

OTHQ: Ochratoxin Hydroquinone

OTα: Ochratoxin alpha

OTβ: Ochratoxin beta

QSAE: Quality and Standards Authority of Ethiopia

SRM: Standard Reference Material

TLC: Thin Layer Chromatography

1. Introduction

Ethiopia is the source of several economically important cultivated crops around the world. Among these crops, the most important gift of Ethiopia is coffee known as *Coffea arabica L.* It is the most important cash crop that has been contributing a lion's share to the Ethiopian economy. In Ethiopia, farmers traditionally grow coffee as an important cash crop under various types of shade trees (Birhanu, 2012).

According to Global Agricultural Information Network, GAIN (2012 & 2013), Ethiopia is famous as the origin of coffee and the major producer and exporter of coffee in Africa; and is the largest producer of coffee in Sub-Saharan Africa and is the fifth largest coffee producer in the world next to Brazil, Vietnam, Colombia, and Indonesia, contributing about 7 to 10% of total world coffee production. The largest volume of coffee is grown in the two large regions of Oromiya and the Southern Nations, Nationalities and Peoples Region (SNNPR). Coffee production is important to the Ethiopian economy with about 15 million people directly or indirectly deriving their livelihoods from coffee.

According to the current context of overproduction and low prices of the coffee market, improvement and valorization of coffee quality could provide the coffee chain with a new impetus (Leroy et al., 2006). Therefore, Production and supply of coffee with excellent quality seems more crucial than ever before for coffee exporting countries (ITC, 2004). In view of the present situation, making effort to overcome challenges and threats only through expansion of production does not seem visible for countries like Ethiopia. Thus, it has been repeatedly mentioned that providing good quality coffee is the only way out and viable option to get into the world market and to remain competitive. However, in Ethiopia the quality of coffee produced by farmers has been deteriorating from time to time (Behailu et al., 2008).

Post harvest processes, one of the factor that affect coffee quality, has significant effects on coffee quality (Barel and Jacquet, 1994). During post harvest process coffee may be infected with micro-organisms. OTA (Ochratoxin A) is a form of mycotoxin, produced as a metabolic product of *Aspergillus ochraceus, Aspergillus. carbonarius and strains of Aspergillus niger* that degrade quality of coffee and causes disease to those who consumed it (Eshetu and Girma, 2008).

Mycotoxins are metabolites of fungi capable of having toxic effects in man and animals. The majority of the mycotoxins are produced by three fungal genera: *Aspergillus, Pencillium* and *Fusarium*. When food and feed contaminated with fungi, and the toxins they may produce, are ingested by humans and/or animals, a wide variety of debilitating diseases (ochratoxicosis) can occur and result in enormous economic losses, both from agricultural losses and medical care costs (De Lucca, 2007)

Ochratoxin A (OTA) is one of the most important mycotoxins. It is produced by a number of fungal species from the genera, *Aspergillus* and *Pencillium* that can colonize a range of food products (Gompa, 2013). Human exposure can occur through consumption of contaminated food products, particularly contaminated grain and pork products, as well as coffee, wine grapes, and dried fruit (Pfohl-Leszkowicz et al., 2007; O'Brien et al., 2005; Blesa et al., 2006). According to European Food Safety Authority, EFSA (2010), OTA is rapidly absorbed from the gastrointestinal tract and bound to plasma proteins (serum albumin, other macromolecule). About 99.9% is distributed in a number of species via blood, mainly to the kidneys where it accumulates. OTA is a substrate for organic anion transporter proteins. Major route of excretion is renal elimination in many species (including monkeys and humans). Major metabolite in all species is ochratoxin *alpha* excreted in urine and faeces, relationship influenced by the extent of the enterohepatic recirculation and it's binding to serum macromolecules.

OTA can have several effects such as nephrotoxic, hepatotoxic, neurotoxic, teratogenic and immunotoxic on several species of animals, and can cause kidney and liver tumors in mice and rats (El Khoury et al., 2010). OTA is able to induce overproduction of nitrogen monoxide (NO), both in kidney and liver, resulting in increased nitrite and nitrate levels. Under normal conditions, nitrogen monoxide presents a broad range of biological activities; conversely, in excess, it may behave as a toxic radical (Sorrenti et al., 2013). Ochratoxin A represents a well-known hazard to human and animal health. According to FAO (2004), the European Union has set legal limits for OTA levels in imported foods, with a maximum of 5μg/kg (ppb) in raw cereal grains, 3μg/kg in processed cereal foods, and 10μg/kg in dried vine fruits (raisins). As of April

2005, the EU imposed limits for ochratoxins in wine, grape juice and coffee. The limits are 2.0 μg /kg for wine and grape juice 5.0μg /kg for roasted coffee, and 10.0μg /kg for instant coffee.

1.1. Statement of the problem

There is a large body of literature documenting OTA contamination of green and processed coffee, and reporting isolation of OTA-producing fungi from coffee, starting in the 1970s. Ochratoxin A contamination of coffee is a worldwide problem. The presence of OTA in green coffee bean has been reported by several authors in wide concentration ranging between 0.2 and 360μg/kg (Taniwaki, 2006). A number of European nations have already set limits for ochratoxin A in green coffee beans (Table 5.)

There is lack of awareness among farmers, processors and consumers' regarding OTA and its potential risk to human and animal health in Ethiopia. Despite the large information generated in the world, there is still lack of information in Ethiopia about consumer exposure to the toxin. Limits on ochratoxins A content in coffee by imported countries increase exposure to ochratoxins A for people in exported countries. What cannot be exported may be consumed domestically. In order to prepare the national coffee sector to meet regulations (export standard) and to prevent health risk of the nation, it is critical to develop quality control system. This study may contribute activities in establishing quality control system and creating awareness among stake holders. Therefore, the main aim of this study was to evaluate ochratoxin A contamination in green coffee obtained by two processing procedures, wet and dry. In addition, screening of fungal load and isolation of ochratoxin producing fungi was also documented.

1.2. Objectives

1.2.1. General Objective

The aim of this study is to evaluate level of OTA in green coffee in relation to drying process and to characterize Ochratoxin A producing isolates in *vitro* so as to put in awareness in order to alleviate the existing problem of coffee quality in the country.

1.2.2. Specific Objectives

1. To evaluate OTA level in relation to drying process in freshly harvested green coffee beans
2. To screen fungal load that colonizes coffee beans at the two drying process
3. To identify the OTA producing strains in vitro

2. Literature Review

2.1. Origin of *Coffee Arabica L. (C. arabica L.)*

The original native population of coffee is thought to have come from East Africa specifically to Ethiopia, and it was first cultivated by Arabs from the 14th century and introduced into the New World and much of the rest of the tropics during the 17^{th} century (Smith 1985, Wrigley 1988). The original native population of coffee was in the highlands of Ethiopia with possible disjunct populations in nearby highland areas of Sudan and Kenya (Berthaud and Charrier, 1988). *Coffee arabica* exist in Ethiopia than anywhere else in the world (Aga *et al.*, 2003), which has led botanists and scientists to agree that Ethiopia is the centre of origin (primary gene centre) for diversification and dissemination of the coffee plant (Fernie, 1966; Zeven & Zhukovsky 1975; Bayetta, 2001).

2.2. Botanical description of *C. arabica L.*

Coffee arabica is a woody, perennial evergreen dicotyledon belonging to the botanical family *Rubiaceae*, which contains some 500 genera and over 6000 species. Most of these are trees and shrubs which grow in the lower storey of tropical forests (Bragulat et al., 2001; Bridson & Verdcourt, 1988).The subgenus *Coffea* consists of approximately 100 taxa so far identified in *African* and Madagascar inter-tropical forests. (Lashermes et al., 1996). The tree grows up to 8-10 m high and its branches are long, flexible and thin. Branches are semi-erect when young and spreading or pendulous when old (Coste, 1992). The architecture of the coffee tree is characteristic of a tree growing in tropical forests: a vertical (orthotropic) stem, with horizontal (plagiotriopic) branches arising in pairs opposite to each other. The growth is by a typical form of monopodial branching where the branches (primaries) remain subsidiary to the main stem, which continues to grow indefinitely by extension of the apical buds (Wrigley, 1988). The coffee plant takes approximately 3 years to develop from seed germination to first flowering and fruit production (Wintgens, 2004). The root consists of a stout central root, often multiple, tapering more or less abruptly. The leaves oriented in opposite pairs on the sides of the branches and ovalshaped and acuminate. It has white, Jasmine-scented flowers grouped together in the axils of the paired leaves, with two to three cymes making up whorls of 8- 15 flowers. Its fruit is sub-lobular, ovoid, oblong or squat-shaped. Fruits are orange-red to red on ripening

2.3. *Coffee Arabica L. (C. Arabica L.)*

Ethiopia is the source of several economically important cultivated crops around the world. Among these crops, the most important gift of Ethiopia is coffee known as *Coffea arabica L.* It is the most important cash crop that has been contributing a lion's share to the Ethiopian economy. (Birhanu, 2012). *Coffee arabica* has its origin in the Southwest highlands of Ethiopia (Lewis, 1969) *Coffea arabica* is predominantly self-pollinating (autogamous) and the only natural allotetraploid (2n=4x=44) species in the genus *Coffea.* The other coffee species are all diploid (2n=2x=22) and out crossing (allogamous) (Charrier & Berthaud,1985).

2.3.1. Taxonomic hierarchy of *Coffee arabicaL. L.*

Kingdom: Plantae

Subkingdom: *Viridaeplantae*

Infrakingdom: *Streptophyta*

Division: *Tracheophyta*

Subdivision: *Spermatophyta*

Infradivision: *Angiospermae*

Class: *Magnoliopsida*

Superorder: *Asteranae*

Order: *Gentianales*

Family: *Rubiaceae*

Genus: *Coffea L.*

Species: *Coffee Arabica L.*

Figure 1. Coffee Arabica, cherry (left), flower (right)

2.4. Coffee Production in Ethiopia

Coffee has for centuries played an important role in the Ethiopian economy and represents the main cash crop cultivated by small scale farmers for social, economic, political and ecological sustainability (Mekuria *et al.*, 2004). In Ethiopia, farmers traditionally grow coffee as an important cash crop under various types of shade trees, mainly dominated by leguminous tree species (Taye, 2001; Gole, 2003). Wide use of tree legumes for providing shade has also been well documented in many coffee growing countries across the globe (Perfecto *et al.*, 1996; Albertin & Nair, 2004; Polzot, 2004). According to Global Agricultural Information Network, GAIN (2012 &2013), Ethiopia is famous as the origin of coffee and the major producer and exporter of coffee in Africa; and is the largest producer of coffee in Sub-Saharan Africa and is the fifth largest coffee producer in the world next to Brazil, Vietnam, Colombia, and Indonesia, contributing about 7 to 10% of total world coffee production. The largest volume of coffee is grown in the two large regions of Oromiya and the Southern Nations, Nationalities and Peoples Region (SNNPR). Only five percent of coffee production is grown on modern plantations, which are owned by private investors or by the government. The rest is grown by smallholder farmers, and about half of that production is in backyards or gardens. In both cases coffee is generally grown under shade.

Coffee production is important to the Ethiopian economy with about 15 million people directly or indirectly deriving their livelihoods from coffee. Coffee is also a major Ethiopian export commodity generating about 25% of Ethiopia's total export earnings. Ethiopia is the birthplace of Arabica coffee and produces mostly Arabica coffee. Coffee has economical, environmental as well as social significance to the country (Boom, 2011).

2.4.1. Coffee processing in Ethiopia

The 2 main methods of processing the harvested coffee cherries are dry processing and wet processing both being sun dried.

2.4.1.1. Dry Processing

The dry process is used to produce natural coffees and is the most traditional method of processing coffee. After harvest and selection, the whole coffee cherries are first cleaned and then placed in the sun to dry. It may take up to 4 weeks before the cherries are dried to the optimum moisture content, depending on the weather conditions. The coffee beans are then removed from the dried cherry.

Ethiopian Harar and Yemeni Mocca are two examples of classic natural coffees.

2.4.1.2. Wet Processing

The wet process is an alternative means of completely separating the coffee beans from the rest of the cherry and in most cases produces the finest results in terms of taste. Firstly, the coffee cherries are washed in clean, fresh water and are pulped to remove the skins and outer flesh from the beans. However, the beans are still covered in the sticky pulp or mucilage that must be completely removed before drying the coffee beans. To do this, the pulped beans are put into fermentation tanks so that the mucilage is fermented for approximately 24-48 hours (depending upon conditions), essentially breaking it down so that it can be washed clear of the bean with clean water. Washing channels are used to help achieve this. The wet processing generally imparts cleaner, fruitier and more acidic flavors to the coffee and results in classic fully washed coffees.

2.5. Coffee Consumption in Ethiopia

Ethiopians have been drinking coffee longer and more consistently than any other people on the planet. There are various legends about how coffee cultivation came about, but what we know for certain is that coffee drinking goes back at least 500 years, and most likely much longer. Coffee drinking is a deep part of Ethiopian culture, and a big part of the identity of the people there. Ethiopians of all classes and ethnicities enjoy coffee. As a result, a very large portion of national production ends up on the local market (Boom, 2011). According to Global Agricultural Information Network, GAIN (2012), Ethiopians are heavy coffee drinkers, ranked as one of the largest coffee consumers in Sub-Saharan Africa. Nearly half of Ethiopia's coffee production is locally consumed. Ethiopian households normally prepare and consume coffee two or three times a day, and the Ethiopian coffee ceremony is a traditional way to welcome guests to one's house. Coffee in Ethiopia has both social and cultural value. It is mainly consumed during social events such as family gatherings, spiritual celebrations, and at times of mourning.

2.6. Coffee Quality

Different coffee producing countries have tremendously expanded their production and export volume (Behailu et al., 2008). According to the current context of overproduction and low prices of the coffee market, improvement and valorization of coffee quality could provide the coffee chain with a new impetus (Leroy et al., 2006). Production and supply of coffee with excellent quality seems more crucial than ever before for coffee exporting countries. Consequently, some countries consider assessment of coffee quality as important as disease resistance and productivity in their coffee variety development program (ITC, 2004). In view of the present situation, making effort to overcome challenges and threats only through expansion of production does not seem visible for countries like Ethiopia. Thus, it has been repeatedly mentioned at various forum that providing good quality coffee is the only way out and viable option to get into the world market and to remain competitive (Behailu et al., 2008). According to the International Organization for Standardization, ISO (2000), Quality is described as "the ability of a set of inherent characteristics of a product, system or process to fulfill requirement of customers and other interested parties". These inherent characteristics can also be called "attributes". For coffee, the definition of quality and the attributes considered have probably evolved through the centuries. But nowadays, this definition varies along the introduction-to-consumer chain (Leroy et al., 2006). . According to the definition of quality and standards authority of Ethiopia (QSAE) (2000) a quality is conformance with requirements or fitness for use in which the parties involved in the industry (customer, processor, supplier, etc) should agree on the requirements and the requirements should be clear to all stake holders involved in the process. On the other hand, Coffee Quality control and auction Center was established with a key objective of maintaining coffee quality control.

However, in Ethiopia the quality of coffee produced by farmers has been deteriorating from time to time. Moreover, factors that determine coffee quality are genotypes, climatic conditions, and soil characteristics of the area, agronomic practices, harvesting methods and timing, post harvest processing techniques, grading, packing, storage conditions and transporting, all contribute either exaltation or deterioration of coffee (Behailu et al., 2008).

Post harvest processes, one of the factor that affect coffee quality, has significant effects on coffee quality can be observed (Barel and Jacquet, 1994). Processing is a very important activity in coffee production and plays a crucial role in quality determination (Mburu, 1999). Coffee is either processed by the wet or dry methods, which vary in complexity and expected quality of the coffee (Wrigley, 1988). During post harvest process coffee may be infected with micro-organisms; the secondary bacterial infection causes a distinct potato flavor. OTA (Ochratoxin A) is a form of mycotoxin, produced as a metabolic product of *Aspergillus ochraceus, Aspergillus. carbonarius and strains of Aspergillus niger* that degrade quality of coffee and causes disease to those who consumed it (Eshetu and Girma, 2008).

2.7. Mycotoxins

Mycotoxins are secondary metabolises produced by fungi, especially by saprophytic moulds growing on foodstuffs or animal feeds. Mycotoxins have been a hazard to man and domestic animals. Although poisonous mushrooms are carefully avoided, moulds growing on foods have generally been considered to cause unaesthetic spoilage, without being dangerous to health. Between 1960 and 1970 it was established that some fungal metabolites, now called mycotoxins, were responsible for animal disease and death. In the decade following 1970 it became clear that mycotoxins have been the cause of human illness and death as well, and are still causing it (Pitt, and Hocking, 1985).

Mycotoxins are a cause of concern during storage, and production of the toxin depends on various factors such as: moisture content, temperature, storage period, contamination rate, broken grain and impurities, insect presence, oxygen concentration, damage during harvest, processing, and grain and seed transport (Rahmani et al., 2009). Fungi often infect crops either in the field after harvesting resulting in considerable economic losses to farmers and producers worldwide. When food and feed contaminated with fungi, and the toxins they may produce, are ingested by humans and/or animals, a wide variety of debilitating diseases, ergotism (due to alkaloids) causes constrictions in blood vessels leading to the hands and feet and in extreme cases death of cells (necrosis), bacterial infections (gangrene) and effects on the mind (hallucinations), renal tubular necrosis (due to ochratoxin A) and hepatic periportal necrosis (due to aflatoxin) and result in enormous economic losses can occur, both from agricultural losses and medical care costs (De Lucca, 2007)

It is important to distinguish between the effects of bacterial toxins and mycotoxins. The classic bacterial toxins are proteins, which produce characteristic symptoms in only a few hours, as the human body recognizes them, and produces antibody mediated reactions to them. Fungal toxins on the other hand, are almost all low molecular weight chemical compounds which are not detected by antigens, and hence produce no obvious symptoms. Mycotoxins are insidious poisons (Pitt, and Hocking, 1997).

Table 1. Moulds and mycotoxins major public health and economic significance

Mould species	Mycotoxins	Occurrence
Aspergillus parasiticus	Aflatoxins B_1, B_2, G_1, G_2	Maize (corn), groundnuts (peanuts), tree nuts, spices, dried fruit, crude vegetable oils, cottonseed and copra
Aspergillus flavus	Aflatoxins B_1, B_2	
+*Fusarium sporotrichiodes*	T-2 toxin	Cereals
Fusarium graminearum	Deoxynivalenol (DON)	Grains, especially wheat, barley, oats, rye and maize. Less often in rice and sorghum
Fusarium graminearum	Zearalenone	Maize, but also lower levels in rice, wheat, barley, malt and soybean
Fusarium moniliforme	Fumonisin B_1	Maize
Penicillium verrucosum	Ochratoxin A (OTA)	Cereals, fresh grapes, dried vine fruit, wine, beer, coffee, cocoa
Aspergillus ochraceus	Ochratoxin A (OTA)	
Penicillium expansum	Patulin	Fresh and processed fruit and vegetables (especially apples)

Source: FAO, Food and Nutrition Papers 73 (2001)

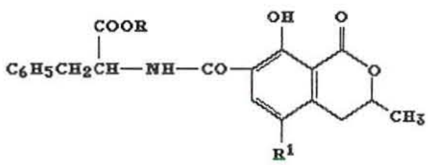
2.7.1. Ochratoxin A

Ochratoxin A (OTA) is one of the most important mycotoxins. It is produced by a number of fungal species from the genera, *Aspergillus* and *Pencillium* that can colonize a range of food products (Gompa, 2013). It was first isolated in 1965 from a culture of *Aspergillus ochraceus*. Ochratoxin A is also produced by other kinds of *Aspergillus*, such as *Aspergillus carbonarius*; within the *Penicillium* species, however, it is only produced by *Penicillium verrucosum*. *Aspergillus ochraceus* grows at temperatures of 8 to 37°C on culture media containing sugar and salt, with an optimum of 24–31°C. *Penicillium verrucosum* grows at temperatures of 0–31°C, with an optimum of 20°C (contamination of foodstuffs in colder climates). *Aspergillus carbonarius* grows well at 32–35°C, a low pH value and high sugar content (International Agency for Research on Cancer (IARC) 1993, WHO, 2001).

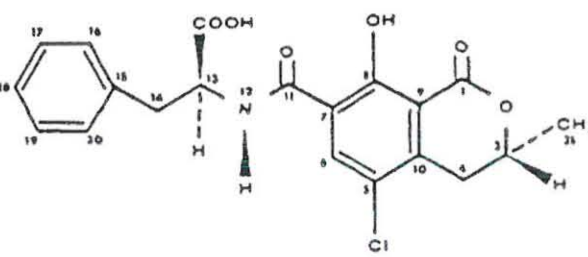
Chemistry of OTA

IUPAC Name

OTA, (R)-N-[(5-chloro-3, 4-dihydro-8-hydroxy-3-methyl-1-oxo-1H-2-benzopyran-7-carbonyl) -L-phenylalanine, is a naturally occurring mycotoxin soluble in organic solvents, in aqueous solution of sodium bicarbonate and slightly soluble in water (Sorrenty et al., 2013)



Chemical structure of Ochratoxin



Chemical Structure of Ochratoxin A

Molecular formula	$C_{20}H_{18}ClNO_6$
Molecular weight	403.8
Melting Point	$169^{0}C$
Boiling point	not stated

Figure 2. Chemical structure of ochratoxin and ochratoxin A

OTA is the most important and most commonly occurring of a group of structurally related compunds. Ochratoxin A consists of a polyketide-derived dihydroiso-coumarin moiety linked through the 12-carboxy group to phenylalanine. There are several analogues of OTA, such as ochratoxin B (which differs in structure from OTA in lacking the chlorine atom), ochratoxin C and ochratoxin ß, which are all fungal metabolites. However, OTA is the major compound found as a natural contaminant of plant material. OTA contamination is commonly associated with cereals, fresh grapes, dried vine fruit, wine, beer, coffee, and cocoa (*The MAK-Collection Part I, 2006).*OTA have been also identified, particularly, ochratoxin B (OTB) the dechloro analog of OTA, ochratoxin C (OTC) its ethyl ester, the isocoumaric derivative of OTA, ochratoxin α (Otα), and its dechloro analog, ochratoxin β (OTβ) (El Khoury et al., 2010)

Table 2.Chemical structures of ochratoxins

Name	R_1	R_2	R_3	R_4	R_5
Ochratoxin A	Phenylalanine	Cl	H	H	H
Ochratoxin B	Phenylalanine	H	H	H	H
Ochratoxin C	Ethyl-ester	Cl	H	H	H
Ochratoxin A methyl ester	Phenylalanine	Cl	H	H	H
Ochratoxin B methyl ester	Methyl- ester, phenylalanine	H	H	H	H
Ochratoxin B ethyil ester	Methyl- ester, phenylalanine	H	H	H	H
Ochratoxin α	Ethyl-ester phenylalanine	Cl	H	H	H
Ochratoxin β	OH	H	H	H	H
4-R-Hidroxyochratoxin A	OH	Cl	H	OH	H
4-s-Hidroxyochratoxin A	Phenylalanine	Cl	OH	H	H
10-Hidroxyochratoxin A	Phenylalanine	Cl	H	H	OH
Tyrosina analog of ochratoxin A	Phenylalanine	Cl	H	H	H
Serin analog ochratoxin A	Tyrosine	Cl	H	H	H
Hidroxyprolina analog ochratoxin A	Serine	Cl	H	H	H
Lysine analog ochratoxin A	Hydroxiprolyne	Cl	H	H	H
Synthetic Ochratoxins					
d ochratoxin A	D phenylalanine	Cl	H	H	H
Ochratoxin A Ethyl-amid	Ethyl-amid phenylalanine	Cl	H	H	H

Source: Robu, Contribution to analytical study of the ochratoxins with applications on food (2011)

2.8. Biosynthesis of ochratoxin A

Ochratoxin A is a chlorinated polyketide molecule containing the amino acid phenylalanine. According to the molecular structure of OTA, several enzymatic activities are required for the biosynthetic process, such as a polyketide synthase for the synthesis of the polyketide dihydroisocoumarin, a chlorinating enzyme, and also a peptide synthetase, which is necessary to link the phenylalanine to the dihydroisocoumarin ring (Gallo et al., 2012).

Biotransformation of OTA into several metabolites, such as Ochratoxin B (OTB), open-ring ochratoxin A (OP-OA), 4 hydroxylated OTA, 10 hydroxylated OTA, OTA without phenylalanine, OTB without phenylalanine, OTA hydroquinone (OTHQ) and a dechlorinated ochratoxin A derivative (Sorrenty et al., 2013).The phenylalanine portion of the molecule is synthesized via the shikimic acid pathway, and the isocoumarin portion could arise either via the shikimate pathway as in coumarin synthesis of higher plants, or from acetate units via an isoprenoid type of condensation as in other fungal biosynthesis pathway (Searcy et al., 1969)

The isocoumarin moiety is formed from acetate units via the pentaketide pathway, carboxylated, and then chlorinated by chloroperoxidase to form ochratoxin-*a*. The final step, linkage through the carboxyl group to phenylalanine, is catalyzed by OTA synthetase. Ochratoxin B may be formed when chlorine concentrations are low, and to some extent by dechlorination of OTA (Bayman & Baker, 2006)

2.9. Ochratoxin A absorption and elimination

According to European Food Safety Authority, EFSA (2010), OTA is rapidly absorbed from the gastrointestinal tract and bound to plasma proteins (serum albumin, other macromolecule). About 99.9% is distributed in a number of species via blood, mainly to the kidneys where it accumulates. OTA is a substrate for organic anion transporter proteins. Major route of excretion is renal elimination in many species (including monkeys and humans). Biliary excretion and entero-hepatic recirculation of OTA-glucuronides. OTA transferred to milk in rats, rabbits and humans (1.2-6.6 ng/ml). OTA crosses the placenta. Major metabolite in all species is ochratoxin *alpha* excreted in urine and faeces, relationship influenced by the extent of the enterohepatic recirculation and its binding to serum macromolecules.

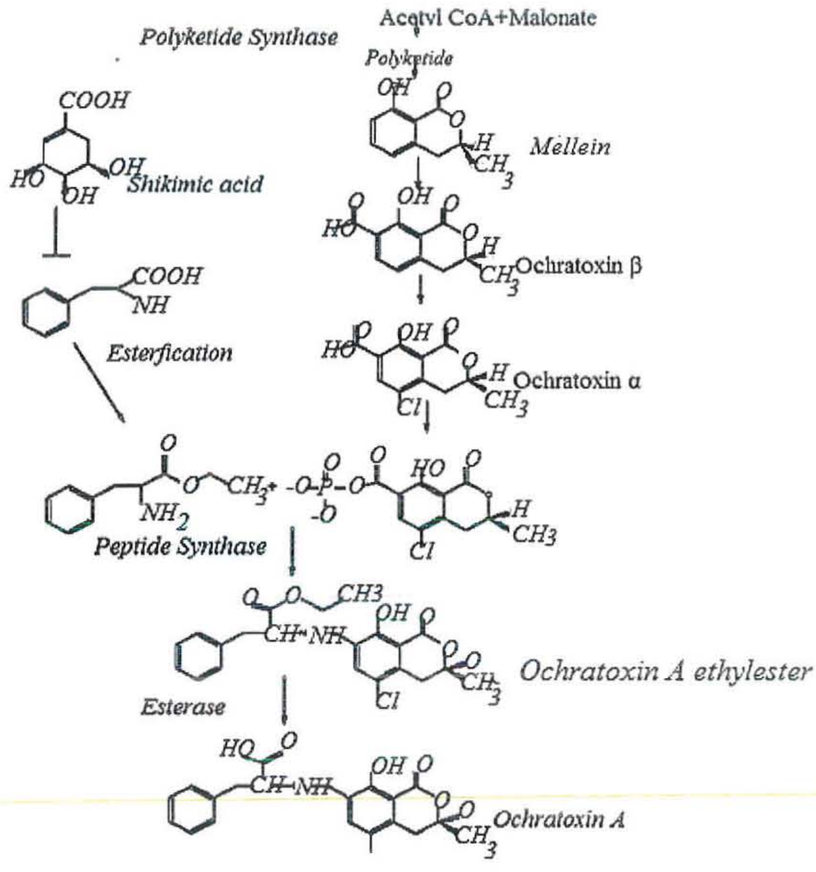


Figure 3. Biosynthesis pathway of ochratoxin A (Huff, et al, 1979)

2.10. OTA Detection Method

There are a number of analytical methods for the detection of ochratoxin A in various food matrices and the vast majority of chemical analytical methods applied for accurate, selective and sensitive mycotoxin determination in various samples come from the group of separation methods: chromatography, electrophoresis. High performance liquid chromatography (HPLC) with different detectors is frequently used both for routine analyses and as confirmatory method for novel or screening techniques (Kralj Cigić et al., 2009).

2.10.1. Thin-layer chromatography (TLC)

Thin-layer chromatography (TLC) is a low-cost, rapid analytical technique, yielding qualitative or semi-quantitative estimations by visual inspection, but with densitometric measurements also reliable quantitative results.TLC determination of ochratoxin A (native fluorescence-densitometric detection) was compared to different HPLC methods and recoveries for the TLC method were lower than average of HPLC methods.These methods were mostly developed before the great development and affordability of HPLC and LC-MS instruments and some were established as the official AOAC methods. Their many advantages include suitability for crude extract analysis, a wide choice of stationary and mobile phases, as well as an array of spraying agents used for the detection. In spite of that, TLC methods are now rarely used for other than screening purposes (Krska et al., 2007; Lin et al., 1998).

2.10.2. High performance liquid chromatography with fluorescence detection (HPLC-FL)

HPLC-FL is one of the chromatographic methods commonly used for the determination of ochratoxin A. Fluorescence detection is by its nature highly specific and sensitive. Several well-established, reliable, robust and sensitive LC methods with fluorescence detection exist for the determination of ochratoxin A with natural fluorescence and has been established as the official AOAC methods. LC-FL might still be superior over LC-MS in the area of quantitative determination, where the influence of matrix is negligible compared to possible problems with LC-MS quantification. In spite of its specificity for the fluorescing compounds, they have to be well separated on the chromatographic column to enable a reliable quantification. Usually, a reverse-phase stationary phase is used, e.g. C18. Base deactivation additionally improves peak shape for polar mycotoxins with carboxylic groups: OTA. Most authors use the octadecyl silica stationary phase and isocratic mobile phase composed of acetonitrile and water acidified with acetic acid in the determination of OTA. Retention time for OTA is below 15 min under these conditions. Fluorescence is usually measured at λex 330-334 nm and λem 460-464 nm. The principle behind HPLC is a test portion is extracted with a solvent solution (e.g. Methanol). The sample extract is filtered, diluted with phosphate buffered saline to a specified solvent concentration and a specified pH (7.2), and a portion is applied on an immunoaffinity column containing antibodies against OTA. The OTA is then eluted from the immunoaffinity column with methanol (HPLC grade). The eluate is evaporated to dryness under stream of nitrogen and the residue dissolved in HPLC mobile phase and then quantified by HPLC (Ahmed et al., 2007).

2.10.3. Capillary electrophoretic (CE)

In spite of the great separation power and versatility of capillary electrophoretic (CE) techniques, they have never gained such popularity as HPLC, although the same analytes can be determined with CE and the same detectors can be used. Possible explanation might be in better detection limits and greater user-friendliness of HPLC methods. Ochratoxin A was determined by CE-laser induced fluorescence in human serum, food samples after immunoaffinity clean-up, and in wine after combined extraction with SPE and supported liquid membrane (Köller et al., 2006)

2.11. Methods used for analysis of coffee samples

There are different studies of OTA in coffee that have used different methods for mycotoxin extraction and cleanup, all followed by detection and quantification by HPLC coupled with a fluorescence detector. These different methods include a combination of various extraction solvents, cleanup methods and mobile phases. A summary of the different methods used for the detection of OTA in coffee by HPLC according to different authors is provided in Table 3. For many methods mentioned in Table 3, Immuno affinity column were used for clean up. For all methods excitation and emission wavelengths used with the fluorescence detector were around 333 nm and 460 nm, respectively.

Table 3. Methods used for HPLC detection of OTA in coffee

Extraction solvent	Mobile phase	Cleanup	Reference
1% aqueous sodium bicarbonate	Methanol: Acetonitrile: Sodium acetate (29:29:42)	MAX catridge	Ventura et al., (2003)
Methanol:3% aqueous sodium bicarbonate solution (1:1)	Acetonitrile: 4 mM sodium acetate (0.5% acetic acid solution) (42:58)	Immunoaffinity columns	Taniwaki et al., (2003)
Methanol: 3% aqueous sodium bicarbonate solution (1:1)	Methanol: acetonitrile: Sodium acetate (29:29:42)	Solid phase and immunoaffinity columns	Lombaert et al., (2002),
Methanol: 3% aqueous sodium bicarbonate solution (1:1)	Acetonitrile: 2% acetic acid (42:58)	Solid phase and immunoaffinity columns	Abdulkar et al., 2004
1% aqueous sodium bicarbonate	Acetonitrile: 2% acetic acid (44:56)	Solid phase and immunoaffinity columns	Vatinno et al., 2008
Methanol: 3% aqueous sodium bicarbonate (1:1)	Water: acetonitrile: glacial acetic acid (51:48:1)	Immunoaffinity columns	Suarez-Quiroz et al., 2004
3% aqueous sodium bicarbonate	Acetonitrile: water: acetic acid (40:60:1)	Solid phase and immunoaffinity columns	Mantle and Chow, 2000

Source: Gompa, 2013

2.12. Toxic Effects of OTA

Ochratoxin A, a toxin produced by *Aspergillus ochraceus*, *Aspergillus carbonarius* and *penicillum verrucosum*, is one of the most-abundant food-contaminating mycotoxin (Al-Anati, et al, 2006). It is also a frequent contaminant of water-damaged houses and of heating ducts (Polizzi, et al, 2009, Richrd, et al, 1999). Human exposure can occur through consumption of contaminated food products, particularly contaminated grain and pork products, as well as coffee, wine grapes, and dried fruit (Pfohl-Leszkowicz et al., 2007; O'Brien et al., 2005; Blesa et al., 2006). The toxin has been found in the tissues and organs of animals, including human blood and breast milk (Clark et al., 2006). Ochratoxin A, like most toxic substances, has large species- and sex-specific toxicological differences (O'Brien et al., 2005).

According to the International Agency Research of Cancer (IARC), OTA is a potent nephrotoxin which may contaminate various foods and feed products (grains, legumes, coffee, dried fruits, beer and wine, and meat). It also exhibits carcinogenic, teratogenic and an immunotoxic property in rats and possibly in humans and it was classified as carcinogenic for humans (group 2B, probable human carcinogen). OTA is receiving increasing attention worldwide because of its wide distribution in food and feed and human exposure that most likely comes from low level of OTA contamination of a wide range of different foods (Petzinger & Weidenbach, 2002). The economically most important OTA producers belong to *Aspergillus* sections *Circumdati* and *Nigri* (Frisvad et al., 2004).

OTA is efficiently absorbed from the gastrointestinal tract, mainly in the small intestine. Information from a number of species shows that it is distributed via the blood, mainly to the kidneys, with lower concentrations found in liver, muscle and fat. Specific transporters may be involved in the cellular uptake of ochratoxin A into the kidney, where it accumulates (Sorrenty et al., 2013). OTA can have several effects such as nephrotoxic, hepatotoxic, neurotoxic, teratogenic and immunotoxic on several species of animals, and can cause kidney and liver tumors in mice and rats (El Khoury et al., 2010). OTA is able to induce overproduction of nitrogen monoxide (NO), both in kidney and liver, resulting in increased nitrite and nitrate levels.

Under normal conditions, nitrogen monoxide presents a broad range of biological activities; conversely, in excess, it may behave as a toxic radical (Sorrenti et al., 2013).

2.12.1. Carcinogenecity

Ochratoxin A is potentially carcinogenic to humans (Group 2B, probable human carcinogen), and has been shown to be weakly mutagenic, possibly by induction of oxidative DNA damage and produced renal adenomas and carcinomas in male mice and in rats (carcinomas in 46% of males and 5% of females). In humans, very little histology data are available, so a relationship between ochratoxin A and renal cell carcinoma has not been found. However, the incidence of transitional cell (urothelial) urinary cancers seems abnormally high in Balkan endemic nephropathy patients, especially for the upper urinary tract (Basic-Jukic et al., 2007).

2.12.2. Neurotoxicity

Ochratoxin A has a strong affinity for the brain, especially the cerebellum (Purkinje cells), ventral mesencephalon, and hippocampal structures (Belmadani et al., 1999). The affinity for the hippocampus could be relevant to the pathogenesis of Alzheimer's disease, and subchronic administration to rodents induces hippocampal neurodegeneration. Ochratoxin causes acute depletion of striatal dopamine, which constitutes the bed of Parkinson's disease, but it did not cause cell death in any of brain regions examined (Sava et al., 2005). Teams from Zheijiang Univ. and Kiel Univ. hold that ochratoxin may contribute to Alzheimer's and to Parkinson's diseases. Nonetheless, their study was performed *in vitro* and may not extrapolate to humans (Zhang et al., 2009). The developing brain is very susceptible to ochratoxin A, hence the need for caution during pregnancy (Doi et al., 2011).

2.12.3. Immunosuppression and immunotoxicity

Ochratoxin A can cause immunosupression and immunotoxicity in animals. The toxin's immunosuppressant activity in animals may include depressed antibody responses, reduced size of immune organs (such as the thymus, spleen, and lymph nodes), changes in immune cell number and function, and altered cytokine production. Immunotoxicity probably results from cell death following apoptosis and necrosis, in combination with slow replacement of affected immune cells due to inhibition of protein synthesis (Al-Anati et al., 2006).

2.12.4. Nephrotoxicity

As a nephrotoxic mycotoxin, ochratoxin A is considered to be associated with Balkan endemic nephropathy, the chronic interstitial nephropathy of unknown origin prevalent in Tunisia, and thus also with the formation of kidney tumors in man. The compound causes kidney tumours in mice and rats and is nephrotoxic in mammals (Shankar, 2010). In Balkan endemic nephropathy (BEN), renal biopsy shows acellular interstitial fibrosis, tubular atrophy, and karyomegaly in proximal convoluted tubules (Djukanovic et al., 2010). A number of descriptive studies have suggested a correlation between exposure to ochratoxin A and BEN, and have found a correlation between its geographical distribution and a high incidence of, and mortality from, urothelial urinary tract tumours (Castegnaro et al., 2006). However, insufficient information is currently available to conclusively link ochratoxin A to BEN.The toxin may require synergistic interactions with predisposing genotypes or other environmental toxicants to induce this nephropathy (Abouzied et al., 2002).

The binding of ochratoxin A to a serum macromolecule of low relative molecular weight may be relevant for the nephrotoxic effects in mammals, as these small molecules can easily pass the normal glomerular membrane, which can lead to the accumulation of ochratoxin A in the kidneys.Research data reveal that OTA is not acting as a direct genotoxic carcinogen and that oxidative stress is implicated in the genotoxicity and cytotoxicity observed in these human renal cells as well as in rat liver and kidney, covalent DNA adducts have been observed and related to OTA biotransformation (Sorrenti et al., 2013)

2.12.5. Detoxification/decontamination of Ochratoxin A

Physical, physicochemical, chemical and biological methods have been developed to reduce and/or eliminate the toxic effects of contaminated products. The process of detoxification is often accompanied with a loss of palatability and nutritional values. The addition of nutrients or additives which has the property of antioxidants counteract the adverse effects of oxygen radicals generated by OTA is one approach that reduces the toxicity of mycotoxins (Sorrenti et al., 2013)

2.12.5.1. Physical methods

Methods used for mycotoxin detoxification include cleaning, mechanical sorting, and separation (e.g., filtering), heat treatment, ultrasonic treatment, and irradiation. During the cleaning process of contaminated food products, dust, husks, hair, and shallow particles are separated from the product. During mechanical sorting and separation, the clean product is separated from mycotoxin-contaminated food products, while washing procedures using water or sodium carbonate solution can also result in some reduction of mycotoxins.(Krogh et al, 1974) Ensiling was found to reduce the OTA content of barley (Rotter et al, 1990). The scouring of wheat can lead to a reduction of more than 50% in OTA concentration, while milling hard wheat to produce white flour resulted in an approximately 65% reduction, and a further 10% decrease occurred during baking (Osborne et al, 1996) OTA is generally stable at temperatures used during ordinary cooking. However, due to the high temperatures used for coffee roasting, a higher percentage of destruction was observed, although contradictory results from different studies have been reported. Initial studies on the influence of the roasting process on OTA levels indicated a reduction of 77–87% (Levi et al, 1974), 80–90% (Gallaz et al, 1976) and 90–100% (Micco et al, 1989), although opposing values of 0–12% (Tsubouchi et al, 1987) and 2–28% (Studer-Rohr et al, 1994) have also been published

2.12.5.2. Physicochemical methods

Another approach for removing mycotoxins from contaminated agricultural products involves the use of adsorbent materials with the capacity to tightly bind and immobilize mycotoxins. Adsorbing agents can be classified into different groups based on their origin: minerals (e.g., aluminosilicates), activated coals, biological adsorbents (e.g., yeast and bacterial cell walls or vegetal fibers), and synthetic adsorbents, including modified natural clays (e.g., grafting of quaternary ammonium groups) and synthetic resins (e.g., polyvinylpyrrolidone, cholestyramine). (Visconti et al, 2008, Castellari et al, 2001).

2.12.5.3. Chemical methods

A wide variety of chemicals have been found to be effective in destroying mycotoxins. The chemicals used include various acids, bases, oxidizing agents, chlorinating or reducing agents, salts, and miscellaneous reagents, such as formaldehyde. Ammoniation is the method that has received the most attention for detoxification of aflatoxin- or ochratoxin-contaminated feeds and has been used successfully in several countries (Chelkowski et al, 1982).Treatments with ethyl acetate, dichloro metane, and methylene chloride supplemented with 2% formic acid were found to be able to reduce OTA levels by up to 80% in coffee beans (Heilmann et al, 1999)

2.12.5.4. Microbiological methods

Microbes or their enzymes can be applied for mycotoxin detoxification. Several reports describe the OTA degrading activities of the microbial flora of the mammalian gastrointestinal tract, including the rumen microbes of cows and sheep (Galtier et al, 1976), OTA in feed was estimated to be converted to ochratoxin α (OTα) in cows (Hult et al, 1976). Sheep also have a good capacity to detoxify OTA before it reaches the blood (Kiessling et al, 1984). The enzymes responsible for hydrolysis to OTα in cows and rodents are carboxypeptidase A and chymotrypsin (Pitout et al, 1969).

2.13. Regulatory limits for ochratoxin A in foods

Ochratoxin A represents a well-known hazard to human and animal health. According to FAO (2004), the European Union has set legal limits for OTA levels in imported foods, with a maximum of 5μg/kg (ppb) in raw cereal grains, 3μg/kg in processed cereal foods, and 10μg/kg in dried vine fruits (raisins). As of April 2005, the EU imposed limits for ochratoxins in wine, grape juice and coffee. The limits are 2.0 μg /kg for wine and grape juice 5.0μg /kg for roasted coffee, and 10.0μg /kg for instant coffee.

As a dietary guide, European food safety authority (EFSA) established in 2006 the "tolerable weekly intake" (TWI) of ochratoxin A (on advice of the Scientific Panel on Contaminants in the Food Chain) at 120 ng/kg (Tsigarida, et al., 2009).

Table 4. Regulatory limits for OTA of different commodities in various countries

Country	Commodity	Limit (ppb)	Country	Commodity	Limit (ppb)
Austria	Wheat, rye, durum whet	5[a]	Romania	All foods, all feed stuffs	5
Brazil	Rice, barley, beans, maize	50	Sweden	Cereals	5
Czech Republic	All foods, children's food, infant's foods	20	Switzerland	Cereals	2
Denmark	Cereals, pig kidney	25	The Netherlands	All foods	10
France	Cereals	5[a]	Uruguay	Rice, barley, beans, coffee, maize	50
Greece	Raw coffee beans	20	European Union	Cereals(raw),	5
Israel	Cereals(product) pulse(product)	50[b]		Cereal(products)	3
	grain for feed	50		Dried vine fruit	10

Source: Lin, L-C., et al (2004). a: guideline level, b: Proposed

Limits for green coffee beans were not imposed, but this policy is to be reviewed in 2006. However, a number of European nations have already set limits for ochratoxin A in green coffee beans (FAO, 2005)

Table 5. Maximum limits of ochratoxin A in coffee set by some European countries.

Country	Green coffee (ppb)	Roasted coffee (ppb)	Instant coffee (ppb)
Czech Republic	10	10	10
Finland	5	5	5
Germany	-	3	6
Greece	20	-	-
Hungary	15	10	10
Italy	8	4	4
Netherlands	-	10	10
Portugal	8	4	4
Spain	8	4	4
Switzerland	5	5	5

Source: International Coffee Organization (ICO, 2005)

2.11. Occurrence in food commodities

The fungi responsible for OTA contamination vary from crop to crop and from place to place. Ochratoxin A is produced by *Penicillium verrucosum* in cereal grains in cold climates, by A. carbonarius in grapes, wines and vine fruits, and by *Aspergillus ochraceus* in coffee beans However, *Penicillum verrucosum* has recently been found in cereals in warmer climates: Italy, Spain, France and Portuga As with aflatoxins, ochratoxin contamination of foods is highly variable. Some of the foods mentioned here rarely contain ochratoxin, or typically contain extremely low levels, or both. Variability in OTA production, combined with the large particle size of some of the foods it contaminates, complicates sampling strategies for detection of ochratoxins (Bayman et al., 2006)

2.11.1. Ochratoxin A in coffee

There is a large body of literature documenting OTA contamination of green and processed coffee, and reporting isolation of OTA-producing fungi from coffee, starting in the 1970s (Bayman & Baker, 2006). In tropical zones, OTA is mainly produced in coffee beans by *A. ochraceus, Aspergillus carbonarius* and *Aspergillus westerdijkiae* (section *Circumdati*), which was recently dismembered from *Aspergillus ochraceus*, due to their important OTA production and occurrence (Pfohl-Leszkowicz et al.,1999). As OTA is not significantly reduced by roasting, the final coffee brew could also be an important OTA source in the human diet (Mariana et al., 2010). The occurrence of OTA in coffee beans can be due to climate, length of storage and transportation and processing conditions (wet, mechanical or dry processes) (Pfohl-Leszkowicz et al.,1999).

Coffee is harvested at different moisture contents varying from 50–70% in ripe cherries, 35–50% in coffee raisons and 16–30% in cherries which are dried on the plant. At the end of drying the moisture content must be <12% (approximately 0.65–0.70 aw) to prevent mould contamination and fermentation. Drying regimes and subsequent storage and transport environmental conditions are critical to coffee quality and to potential for OTA contamination. At <9% the coffee looses flavour, while at >13% there is an increasing risk of OTA contamination. A. ochraceus has been predominantly responsible for contamination of coffee. However, a recent study of green coffee from Africa, Asia and America showed that both *A. ochraceus* and *A. niger* section Nigri species were present in these coffee samples (Pardo et al., 2004). They found that the OTA frequency distribution was higher in African samples (5–10 mg/kg), while for Asian and American samples was often <5 mg/kg. Interestingly, the fungal contamination of African samples was not higher than other samples for both Arabica and Robusta samples.

Ochratoxin A contamination of coffee is a worldwide problem. The presence of OTA in green coffee bean has been reported by several authors in wide concentration ranging between 0.2 and 360µg/kg (Taniwaki, 2006). Extensive sampling of green coffee beans of both Arabica and Robusta types worldwide indicated that although OTA contamination is more frequent in some areas including mainly African countries, no producing country was found to be free of contamination (Taniwaki, 2006).

Bayman & Baker (2006), concluded that *Aspergillus ochraceus*, *Aspergillus carbonarius* and *Aspergillus niger* may all be responsible, though most studies have focused on A. ochraceus and neither of the OTA-producing species of *Penicillium* (*P. verrucosum* and *P. nordicum*) have been isolated from coffee. *Penicillum brevicompactum* is common in coffee and is in the same group as the two producing species, but is not an OTA-producer. For coffee, three species or groups of species, all in the genus *Aspergillus*, are of possible significance:

1. ***Aspergillus niger*** is by far the most common, particularly in *Coffea caneophora* (robusta), but OTA production is rare and usually feeble. One study revealed only one producer amongst the seventy isolates tested

2. ***Aspergillus carbonarius*** is generally rare, but there is some evidence that it can be relatively common in certain locations. Most isolates seem to be capable of OTA production in significant amounts, though over a restricted range of environmental conditions;

3. ***Aspergillus ochraceus*** and related fungi are well distributed in coffee production systems and because OTA production is common (about 80% of isolates readily produce OTA), it comprises the most important OTA-producing species in coffee.

3. Materials and Methods

3.1. Description of the study area

The study area was in Jimma zone, Oromiya region, south western Ethiopia. Jimma is located 360km away from Addis Ababa, the capital of Ethiopia. The altitude of the region is from 1650m up to 2000m which is, according to ministry of agriculture of Ethiopia (2000), located with in tepid to cool sub moist mid highlands agro ecological zone and It has a latitude and longitude of $7^0$40'N36^{0}50'E. Coffee is the major cash crop of the Zone, which is produced in the eight woredas namely, Gomma, Manna, Gera, Limmu Kossa, Limmu Seka, Seka Chokorsa, Kersa and Dedo which serves as a major means of cash income for the livelihood of coffee farming families (JZARDO, 2008). The harvest period is from November until January. Mana, Gomma and Limu kossa were the study weredas. During November, 2013, Coffee Samples were collected from selected weredas, Mana, Gomma and Limu kossa. These weredas were purposefully selected by their coffee production capacity based on information obtained from Jimma zone agricultural bureau.

Limu (Limu kossa)

The altitude of the Wereda is from 1400m to 2100m and harvest period is from November until January. It has a latitude and longitude of8^0 10'N 37^0 10' E. 34.9% of the land is arable or cultivable (24.6% was under annual crops), 20% pasture, 39.7% forest, and the remaining 15.4% is considered degraded or built-up areas. Coffee is an important cash crop of this woreda. Limmu Kosa is bordered on the south by Kersa, on the southwest by Mana, on the west by Gomma, on the northwest by the Didessa river.

Gomma wereda

The altitude of this woreda ranges from 1,380 to 1,680 meters above sea level; however, some points along the southern and western boundaries have altitudes ranging from 2229 to 2870 meters. Harvest period is from November until January. 60.7% of the labd is arable or cultivable (52.7% was under annual crops), 8.1% pasture, 4.6% forest, and the remaining 20.1% is considered swampy, mountainous or otherwise unusable. Coffee is an important cash crop in Gomma; over 50 square kilometers are planted with this crop which is grown under shade trees.

Mana Wereda

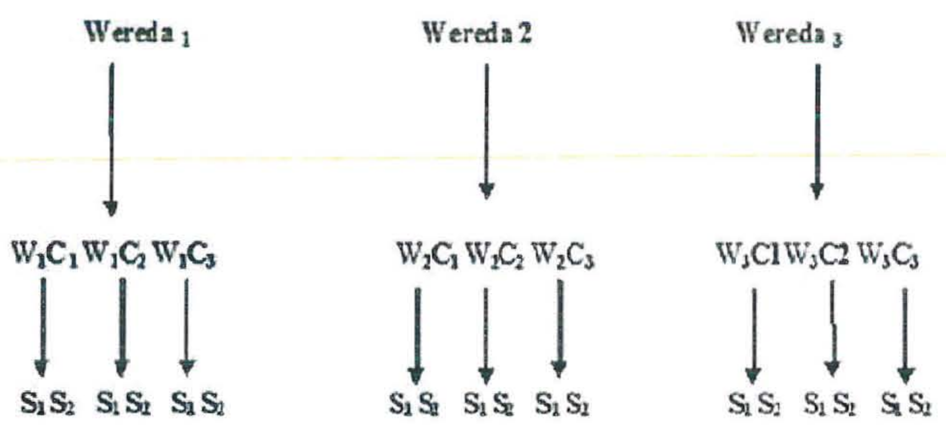
It has a latitude and longitude of 7^0 45' N 36^0 45' Mana is bordered on the south by Seka Chekorsa, on the west by Gomma, on the north by Limmu Kosa and on the east by kersa

3.3. Sample collection

The minimum recommended sample size for ochratoxin A determination in green coffee is 3.0 kg, 100 g incremental sample from 30 sampling points (Coker et al., 1995). From each wereda 3 cooperatives were selected based on ease of access. Each cooperative has more than 2500 members from coffee farmer which channel their coffee to their respective cooperatives. Green coffees processed in dry and wet method each composite sample weighs 3kg (sampling point of 30, 100gm incremental samples) were collected. Each sample was taken with plastic bag covered with paper bag and plastic bag, the later was outer cover to prevent moisture up take from Ice box. All samples were labeled with name of wereda, cooperatives and types of drying process method, and then moved to the laboratory (Bless Agri Food Laboratory Service PLC.) where analysis was conducted and kept in refrigerator under 4^0C until analysis.

Table 6. Sampling location and its respective sample size

Name of District and Kebele or cooperatives		Wet processed	Dry processed
Mana	Afata Wonji	3kg	3kg
	G/Mazorya	3kg	3kg
	Haro	3kg	3kg
Goma	Duromin	3kg	3kg
	Limu Shay	3kg	3kg
	Choche	3kg	3kg
Limu	Dalebo	3kg	3kg
	Ambuyee	3kg	3kg
	Baboo	3kg	3kg



From each cooperatives both dry and wet processed 3kg aggregate samples were taken

Sampling plan, W= Wereda, C= Cooperative, S_1= Dry processed sample, S_2= Wet processed sample

Figure 4. Sampling plan

3.4. Materials and Reagents

Equipments

Centrifuge, Grinder, Refrigerator, Homogeniser/Blender

LC apparatus comprising the following:

Autosampler with valve injection system - 20 μL loop size, mobile phase pump capable of pumping 1 mL/minute, fluorescence detector capable to provide λ = 330 nm excitation and λ = 460 nm emission wavelengths, Reversed Phase (C18) column 150x4.6 mm (length x diameter) with 5 μm particles size (C18) Guard column and Computer based data processing system; with chromatography software

Immunoaffinity columns.

The immunoaffinity column (P/N10515 LCTech GmbH, Germany) contains antibodies raised against ochratoxin A. The column have a maximum capacity of 100 ng of ochratoxin A and give a recovery of 85% when ochratoxin A standard in methanol: 3% sodium bicarbonate (1 + 1, v/v)/PBS solution (4 + 96, v/v) is passed through

Reagents and Media

All reagents were recognized analytical grade.

Acetonitrile, Anhydrous disodium hydrogen phosphate (Na2HPO4), Deionised water. Glacial acetic acid, Methanol, Sodium hypochlorite solution, NaOCl (commercial bleach) 10%, Nitrogen gas (N2) (purity > 99.9%), Potassium chloride (KCl), Potassium dihydrogen phosphate (KH2PO4), Potato Dextrose Agar (PDA), Sodium chloride (NaCl), Sodium hydrogen carbonates (NaHCO3), Toluene and Dichloran rose bengal chloramphenicol (DRBC) agar, Ochratoxin A standard

3.5. Moisture determination of the samples

Moisture of samples was determined by rapid moisture tester instrument Gaucher, model 600 supplied from Witeg, Germany. The instrument was validated by supplier of the instrument and food companies called Nutriset, France. Weighed samples (250 gm) were inserted in to the instrument and the percentage moisture content from the digital reader was recorded as the average moisture content of each sample (Annex 1).

3.6. Isolation of fungi

Isolation of ochratoxin A producing fungi was conducted using direct plating method (as described by Hocking et al., 2006). Analysis of surface disinfection of beans was followed to assess internal invasion of fungi. About 50 gm from each sample were taken aseptically and transferred into 300ml sterile beaker. The samples were covered with 10% NaOCl solution (commercial bleach) for 2 minutes and rinsed with sterile distilled water for 1 minute after the chlorine decanted, after that, samples dried by using two layers of filter paper in a sterilized Petri dishes.30 beans per sample 5 beans in each petridish were plated on Dichloran Rose Bengal Chloramphenicol (DRBC) agar, a medium known to support the growth of targeted fungi. All plates were incubated at 25 ^{0}C for 5-7 days.

3.7. Identification and enumeration of fungi

All fungi isolates growing from the coffee were enumerated. During the incubation period any emerged fungus was isolated on Potato Dextrose Agar, PDA/Czapk Yeast Extract agar, CYA slants (Klich, 2002) and purified by using the single spore technique. The obtained isolates were identified using the microscopic and cultural characteristic according to Raper and Fennel (1965) and Klich (2002). The results expressed as a percentage of infected coffee (% infection) and as a frequency percentage of group/genus (% frequency).

The frequency of isolation of fungal genera and group and total fungal infection were computed based on the following formula.

$$Frequency\ (\%) = \frac{Number\ of\ beans\ from\ which\ the\ genus\ isolated}{Total\ number\ of\ beans\ analysed} x100$$

$$fungal\ infection\ (\%) = \frac{Number\ of\ beans\ from\ which\ fungus\ isolated}{Total\ number\ of\ beans\ analysed} x100$$

3.8. Ochratoxin A producing fungal isolates

The method used to extract OTA from fungal cultures in the studies was based on Bragulat et al. (2001) methodology. It was a simple and clean screening method for detecting OTA production by fungi, based on HPLC detection and quantification of OTA in the extracts obtained from agar plugs cuts from pure Petri dish cultures. After seven days of incubation of the possible ochratoxigenic mould on Czapek yeast Auotolysate (CYA) agar at 25 °C, three agar plugs (diameter 5-6 mm) were removed from the inner, middle and outer area of each colony. Plugs were weighted and introduced into 3-ml vials. Methanol (1 ml) was added, and the vials were shaken for 5 seconds. After being left stationary for one hour, the extracts were shaken again, filtered into another vial and stored at 4 °C until the analysis by HPLC with fluorescence detection.

3.9. Extraction and cleanup of ochratoxin A from samples

Extraction and cleanup procedures of OTA in the coffee samples were performed according to the procedure of Suarez-Quiroz et al. (2004). Coffee samples were frozen at -80°C for 24 hours and then milled to pass through a 0.5-mm sieve for efficient extraction of OTA. Twenty five grams of finely blended samples were extracted for 30 min with a methanol/3% solution of sodium bicarbonate (50:50 v/v). The extract was centrifuged at 3000g for 30 min and 4ml of the supernatant diluted to 100ml with phosphate-buffered saline (PH 7.04) and filtered with pleated filter paper (Whatman Nº 4). The filtrate was then passed through freshly drained immunoaffinity column (OtaCLEAN^R). OTA was eluted with 4mL HPLC grade methanol from the column, which was previously washed with distilled water. The eluate was evaporated to dryness under a stream of nitrogen at 70°C and the residue was dissolved in 0.5mL of HPLC mobile phase to be injected into HPLC.

Figure 5. Sample clean up with otaCLEAN™ immunoaffinity column

3. 10. Ochratoxin A determination using HPLC

Condition of HPLC (as described in section 3.4)

Mobile phase used was a mixture of Acetonitrile: Water: Acetic acid (48: 51: 1)

3.11. Running Ochratoxin A standards

Ochratoxin A standard solution (50µg/ml in benzene acetic acid) was purchased from Sigma Aldrich, Germany (Cat N° 49912) and standard stock solution at 40µg/l in toluene: Acetic acid (99:1, V/V) was prepared according to AOAC official method 2004. Accordingly a working standard solution at 10µg/ml was prepared in toluene-acetic acid (99:1). Standard curves of five solutions at a concentration of (2, 5, 10, 20 and 50 μg/l) were prepared in mobile phase to quantify ochratoxin A content in samples.

3.12. Calculation and Expression of Results of Ochratoxin A content

The mass in ng of OTA in the aliquot of test solution injected onto the HPLC column determined from the calibration curve, then calculated the concentration of OTA in the test portion, using the following formulae:

$$PPB_{\text{OTA}} = \frac{ng}{g} = \frac{M_{\text{OTA}}(ng)}{W(g)} = \frac{M_{\text{OTA}}(ng) x V1 x V3}{Ms x V2 x V4} = \frac{M_{\text{OTA}}(ng)}{0.05}$$

Where:

M_{OTA} = mass of OTA (ng) in the aliquot of extract (50 μl) injected into HPLC

W = equivalent weight of test portion injected into the HPLC system (0.05 g),

25 g sample are extracted with 200 mL extraction solvent; 4 mL from the extract are loaded onto column, taken to dryness and then dissolved with 0.5 mL mobile phase; 20 μL are injected into HPLC. Sample equivalent injected into LC is: $25g\, x \frac{4ml}{200ml} x \frac{0.05ml}{0.5ml} = 0.05g$

Ms = mass of test portion (25 g)

V_1 = volume of extraction solution (200 mL)

V_2 = volume of filtrate loaded onto the immune-affinity column (4 mL)

V_3 = volume of HPLC mobile phase used for taking up the dry residue (500 μl)

V_4 = volume of extract injected onto the HPLC column (50 μl)

3.13. Performance verification of HPLC method used (result discussed in section 4.5)

After the establishment of optimal condition of analysis, (Mobile phase, flow rate, wavelength of excitation and emission), some analytical performance of methods were checked. Validation followed the following parameters: Specificity, linearity, detection limit, lower limit of quantitation, accuracy and recovery.

Specificity

In order to study the specificity of the method, Ochratoxin A was identified by injecting a solution of a known concentration (5μg/l of standard solution) in mobile phase. The solvent (mobile phase) used for the preparation of the standard have also been tested.

Linearity

Standard solutions of OTA with concentrations of 2, 5, 10, 20 and 30μg/l in mobile phase were prepared and correlation Coefficient, r was checked.

Limit of Detection and Quantitation

According to AOAC, limit of detection is the lowest content that can be measured with reasonable statistical certainty; but not necessarily quantified and limit of quantitation is the lowest amount of analyte in a sample which can be quantitatively determined with precision and accuracy appropriate to analyte and matrix considered. Accordingly, limit of detection was determined using the lowest concentration of standard solution yielding a signal to noise ratio, S/N=3, and was confirmed by analyzing five standard solution of this concentration. The limit of quantitation was calculated based on detection limit and percent recovery.

The signal-to noise ratio was determined by the equation:

$S = H/h$

Where:

H: height of the peak corresponding to the component

h = absolute value of the largest noise fluctuation from the baseline of the chromatogram of a blank solution.

Accuracy

According to codex alimentarius (2004) and ISO (1994), accuracy is defined as the closeness of agreement between the reported result and the accepted reference value (certified reference materials); accordingly, the accuracy of the method was confirmed by running standard reference material (SRM). The SRM, ochratoxin A in instant coffee (T17116QT) was purchased from Food

Analysis Performance Assessment Scheme (FAPAS), proficiency testing provider for the food, united kingdom (UK) and determination of its ochratoxin A content was analysed using the entire procedures.

Percent recovery of standards

The recovery of standards was processed with every batch of sample. Ochratoxin A standard at a concentration of 5µg/l was added to 25g of sample which was previously determined its ochratoxin A content; analysis was done by using the entire procedure. The method was evaluated as the percent of recovered concentration recommended by the codex alimentarius.

3.14. Data Analysis

Data were analyzed using simple mean analysis using microsoft excel 2007.

4. Result and discussion

4.1. Moisture content of samples

The moisture content of green coffee beans taken from samples obtained from both dry and wet method of processing was recorded by using digital meter which is presented in Annex 1. The average moisture percentage obtained were compared with the standard moisture range (8-12.5%) recommended by international coffee organization. The result of moisture content revealed that the moisture content of green coffee beans obtained from both dry and wet processing method was above the recommended standard. The mean moisture content of dry and wet processed green coffee beans was 13.07% and 13.57% respectively.

Comparison of moisture content between dry processed and wet processed method showed that the moisture content of wet processed coffee was slightly higher than dry processed. Out of the total samples of wet processed coffee, 88.88% (in the range of 12.90%-14.80%) and of the total samples of dry processed coffee, 77.77% (in the range of 12.90%-14.7%) were found to be above the recommended range. The higher moisture content of green coffee was may be the result of inefficient drying conducted by producers. The moisture content that is higher than the recommended range in coffee is suitable for fungal infestation.

4.2. Mycological analysis and isolation of ochratoxin A producing fungi.

The mycological analysis was performed so as to enumerate and identifying fungi colonizing green coffee beans and to determinate the frequency of potential OTA producing fungi in wet and dry processes. Their ability to produce OTA was also studied. Subsamples of green coffee beans, dry and wet processed were directly plated on DRBC agar after disinfection was done by 10% sodium hypochlorite solution (commercial bleach). After 7 days incubation at 25 ^{0}C, growth of different types of fungi was observed as presented in Figure 6.

Figure 6. Some mixed culture on DRBC agar

The results expressed as percentage of fungal occurrence of different types and percentage of relative dominance among different fungal group (Table 7. and Fig 7). The moulds detected were isolated and sub cultured on Czapek–Dox agar for identification purposes. Predominant moulds were identified according to the identification of synoptic keys (Raper and Fennel, 1965). The fungal genera or group isolated presented in Table 8.

Table 7. Fungal genera or group isolated from total samples

Species	Frequency (%)		Number of Isolates	
	Dry	Wet	Dry	Wet
Aspergillus flavus	29.63	30.74	80	83
Aspergillus niger	40.74	42.59	110	115
Aspergillus chraceus	30	29.26	81	79
Penicillum spp	29.26	26.66	79	72
Fussarium spp	21.11	23.7	57	64
***Rhyzopus* and other undefined spp**	29.26	28.52	79	77
Total			486	490

Results obtained revealed an overall percentage of fungal contamination between 76.67%-100% and 73%-93% in dry and wet processed coffee, respectively. These corresponding to the analysis of 270 coffee beans sample from both drying method. *Aspergillus carbonarius*, one of ochratoxin producing fungi was not isolated in all coffee samples.

There was no drying process method differences in composition of ochratoxigenic species present in three sites. Coffee samples from dry processed had a fungal incidence of 29.63% *Aspergillus flavus*, 40.74% *Aspergillus niger*, 30% *Aspergillus ochraceus*, 29.26% *Penicillium species*, 21.11% *Fussarium* species and 29.26 *Rhyzopus* and other unidentified species. While those of wet processed had a fungal incidence of 30.74% Aspergillus flavus, 42.59% Aspergillus niger, 29.26% Aspergillus ochraceus, 26.66% Penicillium species, 23.70% Fussarium species and 28.52% Rhyzopus and other unidentified species. There was no fungal diversity in types of drying process. The least fungal occurrence in both processed coffee was *Fussarium* species with 23.7% in wet and 21.11% in dry processed.

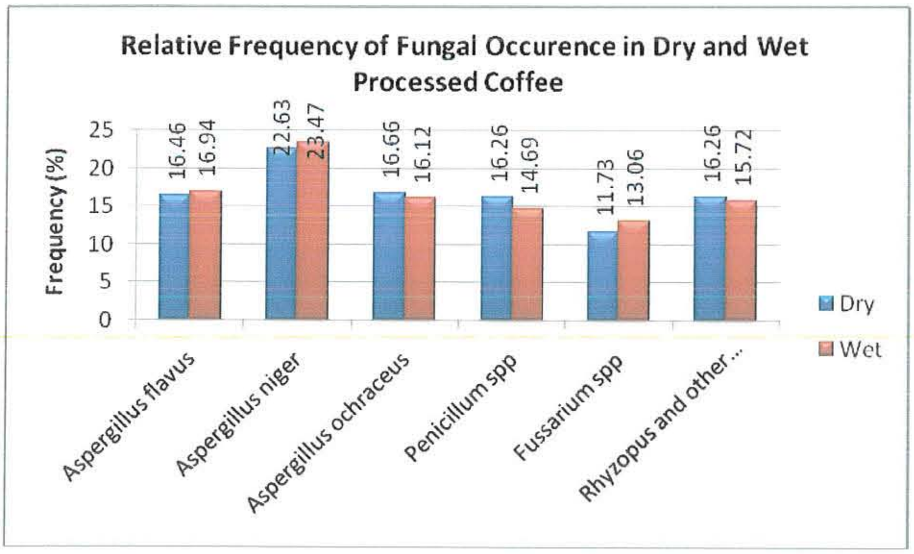


Figure 7. Relative frequency of fungal occurence

The result showed that *Aspergillus niger* was the most predominant in both types of processed coffee with the relative frequency of 22.63% and 23.47% in dry and wet processed respectively. Higher level of infection with *Aspergillus flavus, Aspergillus niger* and *Fussarium* species were observed in wet processed where as the infection with *Aspergillus ochraceus, Penicillum* species and *Rhyzopus* with other unidentified species were higher in dry processed coffee samples

Comparison of our result with other similar research work by Leong, et al, (2007) in Vietnamese green coffee shows congruence in fungal infestation of coffee with different groups of fungi and in the predominance of species. From 65 samples of robusta coffee and 11 samples of Arabica coffee analyzed, hundred percent of coffee beans in all samples were infected with one or more fungi, with *Aspergillus niger* being the dominant species with 65/65, 100% in Robusta and 11/11, 100% in Arabica coffee beans. The difference in percent infection by *Aspergillus niger* in arabica coffee could possibly be from duration. Our analysis was done on freshly harvest coffee sample in contrary to the other research work in that samples were taken from 5 months stored coffee. Fungal infestation would be increased if the storage condition was not kept under the recommended situation. *Aspergillus carbonarius* was less commonly isolated from arabica coffee (1/11, 9%) in the same research which was in agreement with our findings in that no *Aspergillus carbonarius* was isolated in both types processed coffee beans.

Similar study from Ethiopia by Abrham T. (2006), showed highest infection of coffee by *Aspergillus niger* with the range of 28.3% - 39. 9% and lower infection by *Fussarium* species with the range of 8.5%-12.8%.The difference in total percent infection and those of the dominant species, *Aspergillus niger*, could possibly be from the sample size taken. Our aggregate sample size was 3 kg (100gm incremental, 30 sampling points) in contrary to the other research in which 0.5kg was taken for a single sample. Similar studies by Nogaim, et al (2013), in Brazilian green coffee, from the total of 70 samples (unknown size of aggregate sample), the percentage of fungal infection was 41.85% as an average, and here again dominant species were *Aspergillus niger* with 27.5. %. The size of sample may determine the chance of getting fungal infected coffee.

4.3. Study of ochratoxin A producing isolates, *Aspergillus niger* and *Aspergillus ochraceus*.

The result showed that from a total of 54 isolates (Table 8), 36 *Aspergillus niger* and 18 *Aspergillus ochraceus*), only 4 isolates (7.14%) were capable of producing ochratoxin A with the range of concentration of 2.04-3.32μg/kg. From a total of 36 isolates of *Aspergillus niger*, only 8.33% (3/36) were capable of producing ochratoxin A and only 5.55% (1/18) of *Aspergillus ochraceus* were capable of producing ochratoxin A. No *Aspergillus niger* isolated from dry processed produced ochratoxin A and no *Aspergillus ochraceus* isolated from wet processed produced ochratoxin A.

Table 8. OTA content from ochratoxin A producing fungi isolates

Location	sample code	Number of isolates tested		Capable of OTA in *Aspergillus niger*	Capable of OTA in *Aspergillus ochracus*	OTA (ug/kg)
		Aspergillus niger	*Aspergillus ochraceus*			
Mana	DCA-1	2	1	0	0	ND
	DCA-2	2	1	0	0	ND
	DCA-3	2	1	0	0	ND
Gomma	DCA-7	2	1	1	0	3.32
	DCA-8	2	1	0	0	ND
	DCA-9	2	1	1	0	2.35
Limmu	DCA-13	2	1	1	0	2.63
	DCA-14	2	1	0	0	ND
	DCA-15	2	1	0	0	ND
Mana	WCA-4	2	1	0	0	ND
	WCA-5	2	1	0	0	ND
	WCA-6	2	1	0	0	ND
Gomma	WCA-10	2	1	0	0	ND
	WCA-11	2	1	0	0	ND
	WCA-12	2	1	0	1	2.04
Limmu	WCA-13	2	1	0	0	ND
	WCA-14	2	1	0	0	ND
	WCA-15	2	1	0	0	ND

The presence of ochratoxin A producing fungi is not necessarily mean that there will be ochratoxin A. It needs further research to address the factors that trigger the ochratoxin producing fungi to produce toxins.

When we compare our result with reported data by Abrham, T. (2006) from a total of 66 isolates of *Aspergillus ochraceus* from dry and wet method, 12 (18.18%) were capable of producing ochratoxin A and it was not found ochratoxin A producer among black fungi (*Aspergillus niger*). Similarly, in the same research it was not found the black fungi capable of producing ochratoxin A as we could not find the same in dry processed coffee beans. It was also found *Aspergillus* *ochraceus* capable of producing ochratoxin A in lower percentage (18.18%) in the other research. The same fungi were found in our research in lower percentage (5.55%). The other reported data by Taniwaki et al, (2003) on Brazilian arabica green coffee showed *Aspergillus niger* was the species found most commonly (63 % of potential OTA producers), but only 3 % of them produced OTA. *Aspergillus ochraceus* that was also occurred (31 % of isolates), and 75 % of those studied were capable of OTA production, a much higher percentage in *Aspergillus ochraceus* that produced OTA than reported in our research. However, our study was in harmony in the predominant species, *Aspergillus niger* and with its lower percentage OTA production (3%) from total isolates. There may be factors that support the growth of some fungi and suppress the growth of others. There may also be factors that trigger ochratoxin producing fungi to produce toxin (ochratoxin A).

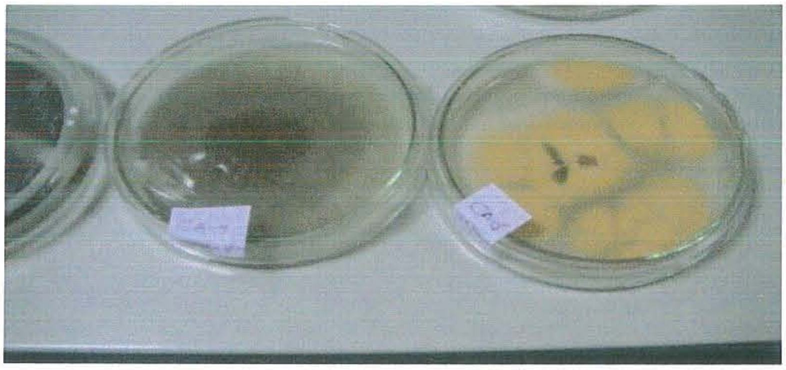


Figure 8. Some isolates of Aspergillus niger (left) and Aspergillus ochraceus (right)

4.4. Ochratoxin A content from total samples

The result showed that from the total of 18 samples analyzed (each sample was extracted and analyzed in duplicate for ochratoxin A content), 44.44% (8/18) were OTA contaminated. As seen on Fig 9,. out of 9 coffee samples from dry processed, 55% (5/9) were contaminated with OTA in the range between 3.42µg/kg and 6.46 µg/kg and Out of 9 coffee samples from wet processed, 33% (3/9) were contaminated with OTA level ranged between 2.74µg/kg and 3.04µg/kg. Higher level of contamination was observed in coffee samples obtained from dry processed with level of contamination of 6.46 μg/kg and 5.62% that were above European regulatory limit (5μg/kg).

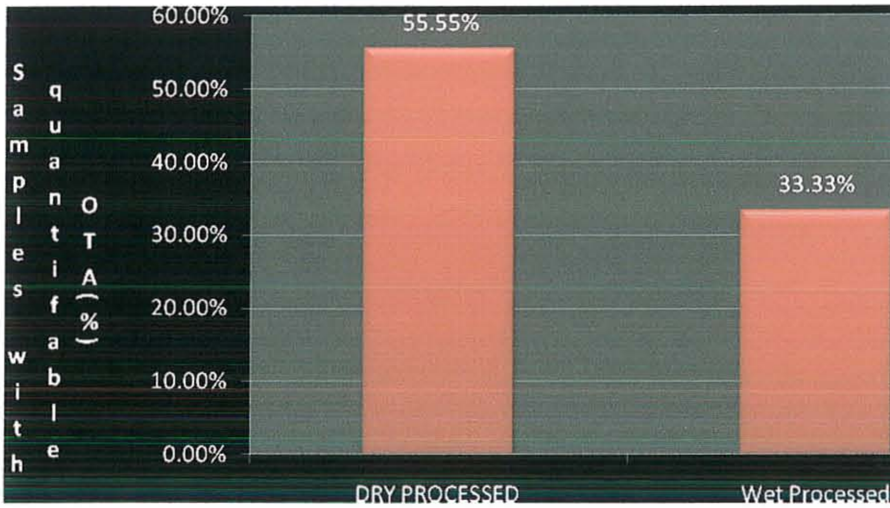


Figure 9. Incidence of contamination of coffee samples with OTA at quantifiable levels

Table 9. OTA content in total samples

Ser N°	OTA content (μg/kg)	
	Dry	Wet
1	ND	ND
2	ND	2.91
3	ND	ND
4	3.42	ND
5	4.22	ND
6	6.46	3.04
7	5.62	2.74
8	4.64	ND
9	ND	ND
Mean	2.71	0.97

ND=Not detected, LOD= 0.5 LOQ=0.6

The contamination level of OTA ranged between 2.74μg/kg and 6.46μg/kg. Table 9. shows the results obtained from analysis of OTA content in coffee samples from the two drying method. Out of 8 samples contaminated, 62.5% (5/8) were from coffee samples processed in drying method and 37.5% (3/8) were from coffee samples processed in wet method.

OTA levels were mostly below the recommended standards by EU (5μg/kg). There was no difference in OTA contamination between wet and dry processing while the mean value is 2.71μg/kg and 0.97μg/kg in dry and wet processed respectively.

Comparing this study result with other reported data by Nganou, N.(2014),the average OTA content of the coffee grains issued via dry processing, fall within the ranges of 0.3 and 5.11 μg/Kg and between 0.11 to 10.1 μg/Kg for the year 2009 and 2010 respectively. With regard to the wet processing, the average OTA content were situated between 0.12 and 124.1 μg/kg for the year 2009, and between 0,1 and 3.85 μg/kg for the year 2010. The result showed that treatment by wet processing had shown a much higher contamination in OTA than by the dry process which is contrary to our result. However our study result was in harmony with other researcher explanation by Suárez-Quiroz et al (2004), the difference in OTA contamination between various processes of coffee processed in dry, mechanical and wet. The differences were explained by the fact that the lowest decrease observed for the mechanical processing was due to the removal of the external layer rich in toxin, whereas the fermentation process much moist and of a long duration can favor the penetration of toxins into the parchment. On the contrary, in the course of drying process, the increase in the OTA content can only be explained by a neosynthesis from the toxicogenic contaminating layer.

4.5. OTA content in samples in relation to different international standard

The upper limit of ochratoxin content for green coffee set by different European countries;20 μg/kg (Greece) and 5μg/kg (Finland and Switzerland), while European regulatory limits (5μg/kg) is the maximum. Hundred percent of contaminated samples were below 20μg/kg and Only two samples 20% of contaminated samples obtained from dry processed method and from different localities (Gomma and Limu saka) were 6.46µg/kg and 5.62 µg/kg, respectively showed contamination with OTA above European regulatory limits (5µg/kg).

4.6. Result from method validation

4.6.1. Specificity

In order to study the specificity of the method, Ochratoxin A was identified by injecting a solution of a known concentration (5µg/l of standard solution) in mobile phase. The retention time for OTA standard was determined, 10.5 minutes. The solvent (mobile phase) used for the preparation of the standard have also been tested and no interference were observed to the retention time corresponding to OTA.

4.6.2. Linearity

The linearity of the methods was measured by calculating correlation coefficient r^2 of concentration versus peak area standard solutions of five different concentrations (2, 5, 10, 20, 30, ppb). The result of correlation coefficient was determined from five point standard curve showed that 0.9978, as shown in Figure 10.

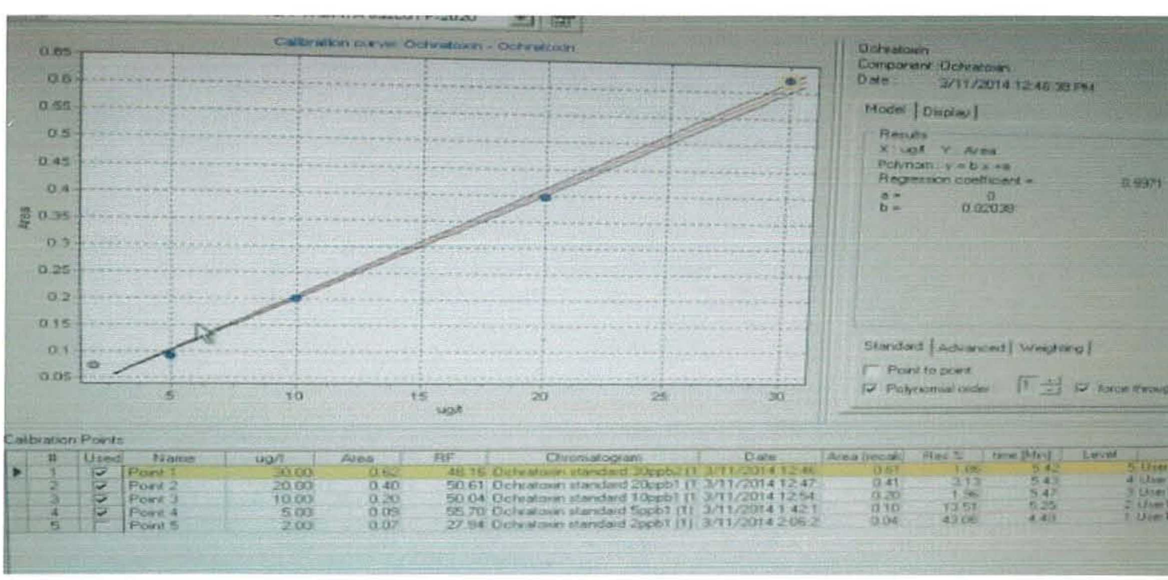


Figure 10. Calibration curve

4.6.3. Limit of detection (LOD) and limit of quantitation (LOQ)

The detection limit was determined by the concentration yielding a signal-to noise ratio of 3:1 and is confirmed by analyzing the lowest concentrations. It was quantified using the equation S = H/h. the results showed that the minimum concentration of the instrument that detected without noisy peak interference was 0.5µg/l and The limit of quantitation was determined by taking the limit of detection (0.5 µg/l) and the percent recovery data (80%) and was found to be 0.6 µg/kg.

4.6.4 Accuracy

Accuracy was determined by running standard reference material (SRM) with assigned value (8.15µg/kg) and range of value (3.1-8.45). The SRM was tested using the entire procedure and measured to be 6.52µg/kg (Plate

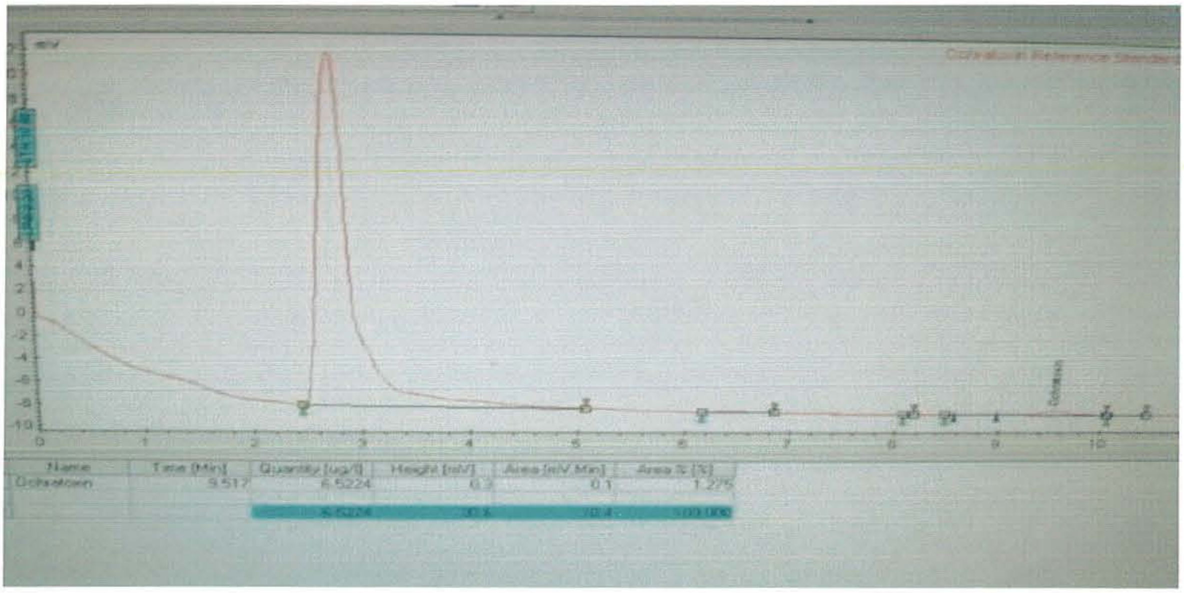


Figure 11. Chromatogram peak from OTA determination of standard reference material

4.6.5. Percent recovery

Ochratoxin A standard at a concentration of 5µg/l was added to 25g of sample which was previously determined its ochratoxin A content as ND (Not Detected); and the analysis was performed using the entire procedures. The recovery rates obtained from five replicates of spiked samples ranged from 79.8% - 81% the result showed that average percent recovery, 80% (Table 10.) which is similar to the recovery results of 64-89% obtained by Lombaert et al. (2002) in coffee samples

Table 10. Percent recovery

	Replication					
Spiking level	**1**	**2**	**3**	**4**	**5**	**Average**
5µg/l	3.99	4.11	3.89	3.96	4.05	4
% Recovery	79.8	82.2	77.8	79.2	81	80

5. Conclusion and recommendation

5.1. Conclusion

The mean moisture content of dry and wet processed green coffee beans was 13.07% and 13.57% respectively, which was above the recommended moisture content of coffee (12%). The moisture content of coffee samples was suitable for growth of moulds and hence the formation of OTA as the high level of fungal infection was observed in both dry and wet processed coffee samples. The time of invasion of coffee by toxigenic fungi is of great importance for the development of OTA in coffee. Inadequate sun drying of coffee leads to OTA formation. The drying stage is the most favorable time for the development of OTA. Therefore, a critical control point could be at the end of the drying process and one critical limit could be the water content.

In samples obtained from the two types of drying method, the three most frequent occurrences of fungi were *Aspergillus niger, Aspergillus flavus* and *Aspergillus ochraceus* with *Aspergillus niger* the predominant species. From 54 isolates of ochratoxin A producing fungi (*Aspergillus niger and Aspergillus ochraceus)*, only 4 isolates were capable of OTA producing ranged between 2.04μg/kg to 3.3μg/kg. It was observed that when a toxigenic strain was isolated from a coffee sample, the sample may not contain OTA. This phenomena suggested that the presence of ochratoxin producing strains is not necessarily to mean that the risk of OTA presence.

From this study it can be concluded that the important fungi with the potential to produce OTA in this region coffee beans were *Aspergillus ochraceus* and *Aspergillus niger*. These two species were predominant in coffee samples obtained from the two types of drying method (dry and wet processed).

Out of 8 OTA contaminated samples, 50% of the samples were obtained from the same locality (Gomma) suggested that coffee samples processed in dry method in this locality were more contaminated than wet method; because all samples obtained from dry method were contaminated with OTA ranged between 3.42μg/kg to 6.46μg/kg, where as only one sample obtained from wet method in this same locality was contaminated with OTA in the level of contamination 3.04μg/kg. OTA was not detected from all coffee samples processed in dry method obtained from Manna and only one samples processed in wet method obtained from this same locality contaminated with OTA in the level of 2.91μg/kg which is much bellow the EU regulatory limit (5μg/kg). Therefore, it can be concluded that there are good drying practices in this locality. OTA was detected from two samples processed in dry method obtained from Limu saka, of which one sample contamination level was 5.62μg/kg which is above EU regulatory limit and one sample processed in wet method obtained from this locality was OTA detected in the contamination level of 2.74μg/kg.

It can also be concluded that Ethiopian green coffee samples (in this region) (*Coffea arabica* L.) under this investigation shows a highly grade of quality. Because though the percentages of fungi infection in coffee samples were high and the toxigenic fungi also was high within these fungi, the occurrence of ochratoxin A was very low comparing with the other results by many researchers in different countries. In this research, the contamination level ranged between 2.74μg/kg and 6.46μg/kg and only two samples 6.46 μg/kg (DCA-9) and 5.62% (DCA-13) out of 18 samples showed contamination with OTA above European regulatory limits (5μg/kg).

5.2. Recommendation

Prevention is currently the only available effective way at farm level to combat OTA although it should be noted that the removal of mouldy cherry, or the reprocessing of moldy coffee, does not guarantee that the clean coffee bean will be free from micro particles or spores.

Adequate drying to uniformly low moisture levels and avoiding local wet spots, caused by uneven drying, rewetting or condensation, is crucial in prevention. Simple and cheap devices for solar drying of coffee can be of great help in improving drying practices, including prevention of rewetting by rain or dew.

Turning and stirring of coffee is essential to promote uniform drying. When harvest coincides with rainy or high humidity season, measures to optimize drying must be adopted. The factors that enhance ochratoxin A producing fungi to form toxin should further be researched.

As the evidence shows that ochratoxin A is formed in raw coffee beans after harvest. OTA contamination can clearly be minimized by following good agricultural practice and good manufacturing practices and hygiene throughout the coffee production and processing chain is highly recommended in order to reduce the risk of contamination of processed coffee.

General strategies to reduce and prevent OTA formation in coffee include the implementation of Good Agricultural Practices (GAP) during pre harvesting and harvesting period, and control moisture and temperature during post harvesting period and storage.

The level of OTA in coffee produced in other regions of the country should be studied. In addition to green coffee beans, the level of OTA in roasted and instant coffee traded in the country should be researched in order to assess the health risk of the nation.

Many countries set maximum limit for OTA contamination in different food commodities including coffee. The government of Ethiopia should impose regulation on maximum limit of OTA in not only coffee but also other food commodities so as to ensure that the required health status of the nation is maintained.

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Appendices

Appendix 1: Moisture content of samples at the time of sampling

Sample code	Name of Wereda	Name of cooperatives	Type of Drying process	Moisture (%)
DCA 001	Mana	Af Wonji	Dry	14.00
DCA002		Harro	Dry	13.60
DCA003		Geroke Mazoria	Dry	11.70
WCA004		Af Wonji	wet	14.80
WCA005		Harro	wet	13.50
WCA006		Geroke Mazoria	wet	14.00
DCA007	Gomma	Dromina	Dry	13.40
DCA008		Limu shay	Dry	14.70
DCA009		Chochea	Dry	13.00
WCA010		Dromina	wet	13.40
WCA011		Limu shay	wet	14.70
WCA012		Chochea	wet	14.00
DCA013	Limu	Dalebo	Dry	12.90
DCA014		Ambuyee	Dry	11.20
DCA015		Baboo	Dry	13.20
WCA016		Dalebo	wet	12.90
WCA017		Ambuyee	wet	11.20
WCA018		Baboo	wet	13.20

Appendix 2: Fungal isolates from dry processed beans on DRBC agar

Sample code	Number of beans analysed	Types of isolates						No of infected beans	% infected beans
		A. flavus	A. niger	A. ochracus	Penicillum Spp	fussarium	Rhyzopus & others		
DCA001	30	11	14	7	7	7	8	28	93.33
DCA002	30	7	11	7	9	3	8	23	76.67
DCA003	30	8	11	6	6	6	8	24	80.00
DCA007	30	10	12	10	9	6	8	27	90.00
DCA008	30	8	14	11	10	5	10	30	100.00
DCA009	30	9	13	10	9	7	10	27	90.00
DCA013	30	8	12	10	9	6	9	26	86.67
DCA014	30	9	12	11	11	8	9	28	93.33
DCA015	30	10	11	10	9	9	9	26	86.67
Total	270	80	110	81	79	57	79	239	796.67
Mean per plate	30	8.90	12.22	9.00	8.77	6.33	8.77	26.55	88.52

Appendix 3: Fungal isolates from wet processed beans on DRBC agar

Sample code	Number of beans analysed	Types of isolates						No of infected beans	% infected beans
		A. flavus	A. niger	A. ochracus	Penicillum Spp	fussarium	Rhyzopus & others		
WCA004	30	8	12	8	7	6	9	25	83.33
WCA005	30	10	11	7	8	6	8	28	93.33
WCA006	30	9	12	6	8	6	9	25	83.33
WCA010	30	9	11	9	8	1	10	26	86.66
WDCA011	30	9	13	10	9	9	9	22	73.33
WCA012	30	10	16	9	8	9	8	25	83.33
WCA016	30	9	12	10	8	10	9	27	90.00
WCA017	30	10	13	11	8	9	8	27	90.00
WCA018	30	9	15	9	8	8	7	26	86.66
Total	270	83	115	79	72	64	77	231	769.97
Mean per plate	30.00	9.22	12.77	8.77	8.00	7.11	8.56	25.66	85.55

Appendix 4: Summary of results for OTA analysis in Coffee samples

OTA= Ochratoxin A, ND= Not Detected, LOD= 0.5, LOQ= 0.6

Ser. Nº	Sample Code/Drying process/sampling location	Mean (OTA μg/kg)
1	DCA-1/Dry/Afata wonji, Mana	ND
2	DCA-2/ Dry/Haro, Mana	ND
3	DCA-3/ Dry/Gerokee mazorya,Mana	ND
4	DCA-7/ Dry/Dromina, Gomma	3.42
5	DCA-8 /Dry/Limu shay, Gomma	4.22
6	DCA-9/ Dry/Choche, Gomma	6.46
7	DCA-13/ Dry/Dalebo, Limu saka	5.62
8	DCA-14/ Dry/Ambuye, Limu saka	4.64
9	DCA-15/ Dry/Baboo, Limu saka	ND
10	WCA-4/Wet/Afata wonji, Mana	ND
11	WCA-5/ Wet/Haro, Mana	2.91
12	WCA-6/ Wet/Gerokee mazorya,Mana	ND
13	WCA-10/ Wet/Dromina,Gomma	ND
14	WCA-11/ Wet/Limu shay, Gomma	ND
15	WCA-12/ Wet/Choche,Gomma	3.04
16	WCA-16/ Wet/Dalebo.Limu saka	2.74
17	WCA-17/ Wet/Ambuye, Limu saka	ND
18	WCA-18/ Wet/Baboo, Limu saka	ND

Declaration

I, the under signed, declare that this thesis is my original work. It has never been submitted to any institution and that all sources of material used for this thesis have been dully acknowledged.

.

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