ADDIS ABABA UNIVERSTIY SCHOOL OF GRADUATE STUDIES

A STUDY ON OCHRATOXIN A AND TOXIGENIC FUNGI ON COFFEE AND SELECTED CEREAL GRAINS CONSUMED IN ETHIOPIA

BY

TESHOME GEREMEW

SUPERVISOR

DAWIT ABATE

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Teshome Geremew Biru

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Approved by Examining Board:

1. Dr. Dawit Abate (Advisor) _____
2. Prof. John Wagacha Maina (External Examiner) _____
3. Dr. Tesfay Alemu (Internal Examiner) _____
4. Dr. Gurja Belay (Chairman) _____

Addis Ababa University
Department Of Microbial Cellular Molecular Biology
College Of Natural Sciences

Signature page

GENERAL ABSTRACT

Ochratoxin A (OTA) is toxic fungal secondary metabolite produced by some filamentous fungi belonging to the genus *Aspergillus* and *Penicillium*. This mycotoxin is commonly found in coffee and cereal grains and total removal of the toxin by various food processing methods is not possible. The aim of this research is to study ochratoxin A and toxigenic fungi in coffee and cereal grains. This dissertation contains five major chapters in which background information, the rationale for the study and research objectives are presented in the first chapter. The second chapter is dedicated to reviewing the available literature on the properties of OTA, the producing fungi, occurrence of OTA in foods, biosynthesis of OTA, analytical methods, toxicity in human and animals, mechanisms of action and prevention strategy. This section also addresses production and processing of coffee and some selected cereal grains. The remaining three chapters are the main experimental part of this study. The third chapter focuses on the occurrence of toxigenic fungi and OTA in Ethiopian coffee for local consumption. In this chapter, fungal incidences in coffee samples and OTA contamination levels in coffee were evaluated using various mycological techniques and ELISA method for toxin analysis. The identities of fungal isolates were revealed using species-specific primers and DNA sequencing techniques. The result indicated that total fungal incidence mounted up to 87%. The predominant fungal genera were *Aspergillus* (79 %), *Fusarium* (8 %) and *Penicillium* (5 %). Ochratosin A producing species of *A. westerdijkiae*, *A. ochraceus*, *A. melleus*, and *A. steynii* were identified for the first time using molecular techniques from locally sold coffee in Ethiopia. *A. westerdijkiae* and *A. ochraceus* were found to be the predominant OTA producers. The median OTA level in the locally sold Ethiopian coffee was 1.53 µg/kg. Although samples from wet processing resulted in low

median levels of OTA, they were not significantly different from other processing types ($p < 0.306$). Significant differences in fungal incidences were observed between the different coffee processing types ($p < 0.001$), coffee sample types ($p < 0.005$), and storage materials ($p < 0.03$). An *in vitro* assessment of OTA and Aflatoxins (AFs) production potential of all *Aspergillus* isolates reviled that *A. westerdijkiae* isolates were clearly the most potent producers of OTA while AFs were only produced by two *A. flavus* isolates. This chapter is published in *Food Control 69 (2016) 65-73*. The fourth experimental chapter deals with the association of coffee bean defects with Ochratoxin A contamination in the beans: A possible implication for visual judgment of OTA contaminated coffee beans. In this chapter coffee bean defects were investigated based on Ethiopian coffee quality and liquoring manual and Specialty Coffee Association of American (SCAA). The predominant coffee bean defects encountered in this study in descending order were black beans (47.5 %), cherry (13.5 %), immature bean (8.1 %), sour bens (7.9%), insect infested (7.9 %), moldy beans (4.4 %), and broken beans (3.7%). Ochratoxin A contamination levels in coffee beans were positively correlated with black beans *($r = 0.33$, $p < 0.03$)*, visible mold overgrowth (*$r = 0.80$, $p < 0.00$*), beans damaged by insects (*$r = 0.71$, $p < 0.00$*), and presence of husk (*$r = 0.67$, $p < 0.00$*). These types of defects could be used as a possible visual marker for OTA presence in locally sold coffee. The fifth chapter deals with High Performance Liquid Chromatography Variable Wavelength Detector (HPLC-VWD) method of detection and quantification of Ochratoxin A in teff (*Eragrostis teff*) and wheat (*Triticum spp.*) flour samples intended for local consumption. In this section, HPLC method was developed and validated to detect and quantify OTA in teff and wheat flour samples. Based on this validated method teff and wheat four samples were analyzed for OTA contamination using HPLC-VWD apparatus.

The results indicate that 20% of the teff and 50 % of the wheat flour samples were contaminated with median OTA level of 0.99 µg/kg (LOD = 0.78) and 6.76 µg/kg (LOD = 0.58 µg/kg) respectively. This validated method for OTA detection and quantification in teff and wheat using HPLC-VWD detector generates meaningful data that satisfy performance criteria set by European Commission, EC 401, 2006 and Eurachem Guideline validation requirements. Moreover, an insight into the occurrence of OTA in teff is very valuable because the cereal might provide a low OTA risk alternative for wheat in Ethiopia.

Keywords: *Aspergillus spp.*, Aflatoxins, toxigenic fungi, coffee, defect count, mycotoxin, ochratoxin, ELISA, HPLC-VWD, teff, wheat

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Teshome Geremew, Addis Ababa, Ethiopia.

"You can't take nine women and make a bay in a month, there is a process. But in the end don't talk about the pregnancy, show the baby." Source: Warren Buffet.

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LIST OF ACRONYMS AND ABBREVIATIONS

AF	Aflatoxin
AFLP	Amplified Fragment Length Polymorphism
AOAC	Association of Official Analytical Chemistry
CEN	Commission Europeenne de Normalization Electrique
CFU	colony forming unites
DDRT-PCR	Differential Display Reverse Transcriptase Polymerase Chain Reaction
ECEA	Ethiopian Commodity Exchange Authority
EFSA	European Food Safety Authority
DNA	Deoxyribonucleic Acid
ELISA	Enzyme Linked Immunosorbent assay
ETOH	Ethanol
EU	European Union
EGTE	Ethiopian Grain Trade Enterprise
FAO	Food and Agriculture Organization
HPLC	High Performance Liquid Chromatography
HPLC-VWD	High Performance Liquid Chromatography with Variable Wavelength Detector
IARC	International Agency for Research on Cancer
ICO	International Coffee Organization

IR	Infrared
IUPAC	The International Union of Pure and Applied Chemistry
LC	Liquid Chromatography
JECFA	Joint Expert Committee on Food Additives
LOD	Limit of Detection
LOQ	Limit of Quantification
MAPKs	Mitogen Activation polyketide synthase
MS	Mass Spectrophotometer
NCRI	Negligible Cancer Risk Intake
NMR	Nuclear Magnetic Resonance
OTA	Ochratoxin A
OTB	Ochratoxin B
OTC	Ochratoxin C
PCR	Polymerase Chain Reaction
PKS	Polyketide Synthase
RAPD	Random Amplified Polymorphic DNA
PTDI	Provisional Tolerable Daily Intake
PTWI	Provisional Tolerable Weekly Intake
ssDNA	single-stranded deoxyribonucleic acid
ssRNA	single-stranded ribonucleic acid
TDI	Tolerable Daily Intake
TLC	Thin Layer Chromatography

UV-VIS	Ultraviolet-Visible
WHO	World Health Organization
YES	Yest extract sucrose

CHAPTER ONE: General introduction

1.1. Background

Ochratoxin A (OTA) is toxic fungal secondary metabolite produced by several species of the genus *Aspergillus* and two species in the genus *Penicillium* (Frisvad *et al*., 2004 (a and b); Palumbo *et al*., 2014). Ochratoxin A has nephrotoxic, hepatotoxic, genotoxic, immunosuppressive, and carcinogenic effects demonstrated in animal models (Reddy and Bhoola, 2010; Zepnik *et al*., 2011; Sorrenti *et al*., 2013). In humans, its effect has been related to the etiology of the Balkan Endemic Nephropathy (BEN) and International Agency for Research on Cancer (IARC, 1993) classified OTA as group IIB (possible carcinogens to human). Because of this negative health impact, several authorities set maximum permitted level of OTA in various food commodities. The European Union sets a maximum allowable limit of 5, 10, and 0.5 µg/kg for raw cereals, roasted and ground coffee and baby foods respectively (EC, 2006). Beside, FAO and EFSA set 100 and 120 ng/kg/bwt/week (tolerable weekly intake) respectively for OTA (EFSA, 2006; JECFA, 2007).

Based on the available toxicological data and the regulatory limits set by the EU and Canada (EC, 2006; Haighton *et al*., 2012) some countries also gave emphasis on mycotoxin regulation including OTA. Egypt, Russia, China and Bosnia and Herzegovina have established limits similar to that of European regulations (Clever and Jie, 2011). Ochratoxin A limits described in the *Codex Alimentarius/code of practice/* of the FAO/WHO have been adopted by the Arab States of the Gulf, Nigeria, Kenya, and India. However, to date, no limits for OTA in foodstuffs have been set in many countries of Africa including Ethiopia.

The sources of OTA are fungal members of the genus *Aspergillus* sections *Circumdati* and *Nigri* (Paterson *et al*., 2014) such as *A. sulphureus, A. carbonarius, A. niger, A. ochraceus, A. westerdijkiae,* and *A. sclerotiorum*. These species are widely distributed all over the world and particularly the major sources of OTA in warmer equatorial countries (Frisvad *et al*., 2004a). Two species in the genus *Penicillium; P. verrucosum* and *P. nordicum* were known to produce OTA in crops of the temperate climate and hence are less likely to occur in tropical areas (Frisvad *et al*., 2004b; Frisvad and Samson, 2004).

Reports from European countries indicated that the mean OTA contamination levels in European food commodities are relatively low (O'Brien and Dietrich, 2005; EFSA, 2006). A certain degree of contamination seems unavoidable irrespective of the preventive measures taken to keep the levels of OTA lower in food and feeds. However, in developing countries where screening of food is rare and traditional storage and transport conditions are still in use, much higher contamination levels may occur (Wagacha and Muthomi, 2008).

Ochratoxin A continues to gain global attention due to its serious human and animal health hazards.

Ochratoxin A has been detected and described in numerous foodstuffs, cereals, coffee and their derivatives and these food items remain the major contributors to OTA exposure (Amare *et al*., 2006; Amare, 2010; Magan *et al*., 2011; Streit *et al*., 2012).

1.2. Statement of the problem

Ochratoxin A is one of the toxic fungal secondary metabolites occurring mainly in coffee and cereal grains. This mycotoxin is categorized as group IIB, possible carcinogen to human by IARC and it is the second most significant mycotoxin following Aflatoxins (IARC, 1993;

Pitt, 2000). Ochratosin A is produced by two groups of filamentous fungi and the warm humid Ethiopian agroecological zones are suitable for ochratoxigenic *Aspergilli* (Abraham, 2006; Amare, 2010). Very few reports from Ethiopia indicated the presence of ochratoxigenic fungi and ochratoxin A in Ethiopian coffee and staple cereal grains (Abraham, 2006; Amare *et al*., 2006; Amare, 2010). On the other hand, international authorities like EU and FAO/WHO are trying to tightening OTA regulation on various foods and feeds due to the rising concern of OTA related health risks. Yet, there is no mycotoxin regulation in Ethiopia due to lack of sufficient occurrence data in local foods and limited awareness on health risks of OTA.

The local communities in all corners of Ethiopia consume coffee beverages and coffee consumption is one of the usual cultural practices in the country. However, neither the coffee growing farmers nor the consumers have adequate information on OTA and associated health risks. Due to lack of information on OTA occurrence in coffee beans and health risks of OTA, almost any kind of deteriorated coffee beans can be collected from the farms and sold in the local market. In addition, national reports indicated that nearly half of the bulk of coffee produced in Ethiopia is consumed locally (Tadesse, 2015) and the majority of this locally consumed portion is either rejected from export or is dry processed low quality supplied by producing farmers. This kind of low-quality coffee beans can be contaminated with OTA and can potentially affect the health of consumers.

In addition, cereal grains also contribute to excessive OTA intake with contaminated foods across the globe (Lin *et al*., 2005; Ibanez-Vea *et al*., 2011). Mycotoxin contamination represents a worldwide problem in various agricultural commodities during pre and post-harvest stages (Amare *et al*., 2006; Magan *et al*., 2011; Streit *et al*., 2012). In particular, in

the countries with warm wet climatic conditions (including Ethiopia), the growth of toxigenic filamentous fungi is most favored. Thus, cereal grains of basic foods of the populations of these countries are often contaminated especially with major mycotoxins *i.e.* aflatoxins and ochratoxins (Amare *et al.*, 2006; Nguyen *et al.*, 2007). In Ethiopia information on the occurrences of OTA in coffee and cereal grains used for human consumption is very scarce.

Based on this background information and personal observation in the field and local markets, it was hypothesized that the level of OTA in locally consumed coffee, teff and wheat flours might be higher than legally allowed limits (5µg/kg) set by harmonized EU regulation (EC, 2006). Secondly, if the level of OTA in locally consumed coffee, teff and wheat flours were higher than the allowed limit and since most food processing methods are unable to completely destroy this heat stable mycotoxin (Khoury and Atoui, 2010) local communities utilizing this low quality coffee and cereal grains might be exposed to higher OTA risks. Based on this hypothesis the following objectives were developed to initiate this study.

1.3. Research objectives

1.3.1. General objectives

The status of OTA in Ethiopian coffee and grains used for local consumption is not well known. Similarly, data on the type of toxigenic fungi associated with Ethiopian coffee and cereal grains consumed by the local people is limited. The major objective of this research is to study toxigenic filamentous fungi and OTA associated with Ethiopian coffee and selected cereal grains intended for local consumption.

1.3.2. Specific objectives of this project

- examine toxigenic filamentous fungi associated with Ethiopian coffee using morphological and molecular methods
- evaluate OTA and aflatoxin production potential of *Aspergillus* and *Penicillium* isolates under in vitro conditions
- determine OTA contamination level of coffee samples using ELISA method.
- examine the types of coffee bean defects that could be used as possible indicators of OTA occurrence in the beans
- suggest visual means of judgment to avoid coffee beans with possible OTA contamination during coffee purchase from local market.
- validate specific and sensitive HPLC-VWD methods for OTA analysis in teff and wheat flour samples.
- determine OTA contamination level in teff and wheat floour samples based on the validated method
- compare the results obtained in this study with OTA tolerable limits in coffee and cereals set by EU and/or FAO/WHO.

2. Literature review

2.1. Mycotoxin overview

The term mycotoxin was coined in 1960 during the outbreak of the mysterious turkey X disease in England when approximately 100, 000 turkey died because of consumption of a groundnut meal contaminated with secondary metabolite, aflatoxin, from *Aspergillus flavus* (Blount, 1961; Kensler *et al*., 2011). Secondary metabolites are low-molecular weight organic compounds that are not essential for normal growth, development and reproduction, but provide a number of fitness benefits to the producing fungus (Moss, 1991; Zinedine *et al*., 2007; Fink-Gremmels, 2008; Zain, 2011). To other organisms, these secondary metabolites can be beneficial (Vaishnav and Demain, 2011) or deleterious. Secondary metabolites which are deleterious are called mycotoxins, referring to their fungal origin and toxic nature (Bennet and Klich, 2003). It is estimated that there may be >20,000 unique mycotoxins, but only a fewer than 50 have been well characterized (CAST, 2003; Jacobson *et al*., 2007). The mycotoxins of most significance from a public health and agronomic perspective are those produced by molds present in feed and food. As mycotoxins are chemically very stable, they are not degraded during normal food processing (Eriksen, 2003) or autoclaving (Wannemacher *et al*., 2000) and thus end up in the feed and food chain. When consumed, they can cause a variety of adverse health effects. The pathological states arising from the consumption of food/feeds contaminated with mycotoxins are referred to as 'mycotoxicoses' (Bouhet *et al*., 2004). The main toxic effects of mycotoxins are carcinogenicity, genotoxicity, nephrotoxicity, hepatotoxicity, reproductive disorders, immunosuppression and dermal effects (Bennett and Klich, 2003). The severity depends, however, on various factors including the type and concentration of mycotoxin, the route

and duration of exposure, mode of action of the toxin, the animal species as well as gender, age, body weight and health status of the animal (Hussein and Brasel, 2001; Avantaggiato *et al*., 2005). They are often very stable molecules produced by molds belonging to several genera including *Aspergillus*, *Fusarium*, *Penicillium*, *Alternaria*, *Chaetomium*, *Cladosporium*, *Claviceps*, *Diplodia*, *Myrothecium*, *Monascus*, *Phoma*, *Phomopsis*, *Pithomyces*, *Trichoderma* and *Stachybotrys* (Kabak *et al*., 2006; Bryden, 2012; Streit *et al*., 2012).

Mycosis refers to a disease caused by the growth of fungi on an animal host (Richard, 2007; Bryden, 2012; Zain, 2011). The effect of mycotoxin on health could be acute, meaning apparent signs of disease are present or even causing death. However, acute manifestation of mycotoxicosis is rare. The effects of mycotoxin ingestion are mainly chronic, implying hidden disorders with reduced ingestion, productivity and fertility (Jouany, 2007; Fink-Gremmels, 2008). Such effects cause severe economic losses through clinically ambiguous changes in animal growth (Mobashar *et al*., 2010; Cheli *et al*., 2013; Gallo *et al* 2013; Giuberti *et al*., 2014).

Mycotoxin contamination represents a worldwide problem for various agricultural commodities both pre and post-harvest (Magan *et al*., 2011; Streit *et al*., 2012). The most studied mycotoxins are regulated mycotoxins including aflatoxins (AFs), ochratoxin A (OTA), Citrinin, trichothecenes (deoxynivalenol, (DON), T-2 toxins, HT-2 toxin, diacetoxyscirpenol, nivalenol), patulin, fumonisins (FBs), zearalenone (ZEA), and Ergot alkaloids (ergot toxins and ergotamine). In African context, the most problematic and wide spreading mycotoxins in the locally consumed and exported agricultural products are the AFs, OTA and FMs (Wagacha and Muthomi, 2008; Udomkun *et al*., 2017). This study

focused on OTA and detail description of this mycotoxin including general property, sources, occurrence in food and feed, biosynthesis, toxicity and prevention methods are addressed hereunder.

2.2. Ochratoxin A

2.2.1. Ochratoxin A and OTA derived metabolites

Ochratoxin A (OTA) is a mycotoxin produced by secondary metabolism of some filamentous fungi belonging to the genera *Aspergillus* and *Penicillium* (Bredenkamp *et al.*, 1989; Paterson *et al.*, 2014). Biosynthetically, this mycotoxin is derived from the dihydrocoumarins family coupled to β-phenylalanine and its chemical formula is $C_{20}H_{18}ClNO_6$ with molar mass of 403.82 g/mol (Renzulli *et al.*, 2004). It was first discovered as a metabolite of *Aspergillus ochraceus* in 1965 from South Africa (Van der Merve *et al.*, 1965) from corn. According to IUPAC, OTA is (N [[(3R) 5-chloro-8-hydroxy-3-methayl-1-oxo-7-isochromanyl] carbonyl] 3-phenyl-L-alanine) (Figure 2.1; Pohland *et al.*, 1992).

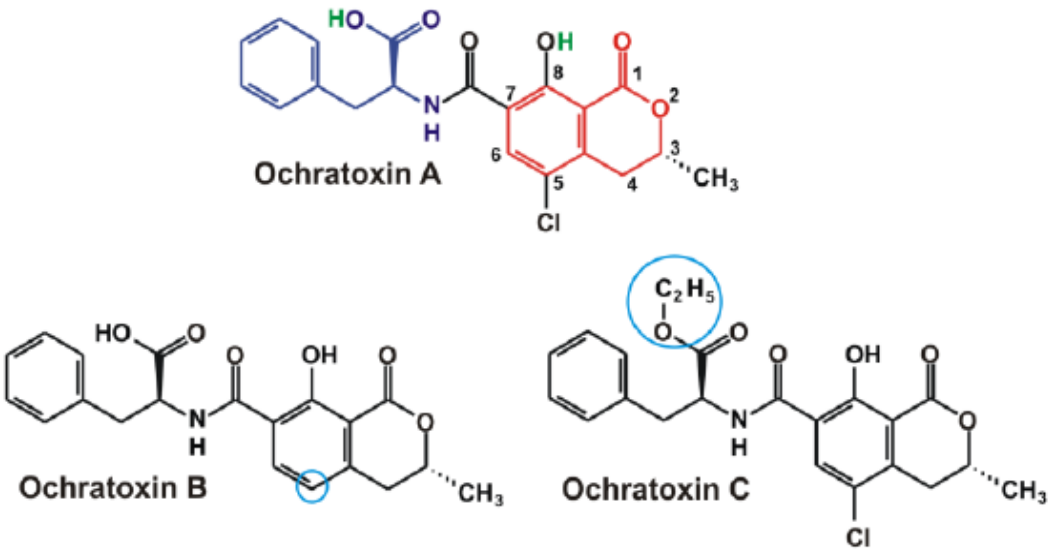

Figure 2.1. Chemical structure of ochratoxin A (blue: phenylalanine part, red: dihydroisocoumarin ring, green: acidic hydrogens), B, and C. The highlighted structures are characteristic to the three different ochratoxin molecules (light blue). Source: Bennett and Klich, 2003.

Several metabolites related to OTA have been also identified particularly, ochratoxin B ($C_{20}H_{19}NO_6$, Mw=369.4 g/mol) and ochratoxin C ($C_{22}H_{22}ClNO_6$, Mw=431.9 g/mol; Figure 2. 1). The general structure common to all ochratoxin metabolites is indicated in Figure 2.2 and Table 2.1 shows the characteristic composition of each one.

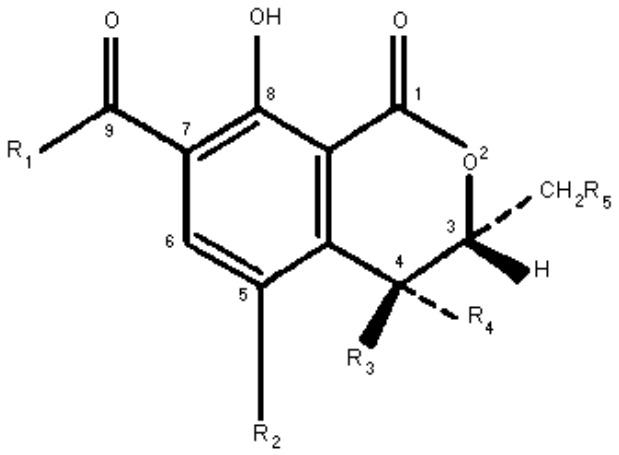

Figure 2.2. General structure of ochratoxin metabolites (Andre and Ali, 2010)

Table 2.1. Characteristic composition of OTA derived metabolites

Name	Abbreviations	R1	R2	R3	R4	R5
Ochratoxin A	OTA	Phenylalanyl	Cl	H	H	H
Ochratoxin B	OTB	Phenylalanyl	H	H	H	H
Ochratoxin C	OTC	Phenylalanyl, ethyl ester	Cl	H	H	H
Ochratoxin A methyl ester	--	Phenylalanyl, methyl ester	Cl	H	H	H
Ochratoxin B methyl ester	--	Phenylalanyl, methyl ester	H	H	H	H
Ochratoxin B ethyl ester	--	Phenylalanyl, methyl ester	H	H	H	H
Ochratoxin α	OTα	OH	Cl	H	H	H
Ochratoxin β	OTβ	OH	H	H	H	H
5′-Hydroxyochratoxin A	5′-OH-OTA	Phenylalanyl, OH at C-5′	Cl	H	H	H
7′-Hydroxyochratoxin A	7′-OH-OTA	Phenylalanyl, OH at C-7′	Cl	H	H	H

9′-Hydroxyochratoxin A	9′-OH-OTA	Phenylalanyl, OH at C-9′	Cl	H	H	H
4R-Hydroxyochratoxin A	4R-OH-OTA	Phenylalanyl	Cl	H	OH	H
4S-Hydroxyochratoxin A	4S-OH-OTA	Phenylalanyl	Cl	OH	H	H
4R-Hydroxyochratoxin B	4R-OH-OTB	Phenylalanyl	H	H	OH	H
4S-Hydroxyochratoxin B	4S-OH-OTB	Phenylalanyl	H	OH	H	H
10-Hydroxyochratoxin A	10-OH-OTA	Phenylalanyl	Cl	H	H	OH
Open lactone of ochratoxin A	OP-OTA	Phenylalanyl	Cl	H	H	H
Ochratoxin hydroquinone	OTHQ	Phenylalanyl	OH	H	H	H
Ochratoxin quinine	OTQ	Phenylalanyl	O	H	H	H
Ochratoxin α glucuronide	--	OH; glucuronide at C-8	Cl	H	H	H
Ochratoxin A phenol-glucuronide	--	Phenylalanyl; glucuronide at C-8	Cl	H	H	H
Ochratoxin A amino-glucuronide	--	Phenylalanyl; glucuronide at N	Cl	H	H	H
Ochratoxin A acyl-glucuronide	--	Phenylalanyl; acyl-glucuronide instead of carboxyl	Cl	H	H	H
Ochratoxin A acyl-hexose	--	Phenylalanyl; acyl-hexose instead of carboxyl	Cl	H	H	H
Ochratoxin A acyl-pentose	--	Phenylalanyl; acyl-pentose instead of carboxyl	Cl	H	H	H
		Synthetic Ochratoxins				
Ochratoxin A, tyrosine analogue	--	Tyrosine	Cl	H	H	H
Ochratoxin A, serine analogue	--	Serine	Cl	H	H	H
Ochratoxin A, hydroxyproline analogue	--	Hydroxyproline	Cl	H	H	H
Ochratoxin A, lysine analogue	--	Lysine	Cl	H	H	H
Ochratoxin A, alanine analogue	--	Alanine	Cl	H	H	H
Ochratoxin A, leucine analogue	--	Leucine	Cl	H	H	H
d-Ochratoxin A	d-OTA	D-Phenylalanyl	Cl	H	H	H
Ochratoxin A, ethylamide	E-OTA	Phenylalanyl, ethylamide	Cl	H	H	H
O-methylated ochratoxin A	OM-OTA	Phenylalanyl, OCH3 at C-8	Cl	H	H	H
Methyl ester of ochratoxin α	M-OTα	Methoxy	Cl	H	H	H
Ochratoxin A, decarboxylated	DC-OTA	Phenylethylamine	Cl	H	H	H

(Compiled from Andre and Ali, 2010; Heussner and Bingle, 2015).

Ochratoxin A derived metabolites have been characterized including a dechlorinated ochratoxin A derivative by nano-spray ESI-MS (Faucet *et al.*, 2006) and a quinone/hydroquinone metabolite showing toxicological properties (Tozlovanu *et al.*, 2006). In addition, it is predicted that OTA will form a benzoquinone electrophile following activation by cytochrome P450 enzymes, and radical species following activation by enzymes with peroxidase activities. These electrophiles react preferentially with deoxyguanosine (dG) to form benzetheno adducts and C8-dG adducts, respectively (Manderville and Pfohl, 2008)

2.2.2. Chemical and physical properties of ochratoxin A

Ochratoxin A has weak acid properties with pKa1 4.2– 4.4 and pKa2 7.0 –7.3, from the carboxyl group of phenylalanine and from the phenolic hydroxyl group of the isocoumarin with a crystalline structure varying from colorless to white (Ringot *et al.*, 2006). This molecule posses an intense green fluorescence under UV light in acid medium and blue fluorescence in alkaline conditions (Pohland, *et al.*, 1992). In acid and neutral pH, OTA is soluble in polar organic solvents (alcohols, ketones, chloroform), slightly soluble in water and insoluble in petroleum ethers and saturated hydrocarbons. While in alkaline conditions, this molecule is soluble in aqueous sodium bicarbonate solution and in all alkaline solutions (Khoury and Atoui, 2010). It has a melting point of about 90 ^{0}C when crystallized from benzene as a solvate. However, non-solvated crystals of melting point 169 ^{0}C have been obtained from xylene, which are suitable for X-ray structural analysis (Khoury and Atoui, 2010).

One of the special properties of OTA is its high stability. It has been shown that it possesses a resistance to acidity and high temperatures. Thus, once foodstuffs are contaminated, it is very difficult to totally remove this molecule. Some authors (Trivedi *et al*., 1992; Boudra *et al*., 1995) showed that the OTA is only partially degraded at normal conditions of cooking. Moreover, this molecule can resist three hours of high pressure steam sterilization of 121 °C and even at 250 °C its destruction is not complete (Mirna *et al*., 2005). Gamma irradiation (up to 7.5 Mrad) of OTA in ethanol does not cause any degradation. However, degradation is observed at low moisture level when OTA has been treated with an excess of sodium hypochlorite (NaOCl).

2.2.3. Biosynthesis of Ochratoxin A

Although large amount of information exists concerning the toxigenic properties of OTA, its biosynthetic pathway is not well known. It is widely believed that the isocoumarin group is a pentaketide synthesised from acetate and malonate via a polyketide synthesis pathway (Jonathan and Peter, 2001; Luis and Robert, 2010). Thus, as demonstrated by some authors (Ertan and Mert, 2010) a polyketide synthase (PKS), which is considered as key enzyme, is involved in the OTA biosynthesis in a similar way of other polyketide mycotoxins such as fumonisins and aflatoxins.

Huff and Hamilton (1979) proposed a biosynthetic pathway based on a mechanistical model according to the structure of OTA and this was further detailed by Renzuli *et al.* (2004) (Figure 2.3). The heterocyclic portion of OTA is structurally similar to mellein, a secondary metabolite produced by many OTA producing species such as *A. ochraceus*, *A. westerdijkiae* and *A. melleus*. Mellein is also produced by non ochratoxigenic species such as *Pezicula spp*. (Varga, *et al*., 2001b).

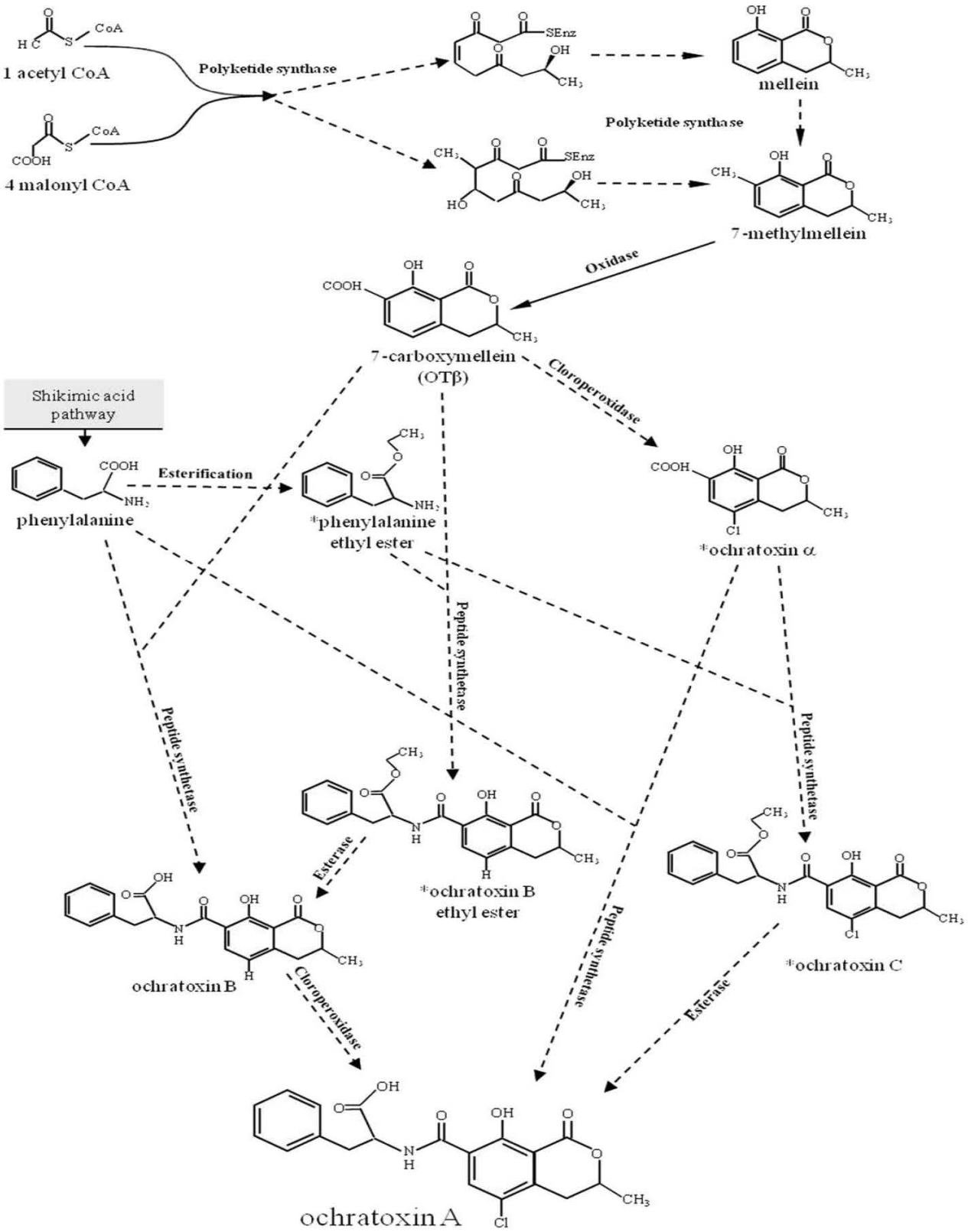

Figure 2.3. Hypothetical OTA biosynthetic pathway according to Huff and Hamilton (1979) and Harris and Mantle (2001). *, hypothetical intermediary compounds.

According to Huff and Hamilton (1979), three separate steps occur in OTA synthesis (Figure 2.3): The initial step is polyketide synthesis of ochratoxin α through mellein involving a polyketide synthase. Then the biosynthesis is followed by acyl activation: methylation and oxidation of mellen to 7-Carboxy-Mellein (OTβ). The adition of chlorine by a chloroperoxidase leads to OTα and then transformed to a mixed anhydride, an activation reaction using ATP. The second precursor phenylalanine is synthesized through the shikimic acid pathway, followed by ethyl ester activation so that it can contribute in the subsequent acyl displacement reaction. In the third step, linkage of those activated precursors through a synthetase takes place, generating OTC, an ethyl ester of OTA: de-esterification by an esterase or transesterification is the last step in this postulated biosynthetic pathway (Figure 2.3).

This schematic pathway has been disputed by Harris and Mantle (2001) who tried to elucidate the pathway using labeled precursors to the growing cultures of OTA producing fungi *A. ochraceus*. Relative incorporation of labeled putative intermediates ochratoxin α and β and mellein indicated a strong preferential role of α, moderate role of β, but found no role of mellein into OTA production. At the molecular level, the study of genetic nature of polyketide has been facilitated by the introduction of molecular techniques such as Genomic DNA bank and cDNA bank construction; polymerase chain reaction (PCR); gene inactivation; differential display reverse transcriptase-PCR (DDRT-PCR) and microarrays (Andre and Ali, 2010). PCR and subtractive PCR have been utilized to identify various PKS genes responsible for the biosynthesis of various polyketides (Nicholson *et al*., 2001; Liou and Khosla, 2003; O'Callaghan *et al*., 2003). According to the explanation of O'Callaghan,

et al. (2003) pairs of degenerated primers targeting KS domain; which is the most conserved domain among different PKSs, have been previously designed to amplify KS domain fragment from different types of PKS genes. These degenerated primers were successfully used in OTA producing fungi. Five different PKS genes have been identified in *A. ochraceus* by Verga *et al*. (2003) and Edwards *et al.* (2002). In addition nine different KS domains in *A. westerdijkiae* NRRL 3174 (*A. ochraceus*) as well as five different KS domain sequences in *A. carbonarius* (2Mu134 = CBS 120167) (Atoui *et al*., 2006) have been isolated.

In an attempt to elucidate the molecular biosynthetic pathway of OTA, O'Callaghan *et al.* (2003) have previously cloned part of the polyketide synthase (*pks*) gene (GeneBank accession number: AY272043) required for OTA biosynthesis in *A. ochraceus*, but no information was obtained concerning the presence or absence of some metabolites like the mellein. Later on, a polyketide synthase gene *otapks*PN (GeneBank accession number: AY196315), from *P. nordicum* that is essential for OTA biosynthesis was reported by Karolewiez and Geisen (2005). Bacha *et al.* (2008) reported the characterization of a PKS gene, awks1 (or aoks1 as named by the authors), (GeneBank Accession Number: AY583209) required for the OTA biosynthesis in *A. westerdijkiae*. Disruption of *awks1* stopped the biosynthesis of OTA, but did not affect the biosynthesis of others metabolites specially the mellein. This finding supports the results of Harris and Mantle (2001) where a mutant in which the PKS gene has been interrupted and cannot synthesize OTA but still produce the mellein.

2.2.4. Fungi producing OTA

Ochratoxin A was isolated in 1965 from a culture of *Aspergillus ochraceus* (section *Circumdati)* (Van der Merve, 1965), but subsequent studies have revealed that a variety of fungal species included in the genera *Aspergillus* and *Penicillium* are able to produce ochratoxin (Varga *et al*., 2001b; Frisvad *et al*., 2004a; Frisvad *et al*., 2004b). The study of these authors indicated that *A. ochraceus* has been shown to consist of two species. The second and new species producing large amounts of OTA consistently has been described as *A. westerdijkiae* (Frisvad *et al*., 2004a). The study of Leong *et al*. (2004) revealed that in *Aspergillus* section *Nigri, A. carbonarius* is a major OTA producer. It occurs in grapes, producing OTA in grape products, including grape juice, wines and dried vine fruits and sometimes in coffee beans (Taniwaki *et al*., 2003). *A. niger* aggregates have been reported as OTA producers (Dalcero, 2002; Magnoli, 2003; Abarca, 2004). The reported percentage of ochratoxigenic isolates belonging to the *A. niger* aggregate is much lower than *A. carbonarius* species. *A. lacticoffeatus and A. sclerotioniger* are also reported to produce OTA (Abarca, 2001).

Others *Aspergilli* can produce OTA in large amounts, but they appear to be relatively rare. In *Aspergillus* section *Circumdati* (formerly the *Aspergillus ochraceus* group) OTA producing species includes: *Aspergillus cretensis*, *A. flocculosus*, *A. pseudoelegans*, *A. roseoglobulosus*, *A. sclerotiorum*, *A. sulphureus* and *Neopetromyces muricatu* (Frisvad *et al*., 2004a). According to Ciegler (1972) *A. melleus*, *A. ostianus*, *A. persii* and *A. petrakii* may produce trace amounts of OTA, but this has not been confirmed since publication of those papers. Strains of these species reported to produce large amounts of OTA were re-identified by Frisvad (1985).

In the genus *Penicillium*, it has been shown that two species of the genus have this capacity, namely *P. verrucosum* and *P. nordicum*. *P. verrucosum* is the major producer of OTA in stored cereals (Geisen *et al*., 2004). *P. nordicum* (Larsen *et al*., 2001) is the main OTA producer found in meat products such as salami and ham.

Table 2.2. Fungal growth condition for OTA production by two fungi

Growth condition	*Aspergillus ochraceus*	*Penicillium verrucosum*
Optimum temperature for growth	24-37^{0}C	20^{0}C
Optimum temperature for OTA production	31^{0}C	20^{0}C
Optimum growth pH	3.0 to 10	6.0 to 7.0
Minimum water activity for OTA production	0.8	0.86

(Source: Lalini and Kanti, 2010). Sutable media for funal isolation, maintainace, and macro and microscopic study were described will in the article published by Samson *et al*., 2014.

2.2.5. Analytical methods of OTA

Due to the adverse health effects of OTA, detection and quantification are very import in the process of analysis. Quality control of food and feed requires extraction, purification and accurate quantification of the toxin. TLC, HPLC, MS, and immunochemical methods can be used with their own advantages and disadvantages.

2.2.5.1. Chromatographic methods

The analysis of OTA in most foodstuffs is relatively straightforward and generally reliable results are obtained. Samples need to be acid or alkali extracted from the matrices (foodstuffs), with alkaline extraction from most matrices showing generally better recoveries

(Senyuva *et al*., 2005). Once the toxin is extracted and purified detection and quantification can be possible using TLC or HPLC with various facilities.

2.2.5.1.1. Thin layer chromatography (TLC)

TLC was used in earlier AOAC methods, which employ a silica gel adsorbent and an acidic solvent system. Since then, this method has been very commonly used in many laboratories around the world for identifying and quantifying OTA in foodstuffs. The TLC consists of an OTA visual detection by its greenish fluorescence under long wave ultraviolet light, which changes to blue fluorescence after spraying the chromatographic plate with methanolic sodium bicarbonate solution or exposing it to ammonia fumes. However, the detection limit makes this method inadequate for present day monitoring and compliance purposes.

2.2.5.1.2. Liquid chromatography (LC)

Conventionally liquid/liquid extraction and solid phase clean-up have been used prior to HPLC determination with fluorescence detection (El Adlouni, *et al*., 2006). However, over the past decades, most laboratories have tended to move towards using immuno-affinity column clean-up because they considered it is relatively simple to carry-out and provides sample extracts generally free of interferences.

Several authors developed good methods for dried fruit (Mac Donald *et al*., 1999), beer (Legarda and Burdaspal, 1998), green coffee (Stegen *et al*., 1997; Leoni *et al*., 2000), dried figs (Senyuva *et al*., 2005), milk and wine (Valenta and Goll, 1997). Roasted coffee tends to be the most problematic of foodstuffs to analyze for OTA and in some methods an additional clean-up step is needed prior to the affinity column stage (Entwistle *et al*., 2001). Similar methodology has been used successfully to analyze blood and urine for OTA, when

this biomarker approach has been employed for exposure assessment. There are CEN standards for determining OTA in cereals, roasted coffee, wine and beer with limits of quantification (LOQ) ranging from 1.3 ng/g for barley (Gilbert *et al*., 2001) and coffee to 5 pg/mL (Entwistle *et al*., 2001). Particular attention was paid to validating a method for OTA in baby food at an LOD and LOQ of 0.05 ng/g and 0.22 ng/g respectively. Additional sensitivity being obtained by extracting larger sample sizes and enhancing fluorescence detection by using post-column ammoniation (Burdaspal *et al*., 2001). Whereas affinity column clean-up for confirmation of results is not essential, verification of results can be undertaken by methylation of OTA followed by the observation of the shift in retention time, or by LC/MS (Senyuva *et al*., 2005).

2.2.5.2. Immunological methods

Immunochemical methods have gained much interest because of cost and rapidity of the procedure. The use of ELISA (Enzyme Linked Immuno Sorbent Assay) for OTA analysis is considered as an important and very rapid method. It is easy to use, large number of samples can be processed at the same time and it does not require any clean-up procedure. ELISA methods have been applied to quantify OTA in coffee, cereals, food, feed, animals' tissues and serum (Fukal and Reisnerova, 1990). However, an important consideration with ELISA should be taken: the specificity of the antibody. Cross reactivity with related molecules can vary widely given over-estimated values. Radio-immunoassay (RIA) using for OTA has been applied to surveys of cereals, cereal products, feedstuffs, pig serum and tissues (Fukal and Marek, 1991). Ochratoxin A has been also determined by an enzyme immunosensor with an oxygen electrode (Aizawa, 1987). However, these methods do not appear to have been used recently due to health hazards of radiolabel compounds (Meulenberg, 2014). A

very recent method of OTA detection was reported by Sharma *et al.* (2015) by using titanium dioxide nanoparticles as quenching aptasensing (ssDNA or ssRNA) platform. The optimization result of Sharma *et al.* (2015) fluorescein labeled aptamer showed high selectivity of assay towards OTA without interference to structurally similar analogue Ochratoxin B.

2.2.5.3. PCR method

DNA-based techniques such as real-time PCR (RT-PCR) are providing tools for fungal detection and quantification by detecting and quantifying their DNA. RT-PCR can be performed using different chemistries, such as SYBR® Green I dye (Witter *et al.*, 1997; Gonzalez-Salgado *et al.*, 2009) and TaqMan® (Livak *et al.*, 1995). Both systems have proven useful in monitoring and quantification of OTA producing fungi in many food commodities (Farber and Geisen, 2004; Geisen *et al.*, 2004; Schmidt *et al.*, 2004).

One of the major motivations for the development of PCR based detection systems in many publications is the prospect of using this kind of analysis to estimate OTA concentrations in sample material. One might, therefore, anticipate that assays based on OTA biosynthetic genes might better fit that purpose as compared to systems based on genes unrelated to their biosynthesis. By using real-time PCR, a positive correlation between OTA content and DNA quantity has been indicated for *P. nordicum* and *A. ochraceus* (Farber and Geisen, 2004) and more recently, in *A. carbonarius* (Atoui *et al.*, 2007; Selma *et al.*, 2008). Such a correlation has been established with quantitative real-time PCR on mycotoxin biosynthesis genes or when using primers targeted sequences of housekeeping genes (Mule *et al.*, 2006).

Currently, RT- PCR quantification of *A. carbonarius* in grapes is clearly the best alternative to conventional methods in order to investigate the relation between OTA producers and

OTA content. With regards to food safety Atoui *et al.* (2007) established, according to their correlation, that *A. carbonarius* DNA content has to be lower than 10 ng DNA g−1 grape berry to fulfill the maximum OTA permitted levels in the European Union (EC, 2005). All the systems described above are based on PCR with genomic target DNA as template to estimate OTA concentrations in sample materials. Given the fact that biosynthesis of most mycotoxins is a highly complex process with poorly understood regulation at the transcriptional level as well as being highly influenced by environmental factors; it is important to find correlation between gene expression and concentrations of compounds. A RT-PCR system based on the *otapksPN* sequence has been used to monitor growth and OTA production of *P. nordicum* in wheat (Farber and Geisen, 2004; Geisen *et al*., 2004). A strong correlation between the copy numbers of the *otapksPN* gene and the colony forming units (cfu) was observed. In addition, there was a strong congruence between *otapksPN* gene expression and OTA production in wheat. It can be used for the rapid quality assessment of food products by quantitative determination of the fungal biomass. It can further be used for HACCP (Hazard Analysis Critical Control Point) purposes to determine the critical control points (CCP´s) during the production chain under which *otapks*PN gene expression and thereby OTA production is possible. With the available systems of PCR-based detection and quantification of ochratoxin A described in this section, the choice of the best method depends on the goal of the study to be conducted. For example, AFLP and RAPD could be the best choice when the purpose of the study is to show the polymorphism of some isolated strains belonging to the same species and to discriminate between relevant OTA producer species. On the other hand, when dealing with the detection of the fungus, the best way is to conduct conventional PCR using primers designed from housekeeping genes or mycotoxin

biosynthetic genes. Concerning the application of the described molecular techniques in food, AFLP and RAPD show limitations such as fungal isolation and preparation of DNA of very high quality. For this reason Real time PCR technology provides an insight into the mycotoxigenic status of food sample as well as it has the power to estimate its mycotoxin content.

2.2.6. Occurrence of OTA in food commodities

Ochratoxin A has been found in barley, wheat, rye, maize, coffee, teff, rice (Amare *et al*., 2006; Hussaini *et al*., 2011) in legumes, grapes, raisins and wines (Zimmerli and 1996) in nuts, beers, cacao, coffee and in spices (Ertana and Mert, 2010). Ochratoxin A is also found in animal products, including cow's milk, and pork, particularly pork kidneys, liver and sausages (Monaci *et al*., 2004).

Unlike aflatoxins, which are found mainly in cereal grains from warmer regions, ochratoxin A is found in cereal grains from all regions because it can be produced by *Aspergillus spp.* in warmer regions and by *Penicillium spp.* in the temperate climates (Table 2.3).

Table 2.3 Presence and contamination of ochratoxin A in different food items

Food product	Contamination level	References
Beans	0.25–0.92 µg/Kg	Domijan *et al*., 2005
Cocoa beans	0.35–14.8 µg/Kg	Amezqueta *et al*., 2004
Coffee	0.5 – 22 µg/Kg	Geremew *et al*., 2016
Corn	0.11–0.15 µg/Kg	Daniela *et al*., 2009
Teff	1.0 – 80 µg/Kg	Amare *et al*., 2006
Dried figs	<0.1–35.1 µg/Kg	Senyuva *et al*., 2005
Dried fruits	0.1–30 µg/Kg	Iamanaka *et al*., 2005
Grapes	0.008–1.6 µg/Kg	Serra and Mendonca, 2006
Green coffee beans	0–48 µg/Kg	Romani *et al*., 2000
Milk	0.011–0.058 µg/L	Skaug, 1999
Pork kidneys	0–15 µg/Kg	Jorgensen and Petersen, 2002

Pork meat	0–2.9 µg/Kg	Jorgensen and Petersen, 2002
Raisins	0.2–53.6 µg/Kg	Daniela *et al.*, 2009
Rice	1.0–27.3 µg/Kg	Gonzalez *et al.*, 2006
Spices	4.2–103.2 µg/Kg	Thirumala-Devi *et al.*, 2001
Wheat, Barley, oats	0.1–17.8 µg/Kg	Daniela *et al.*, 2009
Wheat, oats and rye	0.03–27 µg/Kg	Daniela *et al.*, 2009
Baby food	0.06–2.4 µg/Kg	Lombaert *et al.*, 2003
Beer	<0.01–0.135 µg/L	Visconti *et al.*, 2000
Breakfast cereals	0.4–8.8 µg/Kg	Molinie *et al.*,2005
Cocoa products	0.22–0.77 µg/Kg	Tafuri *et al.*, 2004
Grape juice	<0.003–0.311 µg/L	Daniela *et al.*, 2009
Pork products	<0.03–10.0 µg/Kg	Daniela *et al.*, 2009
Roasted coffee	3.2–17.0 µg/Kg	Romani *et al.*, 2000
Salami	<0.006–0.40 µg/Kg	Ertana and Mert, 2010
Wine	<0.003–0.388 µg/L	Ertana and Mert, 2010
potatoes	0.32 µg/Kg	Baydar, 2005
Lentil	0.83 µg/Kg	Baydar, 2005

The foods produced from cereals contain sometimes ochratoxin A but generally in low levels (Ngundi, 2006). *Aspergillus* and *Penicillium* proliferate most often at cereal surface and a large amount of mycotoxin is removed during the technological processes. The figs and raisins (Tjamos, 2006; Zinedine, 2007b), peanuts (Zinedine, 2007b), coffee (Fazekas, 2002; Fujii, 2006) are often contaminated with ochratoxin A. Small amounts of this mycotoxin have also been found on potatoes and lentil (Baydar, 2005).

Concentrations found in the final food products are lower than those found in raw materials since some processing steps can contribute actively to its reduction. For example, malting, white bread and whole bread production can contribute to reduce OTA by 56%; 80% and 40%, respectively (Mayura *et al.*, 1984). Additionally, reductions of 35%, 71% and 83% for mild, medium and strong coffee roasting, respectively, were reported by Hayes *et al.* (1974).

The wine-making process contributes to almost 90% reduction of OTA (Hood *et al*., 1976). All these literatures indicated that OTA is found in majority of food commodities and this mycotoxin is a serious problem.

2.2.7. Occurrence of OTA in animal feeds

Ochratoxin A is also detected in feed with the concentrations usually being higher than those in food. Ochratoxin A has been found in hay and mixed feed (Domijan *et al*., 2005; Gonzalez *et al*., 2006; Daniela *et al*., 2009,). The amount OTA in animal feed varies from country to country (WHO, 2002). High frequencies were described in Denmark (57.6%), Canada (56.3%), Yugoslavia (25.7%) and Spain (Muzaffer and Jose, 2014) found OTA above the levels recommended by the EU legislation <50 µg/kg (EC, 2006). In Brazil, Rosa *et al.* (2009) described the OTA occurrence in corn, brewers' grain and finished swine feed samples collected from different factories. Corn samples (44%) were contaminated with 42–224 µg/kg of OTA. The animal feed (31%) and samples of brewer's grain (13%) were contaminated with 36–120 µg/kg and 28–139 µg/kg of OTA, respectively. In Argentina, Dalcero *et al.* (2002) also detected OTA in 38% of the poultry feed samples tested with levels ranging from 25 to 30 µg/kg. Data on OTA contamination in African feed including Ethiopia is not available or nonexistent.

2.2.8. Human Exposure to OTA

Ochratoxin A occurrence in human food commodities of plant and animal origin has been recognized as a potentially global human health hazard. A general maximum OTA limit of 5 µg/kg in cereals and 3 µg/kg in cereal derived products were set by the EU and WHO (CE, 2006). Ochratoxin A has been detected in human blood and human milk samples. The

increase of OTA in several human fluids in the various populations of endemic regions may describe the human exposure to OTA contaminated food (Pfohl-Leszkowicz and Manderville, 2007; Peraica *et al.*, 2008; Pfohl-Leszkowicz, 2009). Thuvander *et al.* (2001) determined blood levels of OTA in 406 Scandinavian blood donors. The authors described the strongest correlations for women in relation to the consumption of beer or medium brown bread. Consumption of several foodstuff, including cereal products, wine, beer and pork, were to a minor degree related to high plasma levels of OTA. In breast milk, Skaug *et al.* (2001) found 17 (21%) out of 80 human milk samples containing OTA in the range 10–182 ng/L. However, the highest values were observed in a survey with samples from 75 mothers in Turkey (Gurbay *et al.*, 2009). Ochratoxin A was found in all samples tested in the range of 0.620–13.11 μg/L. Galvano *et al.* (2008) also detected OTA in 61 (74%) of 82 milk samples collected in Italian hospitals (ranging from < 5 ng/L to 405 ng/L; mean level: 30.43 ng/L). Ochratoxin A levels were significantly higher ($p < 0.05$) in the milk of habitual consumers of bread, bakery products and cured pork meat. These results confirm the occurrence of OTA in human milk and its likely association with maternal dietary habits. The strongest associations were observed with foodstuff sources of plant origin and, to a lesser extent, with food of animal origin. The findings also support the possibility of issuing dietary recommendations to women during pregnancy and lactation, aimed at reducing the OTA contamination of human milk.

Following the discovery of human and animal spontaneous nephropathies (the inability of the kidney to properly filter toxins from the blood), many experimental studies were carried out in order to show the implication of OTA in these diseases. These studies showed that OTA can have several effects such as nephrotoxic, hepatotoxic, neurotoxic, teratogenic and

immunotoxic on several species of animals. Brief description of OTA toxicity and its mechanism of action are presented hereunder.

2.2.9. Ochratoxin A toxicity and mechanism of action

2.2.9.1. Ochratoxin A toxicity

Nephrotoxicity

Nephropathy is the major toxic effect of OTA. Several detailed risk assessments have linked kidney damage incidence to estimated OTA consumption in the diet (Abouzied *et al*., 2002; Vrabcheva *et al*., 2004; Peraica *et al*., 2008). Ochratoxin A is associated with the Balkan Endemic Nephropathy and was also linked to human renal disease (Abouzied *et al*., 2002; Peraica *et al*., 2008) an interstitial chronic disease affecting the south-eastern population of Europe (Croatia, Bosnia, Bulgaria and Romania) (Pfohl-Leszkowicz, 2009). Ochratoxin A is shown to be potentially nephrotoxic in all non-ruminant mammals (Ribelin *et al*., 1978). Epidemiological studies carried out in Denmark, Hungary, Scandinavia and Poland, showed that OTA plays an important role in the etiology of porcine nephropathy (Elling *et al*., 1985; Varga *et al.,* 2001b). It is also considered to be the major cause of the Tunisian Nephropathy (TCIN) affecting the population in Tunisia (Hassen *et al*., 2004).

Neurotoxicity

It has been shown that the administration of OTA at gestation period in rats induced many malformations in the central nervous system and Sava *et al.* (2006) reported that OTA can be regarded as a possible cause of certain lesions as well as damage at the cerebral level. Thus, this substance seems to be highly toxic for the nervous cells and able to reach at any time the neural tissue (brain, retina) (Brown *et al*., 1976).

Teratogenicity

Ochratoxin A is a potent teratogen to laboratory animals. It can cross the placenta and accumulate in fetal tissue causing various morphological anomalies. It has been reported to elicit prenatal dysmorphogenesis in rats, mice, hamsters and chick embryos (Mayura *et al*., 1982; Mayura *et al*., 1984). The mechanism of OTA induced teratogenesis has not been clearly defined and may involve an indirect effect through maternal action and/or a direct effect on the developing embryo (Petzinger and Weidenbach, 2002). Thus, the gravity of malformations depends on the route of administration and the gestation period.

Immunotoxicity

Under certain conditions, OTA presents a powerful immunosuppressor effect, which is observed at low or high doses. Necroses of lymphoid tissues, humoral and cellular immunity effects indicating there high sensitivity to the OTA were described (Holmberg *et al*., 1988; Lea *et al*., 1989). Ochratoxin A seems to play a role in the inhibition of the peripherals T and B lymphocytes proliferation and stops the production of interleukin 2 (IL2) and its receptors (Lea *et al*., 1989). Moreover, it blocks the activity of killer cells as well as the production of interferon (Lea *et al*., 1989). The administration of OTA into many animal species causes variable effects on the bone marrow and immunity response. Thus, this molecule is considered to be the origin of Lymphopenia /reduced lymphocyte/, Regression of the thymus, and suppression of the immunity response (Petzinger and Weidenbach, 2002).

Carcinogenesis

Ochratoxin A is anticipated to be a human carcinogen based on evidence of carcinogenicity in experimental animals. When this molecule was administered in the diet, hepatocellular tumors, renal cell tumors, hepatomas, and hyperplastic hepatic nodules were observed in male mice (Petzinger and Weidenbach, 2002). In another study, administration of OTA in the diet induced hepatocellular carcinomas and adenomas in female mice (Pfohl-Leszkowicz *et al*., 1998). Gavage administration of OTA to male and female rats resulted in a dose-related increase in the incidence of renal-cell adenomas and adenocarcinomas. Further, metastasis of the renal-cell tumors was also observed in male and female rats. When administered by gavage, OTA increased the incidence and multiplicity of fibroadenomas of the mammary gland in female rats (Feier and Tofana, 2009). On the contrary Gagliano *et al*. (2006) after oral administration constant dose of 289 μg/kg body weight every other day for 90 days by gastric gavage for male rates found out that there is no major morphological difference between rats feed with OTA and the control groups. Similarly in this study, Light Microscopy of Sirius red-stained liver sections indicated similar collagen content in controlled and OTA treated rats, suggesting that the toxin did not trigger a fibrotic process, unlike in the renal cortex (Gagliano *et al*., 2005). These findings enlighten that the molecular mechanisms underlying OTA hepatotoxicity are different in the liver and the kidney, and that fibrosis is not a histopathological lesion typical of OTA-induced liver injury. This finding is supported by the similar αSMA(S-adenosylmethionine) expression in both experimental groups, indicative of a lack of activated myofibroblasts in the liver of OTA-treated rats (O'Brien *et al*., 2001).

However, no adequate human studies of the relationship between exposure to OTA and human cancer have been reported. Incidence and mortality from urothelial urinary tract tumors have been correlated with the geographical distribution of Balkan endemic nephropathy in Bulgaria and Yugoslavia (Feier and Tofana, 2009).

2.2.9.2. Mechanism of action

The mode of action of OTA is not clearly understood yet, and it seems to be very complex. Inhibition of protein synthesis, inhibition of cellular energy production, generation of oxidative stress, DNA adduct formation, as well as apoptosis/necrosis and apprehending cell cycle are possibly involved in its toxic action and brief explanation based on the recently available literature is presented hereunder.

Protein synthesis inhibition

Ochratoxin A has been shown to inhibit protein synthesis in all experimental systems tested including bacteria, yeast cells and mammalian cells in vitro and vivo. It is hypothesized that the mechanism of OTA-mediated inhibition of protein synthesis may involve competition with phenylalanine in the reaction catalyzed by phenylalanyl-tRNA synthetase (Bentoit *et al*., 2005; Vettorazzi *et al*., 2013). This hypothesis was supported by in vivo and in vitro data showing that co-administration of phenylalanine with OTA prevented the OTA-mediated protein synthesis inhibition (Bentoit *et al*., 2005; Vettorazzi *et al*., 2013). Many studies have indicated that inhibition of protein synthesis is likely to be involved in most of the acute toxic effects of OTA. When injected simultaneously, phenylalanine prevented OTA lethality (Creppy *et al.,* 1979), immunotoxicity (Haubeck *et al.,* 1981) and teratogenicity (Mayura *et*

al., 1984). The role of protein synthesis inhibition in effects observed at lower doses is less obvious. In vitro, cell-signalling disturbances were observed at concentrations in the nanomolar range which did not affect protein synthesis (Gekle *et al.,* 2005). Indirect data, exploiting the preventive activity of aspartame, a structural analogue of phenylalanine, and thus of OTA, suggested such a possibility. Aspartame was found to exhibit protective effects against the nephrotoxicity of OTA administered to rats at a level of 289 mg/kg bw/48 h over 6 weeks (Creppy *et al.,* 1995). The mechanism of protection was assumed to involve a prevention of protein synthesis inhibition.

Inhibition of cellular energy production /Mitochondrial dysfunction/

The nephrotoxic potential of OTA is assumed to be related to mitochondrial dysfunction leading to energy shortage and to the production of reactive oxygen species (Poor *et al*., 2014). The treatment of isolated renal proximal tubules with large concentrations of OTA resulted in an inhibition of mitochondrial respiration (Aleo *et al.,* 1991). Other in vitro studies applying direct treatments of mitochondrial preparations showed that OTA affected respiration and oxidative phosphorylation through an impairment of the mitochondrial membrane and an inhibition of the succinate supported electron transfer activities of the respiratory chain (Wei *et al.,* 1985). In addition, ATP synthesis in mitochondria isolated from renal cortex was significantly inhibited by micromolar concentrations of OTA (Jung and Endou, 1989). In contrast to the data outlined above, nanomolar concentrations of OTA produced a stimulation of mitochondrial activity in cell cultures (Gekle *et al.,* 2005). Based on the data available, a role for mitochondrial dysfunctions in OTA toxicity and carcinogenicity is possible although more research will be necessary to clarify its relevance at chronic, low dose exposure in vivo.

Oxidative stress

Several reports have suggested a potential role for oxidative stress in OTA toxicity and carcinogenicity. For example, an increased formation of the lipid peroxidation product malondialdehyde (MDA) was observed in rats treated orally with 120 mg/kg bw/day of OTA over 60 days (Petrik *et al.,* 2003). In cell culture, an OTA-dependent increase in DNA damage (such as formation of 8-oxoguanine) was correlated with a production of reactive oxygen species (Schaaf *et al.,* 2002; Kamp *et al.,* 2005). Antioxidants were shown to prevent OTA-mediated increases in MDA production in vitro and in vivo (Meki and Hussein 2001). In rats, the injection of superoxide dismutase and catalase provided protection against OTA-induced nephrotoxicity in vivo and in mice, antioxidant vitamins reduced an OTA mediated increase in chromosomal aberrations and the formation of ^{32}P-postlabelling spots (Grosse *et al.,* 1997).

Several potential relevant oxido-reduction mechanisms have been identified and proposed. In a reconstituted system consisting of phospholipid vesicles, the flavoprotein NADPH-cytochrome P450 reductase and Fe^{3+}, OTA was found to chelate ferric ions (Fe^{3+}), facilitating their reduction to ferrous ions (Fe^{2+}), which in the presence of oxygen, provided the active species initiating lipid peroxidation (Omar *et al.,* 1990). Other authors have reported that the OTA hydroquinone/quinone couple was generated from the oxidation of OTA (phenol oxidation) by electrochemical, photochemical and chemical processes (Gillman *et al.,* 1999; Dai *et al.,* 2002). The quinone is thought to be a likely candidate for covalent binding to DNA. It can also undergo reductions to form semiquinone and hydroquinone. Such events are likely to result in redox cycling and in the generation of

reactive oxygen species (Gillman *et al.,* 1999; Dai *et al.,* 2002). The actual relevance of these reactions for the physiological in vivo situation has still to be elucidated.

Alteration of signal transduction/Calcium homeostasis/

Cytosolic calcium is maintained at low concentrations through active sequestration in extracellular medium, mitochondria and endoplasmic reticulum. Disruption of calcium homeostasis, leading to sustained increase in cytosolic level, has been associated with toxic cell injury. In addition, calcium is known to be one of the major players in cell signaling through the activation of regulatory pathways involving calmodulin and protein kinase C. In immortalized human kidney epithelial cells-1 (IHKE-1), nanomolar concentrations of OTA induced rapid and reversible oscillations in intracellular calcium (Benesic *et al.,* 2000). This effect was dependent upon extracellular calcium, phospholipase C and cAMP-dependent protein kinase. Interestingly, in the same system, OTA potentiated the calcium-dependent stimulation of cell proliferation induced by epidermal growth factor and angiotensin II (Benesic *et al.,* 2000). These effects were observed in the absence of cytotoxicity, suggesting that OTA may interfere with calcium-dependent cell signalling to lead to increased cell proliferation. There are only limited and inconsistent data regarding OTA and calcium homeostasis in vivo. In rat, a single high dose (intraperitoneal administration, 10 mg/kg bw) or repeated lower doses (0.5–2 mg/kg bw) were found to stimulate calcium uptake by the endoplasmic reticulum, while moderate doses seemed to inhibit calcium sequestration (Rahimtula and Chong, 1991). Because in vivo data was only derived from experiments using high doses of OTA, it is currently difficult to link in vitro and in vivo data. The elucidation of the actual role of calcium in OTA toxicity and carcinogenicity requires further investigation.

Mitogen-activated protein kinases (MAPKs)

MAPKs are key components of signaling cascades involving phosphorylation-activation reactions aimed at transducing signals from the cellular membrane to the nucleus. Extracellular regulated kinases isoforms 1and 2 (ERK1/2) are part of the most studied MAPKs. ERK1/2 is generally thought to regulate cell proliferation. It has been implicated in cancer development including renal carcinoma (Oka *et al.,* 1995). In Madin-Darby canine kidney-C7 cells (MDCK-C7, from collecting duct) and in renal proximal tubule cell lines (OK cells, NRK-52E), OTA was found to increase the phosphorylation and activity of ERK1/2 (Gekle *et al.,* 2005; Sauvant *et al.,* 2005). In MDCK-C7 cells, this effect was correlated with cell dedifferentiation (Gekle *et al.,* 2005). In the same cellular systems, other MAPKs, JNK (c-jun amino terminal kinase) and the extracellular regulated protein kinase 38 (p38), were also activated by OTA treatments (Gekle *et al.,* 2005; Sauvant *et al.,* 2005). Unlike ERK1/2, JNK and p38 do not seem to act as promitotic signals, but are predominantly implicated in stress response and apoptosis. Altogether, these data indicate that in renal cells OTA is able to stimulate MAPK activities promoting opposite effects, either proliferation or apoptosis. It is believed that the balance between the different MAPKs will determine the fate of the exposed cells.

Apoptosis

Apoptosis refers to programmed cell death, an active process to be differentiated from necrosis. As suggested above, in MDCK-C7 and OK cell cultures, OTA activated JNK and p38 pathways leading to apoptosis (Gekle *et al*. 2005*;* Sauvant *et al*. 2005). In contrast, in other in vitro systems, OTA did not induce apoptosis or only at a very low rate (O'Brien and

Dietrich, 2005). Studies addressing the presence of apoptosis in OTA-treated animals provided some controversial data. Some authors did not find any apoptotic cells in the kidney of OTA-treated animals (Mantle *et al.,* 1998) while others obtained results compatible with the presence of apoptosis (WHO, 2001; O'Brien and Dietrich, 2005). For example, using the TUNEL assay, apoptotic cells were observed in rats given OTA (120 mg/kg bw/day) for 60 days (Petrik *et al.,* 2003). However, in this study, DNA electrophoresis did not show any characteristic fragmentation (DNA laddering). In another study, some apoptotic bodies were found in proximal convoluted tubules of rats given 1mg OTA/day for 5 days by gavage, while no apoptosis was observed when the same dose of toxin was administered in feed (Miljkovic *et al.,* 2003). With respect to tumor development, the contribution of apoptosis is difficult to define. It is generally thought that apoptosis prevents tumor development while failure of apoptosis may be a causative factor for cancer. However, some authors have advocated that OTA-mediated apoptosis could lead to the selection of apoptosis-resistant cells characterized by a higher probability of transformation into tumor cells (O'Brien and Dietrich, 2005).

In summary, the mechanism of action of OTA is still unclear. As a result, effective preventive strategies have not been developed to alleviate OTA related toxicity and the problem is a growing concern all over the world.

2.3. Coffee and grain production and processing in Ethiopia

Agriculture is one of the most important sectors of Ethiopia's economy. Ethiopia's crop agriculture is dominated by the country's numerous small farms that cultivate mainly cereals and coffee for both own-consumption and sales. These small holding farmers practiced traditional methods and increase cultivated land to increase yield. To what extent expansion can continue remains a question.

2.3.1. Coffee production, harvesting and processing in Ethiopia

Coffee production in Ethiopia is a longstanding tradition. Ethiopia is where *Coffea arabica* the coffee plant, originates and traditional production, harvesting and processing are practiced yet.

2.3.1.1. Coffee production systems

According to CSA (2013) more than four million primarily smallholder farming households cultivate coffee in Ethiopia, Africa's most important coffee producing country. These Smallholder farmers produce 95 % of Ethiopia's coffee (Abu and Teddy, 2013). There are four categories of coffee production systems in the country based on management level, vegetation, structural complexity, and agronomic practices (Tadesse *et al*., 2002; Tadesse, 2003; Senbeta and Denich, 2006).These are the forest coffee (8-10%), semi - forest coffee (30-35%), garden coffee (50-57%), and modern plantation coffee (5%) production systems (ECEA, 2008). Forest coffee is wild coffee grown under the shade of natural forest trees, and it doesn't have a defined owner. Semi-forest coffee farming is a system where farmers clear and select forest trees to let sufficient sunlight to the coffee trees and to provide

adequate shade. It is normally fertilized with organic material and usually inter-cropped with other crops. Plantation coffee is planted by the government or private investors for export purposes. Areas covered by coffee production are estimated to be about 800,000 ha with a production of about 400,000 tons of green coffee. For instance, in 2014/15 production year, Ethiopia produced 397,500 tons of green coffee (Abu, 2015a).

2.3.1.2. Coffee harvesting methods

Coffee cherries mature from May to October, and the main harvest season is from October to December depending on the climatic zone and the cultivars. The berries are mature when they are red and soft. The coffee berries are ready eight to nine months after the plant flowers. According to ICO (2006) there are four different methods of harvesting coffee, the first is stripping method. This form of harvesting is done by hand, and it removes all of the berries, flowers, green berries and deeply over ripened berries. The second method uses a comb to brush the trees. This method does remove all ripe berries, leaving the unripe berries as well as the green leaves that are still connected to the branches of the tree. This is a time consuming process, but it is worth the time invested. However, this process of harvesting would be more profitable because the unripe berries will eventually become ripe, increasing the future yield. The third process used for harvesting is mechanical method where a vibrator fixed to the trunk of the tree is used to shake the ripe berries loose so that they fall to the ground where they can be reached with ease (ICO, 2006).

2.3.1.3. Coffee processing methods

Coffee processing is a critical operation in the production of quality coffee. The quality of coffee can be enhanced or compromised in the course of processing. Generally, there are two coffee processing methods: the dry (natural) and the wet (washed) methods.

2.3.1.2.1. Dry method of coffee processing

This method involves the drying of red cherries without using water at any stage. The harvested ripe cherries spread over a raised bed, concrete, or on any other suitable drying material and raked at regular intervals to prevent fermentation and to ensure even drying. The cherries dried from moisture content of about 65% to 12%. The cherries are dried on beds constructed from chicken wire and fixed on wooden frames raised about 80 cm above the ground. A synthetic black shade net is then placed over the chicken wire before the cherries are spread on top of it. A Hessian cloth is used to cover the drying coffee during mid-day to protect from strong sun. In the night and during rain, the Hessian cloth also serves to protect the coffee from coming in contact with the overlying polythene sheet. Local coffee growers dry the cherries on “selen” a manually woven carpet made of palm tree leaves, on bamboo mat and other locally available spreading materials.

2.3.1.2.2. Wet processing method

The wet method involves use of water in most stages. This method involves several stages whereby ripe cherries are transformed into parchment coffee. In fully-fledged conventional wet processing, red cherries are pulped, fermented under water until the mucilage is degraded so that it can be easily washed off. Fermentation period in most cases varies from

12 to 48 hours depending on the temperature of the locality. The parchment is then washed and dried to attain a moisture content of 10-11.5%.

Stages in wet processing

Selective picking of cherries: Coffee cherries for wet processing should be mature and fully ripe. Ripe cherries have adequate pulp and mucilage which facilitate pulping. Cherries in mixed stages of maturity would cause pulping and fermentation problems, which have series of negative consequences for the quality of the product (Tadesse, 2015).

Sorting: The first operation in coffee processing following picking is sorting, the purpose of which is to remove undesirable objects such as leaves, twigs, stones, as well as diseased or pest infected, immature, over ripe and dry cherries. Under-sized cherries which would escape pulping are also removed and processed by dry method, and may be used for own consumption. Use clean material such as canvas, drying trays or mesh wire beds; cherries should not come into contact with the soil during sorting.

Pulping: Pulping is the mechanical removal of the red outer skin and pulp from the cherry to produce parchment coffee. According to Tadesse (2015) this is performed by squeezing the cherry to release the parchment coffee out of the pulp, and facilitated by the lubrication from the mucilage formed between the pulp and the parchment. But in over-ripe cherries, the mucilage layer is dried up, while in the green cherries, it is not fully developed. So, pulping under both conditions will result in large number of damaged beans due to lack of adequate mucilage. Pulping is done via a stream of water which helps the cherries to be fed to the pulper. The water also facilitates separation of the parchment coffee and the pulp.

Drying parchment: Freshly harvested and pulped coffee has high moisture content. For example, after the parchment coffee has been washed and drained, it will have a moisture

content of 50-65%. Drying is thus the process of reducing the moisture content of this product down to 10-11.5%. Green coffee that is high in moisture (greater than 12 %) can deteriorate due to bacteria, mold, or yeast, especially if the seed is killed. If the seed remains alive, enzymatic activity will cause the cupping quality to change (Gautz *et al*., 2008). Drying of pulped coffee is a critical operation and is done with care, as coffee of excellent origin can lose its quality if drying is not done properly. Under-drying causes rapid fading of bean color while over-drying leads to unnecessary weight losses and quality degradation. At 10.5% moisture content the parchment is fully dry and safe for storage. At this moisture content and 60% relative humidity, the coffee suffers no quality losses if properly stored (Gautz *et al*., 2008). Though similar methods are used for drying both cherry and parchment coffee, drying area requirement for dry method is, however, larger for the same quantity of drying cherries.

2.3.1.2.3. Storage of dry coffee

Coffee storage and handling is one of the crucial processes influencing quality, and thus needs due care. Storage operations are also important from the point of view of OTA prevention and quality assurance. The dried coffee should not be stored in an environment where it can pick up or lose moisture and undesirable smell. Dry coffee should be stored in sisal bags placed on wooden battens raised 15-20 cm above the ground level, and about 30 cm away from the wall or roof to encourage ventilation, as well as about 100 cms away from corrugated iron sheet roof. In general, storage facilities should be clean, cool, shaded, dry and well ventilated. In conditions of high relative humidity and temperatures, coffee beans will absorb moisture and develop mould. They may be bleached out in color and lose some desirable flavor. Storage temperature of about 20 ^{0} C, and relative humidity of 50-60% is

recommended. Dry cherry coffee can be stored longer in relatively similar conditions than parchment without deteriorating in quality, since they are still covered with the husk.

Given the small scale coffee production in the area, it is difficult to build such storage facilities by individual farmers. However, such storage facility can be built by cooperatives if the farmers are organized. It is also possible to arrange collection of dried coffee by coffee buyers immediately, so as to avoid quality deterioration due to poor storage at farmers' homes.

2.3.2. Teff (*Eragrostis tef*) and wheat (*Triticum* spp.) production and consumption in Ethiopia

Out of the 86.68% total cereal production in 2015/2016, maize, teff, wheat, and sorghum makes up 26.80%, 16.76%, 15.81%, and 16.20% respectively (CSA, 2016). This CSA data indicates teff and wheat are the second and fourth major cereal crops in terms of production.

2.3.2.1. Teff production

Teff is a cool weather crop grown predominantly in the Ethiopian highlands and neighboring Eretria (D'Andrea, 2008) at optimum altitude range of 1800 to 2200 meters. This crop occupies the largest area (about 1.4 million hectares of land) and has relatively large amount of grain production (Trade Economics, 2011; Bekabil *et al*., 2011). It is an indigenous to Ethiopia, forms the staple diet of many Ethiopians and it furnishes the flour to make *injera*, leavened bread that is consumed in the highlands and in urban centers throughout the country.

2.3.2.2. Wheat production

Ethiopia is the second largest wheat producing country in Africa after South Africa . Most wheat production in Ethiopia comes from small holder farmers. Wheat is mainly grown in the central and southeastern highlands during the main rainy season (June to September) and harvested in October-November. The majority of wheat grown in Ethiopia is bread wheat. However, durum and bread wheat (mixed together) is grown in some parts of the country. Eight percent of wheat is produced on large state-owned farms consisting of roughly 124,000 ha of land. The remaining ninety two percent (1,426,000 ha) of production is from small farms (Abu, 2014).

In 2013/14, more than 1.5 million hectares were dedicated to wheat cultivation with estimated annual yield of 2.2 tons per hectares. Annual yield has risen steadily in Ethiopia since 2002 owing to increased investment in wheat production by both the government and various stakeholders. In 2013/14, Ethiopia produced about 3.32 million tons of wheat, almost double the quantity produced in 2002 (1.07 million tons) (EGTE, 2014). The GOE estimates that over 4.5 million households are involved annually in wheat production, but that still does not satisfy the country's annual domestic demand. Hence, a large quantity of wheat is imported every year to meet the rising domestic consumption demand.

Threshing of majority of the harvest is carried out using herds of animals on soil ground (Figure 2.4). This kind of traditional threshing practice using anima overriding on soil ground allow soil fungi easy access to the cereals and leads to OTA contamination in the grains.

Figure 2.4. Traditional grain threshing using herds of animals in Ethiopia. Contaminate the grains with urine and feces besides leaving 30% of the crop behind.

Wheat and teff Consumption

Wheat is used in the preparation of a wide range of products such as the traditional fermented thin bread ("*injera*"), regular bread ("*dabo*"), local beer ("*tella*"), and several other local food items. Additionally, wheat straw is commonly used as a roof thatching material and as animal feed.

Wheat's share of total cereal consumption increased by 20 percent in recent years, making it the second most consumed cereal in Ethiopia after maize. In the average Ethiopian

household diet, wheat accounts for approximately 200 kcal/day in urban areas compared to 310 kcal/day in rural areas (CSA, 2015/16). Wheat accounts for about 11 percent of the national calorie intake in the country. In most parts of the country, families prefer to use teff to make 'injera' (fermented bread) and occasionally to make porridge. Because of the price escalation of teff compared to wheat, most middle and lower class populations are consuming more wheat.

According to GAIN report, there are around 216 flour mills in Ethiopia, with a total production capacity of about 4.2 million tons of wheat flour a year and almost 57% of these mills are located in Addis Ababa (Abu, 2014).

2.4. Prevention of Mycotoxin contamination

Since the discovery of mycotoxin in 1960s, researchers have been thoroughly researching ways to eliminate or minimize the effects of these contaminants at different stages of agricultural products.

2.4.1. Pre-harvest strategies

The steps of prevention that are the most effective are those carried out before the fungal infection and before mycotoxin production occurs on plant material. Pre-harvest strategies primarily consist of tactics designed to reduce infection of crops by mycotoxin-producing fungi. It includes biological control, development of resistant varieties of crops through new strategies and good agricultural practices, which includes crop rotation, tillage practices, cropping pattern, reduction in plant stress through irrigation, timely planting and harvesting and protection of insect damage by the use of biopesticides (Choudhary and Koumari, 2010).

2.4.2. Post-harvest strategies

Prevention of mycotoxin contamination in the field is the main goal of agricultural food and feed industries, but even the best management of agricultural strategies cannot totally eradicate mycotoxin contamination (Jouany, 2007). Therefore several physical, chemical and biological methods have been developed in order to remove mycotoxins from contaminated feed (Kabak and Dobson, 2009). Physical methods like cleaning, mechanical sorting and separation, washing, density segregation, thermal inactivation, irradiation, solvent extraction, and chemical procedures like treatment with acid/base solutions or other chemicals, ammoniation and ozonation have been tested (CAST, 2003). However, these physical and chemical detoxification methods often do not work, and often destroy or remove essential nutrients from the feedstuff, reduce palatability, and not feasible for large scale. Biological detoxification, which comprises binding of mycotoxins by adsorptive materials as well as microbial inactivation by specific microorganisms or enzymes (biotransforming agents), is the most prominent approach to reduce the risk for mycotoxicosis in farm animals (Upadhaya, 2010).

CHAPTER III. Occurrence of toxigenic fungi and ochratoxin A in Ethiopian coffee for local consumption

Abstract

Ethiopia is the center of origin of Arabica coffee and coffee has been produced ever since. Information on the presence of toxigenic fungi and ochratoxin A (OTA) in the locally sold Ethiopian coffee is scarce. Therefore, the objective of this study was to identify the fungal and mycotoxin contamination levels in Ethiopian coffee consumed by the local community. Seventy-four coffee samples (one kilogram each) were collected from six major coffee growing districts in Jimma zone of Oromiya Regional State in Ethiopia. All samples were evaluated for fungal presence and total fungal incidence mounted up to 87%. The predominant fungal genera were *Aspergillus* (79%), *Fusarium* (8 %) and *Penicillium* (5%). Ochratoxin A producing species of *A. westerdijkiae*, *A. ochraceus*, *A. melleus*, and *A. steynii* were identified for the first time using molecular techniques from locally sold coffee in Ethiopia. *A. westerdijkiae and A. ochraceus* were found the predominant OTA producers. The median OTA level in the locally sold Ethiopian coffee is 1.53 µg/kg. Despite this low median value, significant differences in fungal and toxin incidences were observed between the different coffee processing types, coffee sample types, and storage characteristics. Using an *in vitro* approach, the OTA and Aflatoxins (AFs) production potential of all *Aspergillus* isolates was assessed with Enzyme Linked Immuno Sorbent Assay (ELISA) method under standardized conditions. Based on this experiment, it was concluded that *A. westerdijkiae* isolates were clearly the most potent producers of OTA while AFs were only produced by two *A. flavus* isolates.

Keywords: aflatoxins, *Aspergillus spp.,* coffee, ELISA, ochratoxin A, toxigenic fungi

3.1. Introduction

Ethiopia is the center of origin of Arabica coffee and coffee has been produced ever since. Coffee production is important to the Ethiopian economy and about 15 million people directly or indirectly make a living on it (Nicolas, 2007) and this number is still on the rise. Coffee is also a major Ethiopian export product contributing up to 25% of the country's total export earnings (Abu and Teddy, 2013; Girma *et al.*, 2008, Nicolas, 2007). A market share of 7-10 % makes Ethiopia the major coffee producing country in Sub-Saharan Africa and the fifth producer worldwide, next to Brazil, Vietnam, Colombia, and Indonesia (Rao, 2013; Girma *et al.*, 2008). The Ethiopian government has set an objective to increase coffee production to 600,970 tons in 2015 (FDRE GTP, 2010). This objective has been an incentive for an increased growing area of coffee from 520,000 hectares in 2013 to 662,000 hectares now (FDRE GTP, 2010).

Coffee is cultivated by over 4 million primarily smallholder farming households, producing 95 % of Ethiopia's coffee (Abu and Teddy, 2013) and the production is almost exclusively situated in the regions of Oromiya and the Southern Nations Nationalities and People Regions (SNNPR). It is produced by several types of production systems, including plantation coffee, forest, semi-forest, and garden coffee. These different production systems make up about 5, 10, 35 and 50% respectively of the total production (Taye, 2012). Several factors from the field to post-harvest processing and secondary processing determine the quality of coffee (Musebe *et al.*, 2007). For this reason, quality control is necessary for all production and processing stages as the final quality will determine the market price.

There is a major discrepancy between coffee destined for the local market and coffee for export as the former one has to meet the highest standards set by several control authorities. At farm gates and at district level, quality inspection is carried out by regional Agricultural Offices, by Ethiopian Commodity Exchange and by the Ministry of Agriculture. Quality assessment is conducted by visual examination (defect count, shape and make, color and odor) and liquor analysis (aroma, acidity, body, and flavor) by trained expert panels. Any coffee product that fails to pass the rigorous evaluation at various levels is redirected to the local market (Boot, 2011; Abu and Teddy, 2013; Minten *et al.*, 2014).

One of the main constraints in coffee bean production is contamination by fungi. Temperature, moisture content, storage conditions, and durations of storage can be important factors for mold development (Amezqueta *et al.*, 2009). In addition, field-related factors such as insect infestation, susceptibility of coffee variety, and nutrient composition of the crop are predisposing factors for fungal invasion (Amezqueta *et al.*, 2009). The presence of fungi in coffee beans does not only affect the sensorial quality of the coffee beverage but also presents a health risk attributed to the production of mycotoxins by some of these fungal genera (Rezende, *et al.*, 2013). Aflatoxins (AFs) B1, B2, G1, and G2, OTA, patulin and sterigmatocystin are mycotoxins often occurring in coffee (Soliman, 2005; Bokhari, 2007; Bokhari and Aly, 2009; Paterson *et al.*, 2014; Culliao and Barcelo, 2015). A recent analysis of coffee however showed convincingly that coffee can be contaminated by a much broader range of mycotoxins including the trichothecenes, nivalenol, deoxynivalenol, T-2 and HT-2 Toxin, diacetoxyscirpenol, the fumonisins B1 and B2, and the emerging mycotoxins cyclodepsipeptides enniatin A, enniatin A1, enniatin B, enniatin B1, and beauvericin (Moraleja *et al.*, 2015).

To date, the major mycotoxin in coffee is OTA although it is expected that other currently less frequently detected mycotoxins such as AFs might become an issue in several geographic regions the coming decennia due to climate change (Paterson *et al*., 2014).

Ochratoxin A which is produced by several species of the genera *Aspergillus* and *Penicillium* has nephrotoxic, hepatotoxic, genotoxic, immunosuppressive, and carcinogenic effects as demonstrated in animal models published by several authors (Pfohl-Leszkowicz *et al*., 2007; Reddy and Bhoola, 2010; Zepnik *et al*., 2011; Sorrenti *et al*., 2013). The producers of OTA in coffee are members of *Aspergillus* sections *Circumdati* and *Nigri* (Paterson *et al*., 2014) such as *A. sulphureus, A. carbonarius, A. niger, A. ochraceus, A. Westerdijkiae,* and *A sclerotiorum*. Two *Penicillia* known to produce OTA, *Penicillium verrucosum* and *Penicillium nordicum*, are associated with crops of temperate climates so are less likely to occur in coffee. In humans, its effect has been related to the etiology of the Balkan Endemic Nephropathy (BEN) and International Agency for Research on Cancer (IARC, 1993) classified OTA as group 2B (possible carcinogens to human). Because of its negative health impact EU set maximum permitted level of OTA to 5 ng/kg for roasted and ground coffee and 10 ng/kg for soluble coffee (EC, 2006)

AFs are toxic secondary metabolites produced mainly by *A. flavus* and *A. parasiticus* (Abdel-Azeem *et. al*., 2015; Arroyo-Manzanares *et al*., 2015). AFs are classified as B1, B2, G1 and G2 and various products such as grains, nuts, almonds, dried fruits, legumes, coffee, peppers and meat (Ventura *et al*., 2004; Turner *et al*., 2009). Milk products can be contaminated with AF M1 which is less toxic than the other AFs and is a metabolite produced by the microorganisms in the rumen of the cow (Malhotra *et al*., 2013). Among all mycotoxins, AFs B1 is the most toxic. It is carcinogenic, mutagenic, and teratogenic. Lethal

dose (LD50) values of AFB1 vary from 0.3 µk/kg for rabbit to 17 µk/kg for female rat (Saini and Kaur, 2012). Therefore, IARC classified this substance as Group I, carcinogenic to human (IARC, 1993). Other AFs have been classified as possible carcinogen (Group 2B) (IARC, 2002). The limit for total AFs varies from 0.1 µk/kg for processed cereal based foods to 15 µk/kg for peanuts and other oilseeds to be subjected to sorting (EC, 2006).

3.2. Objectives

3.2.1. General objective

The general objectives of this study was to determine toxigenic fungal species associated with Ethiopian coffee and to depict post-harvest environmental factors influencing fungal contamination and toxin production in Ethiopian coffee for local consumption.

3.2.2. Specific objectives of this study were to:

- examine filamentous fungi associated with *Arabica* coffee using morphological and molecular methods
- evaluate OTA and aflatoxin production potential of *Aspergillus* and *Penicillium* isolates under in vitro conditions
- Determine OTA contamination level of coffee samples using ELISA method

3.3. Material and methods

3.3.1. Sampling area and coffee sample collection

Jimma zone (Figure 3.1) is one of the most important coffee producing zones in Oromiya Regional states of Ethiopia and the capital of this zone is located at 7°40′0″N latitude and 36°50′0″E longitude. Detail meteorological data of sampling sites are given in Appendix B1. This zone is bordered in the South by the Southern Nations, Nationalities and People's

Region (SNNPR), in the North West by Illubabor in the North by East Welega, and in the North East by West Shewa where part of the boundary with East Showa is defined by the Gibe River. Of all coffee produced in Ethiopia, 61.5 % (164,000 tons) is produced in Oromiya Regional State. Jimma represents 23.2 % of this Region's coffee production (CSA, 2013).

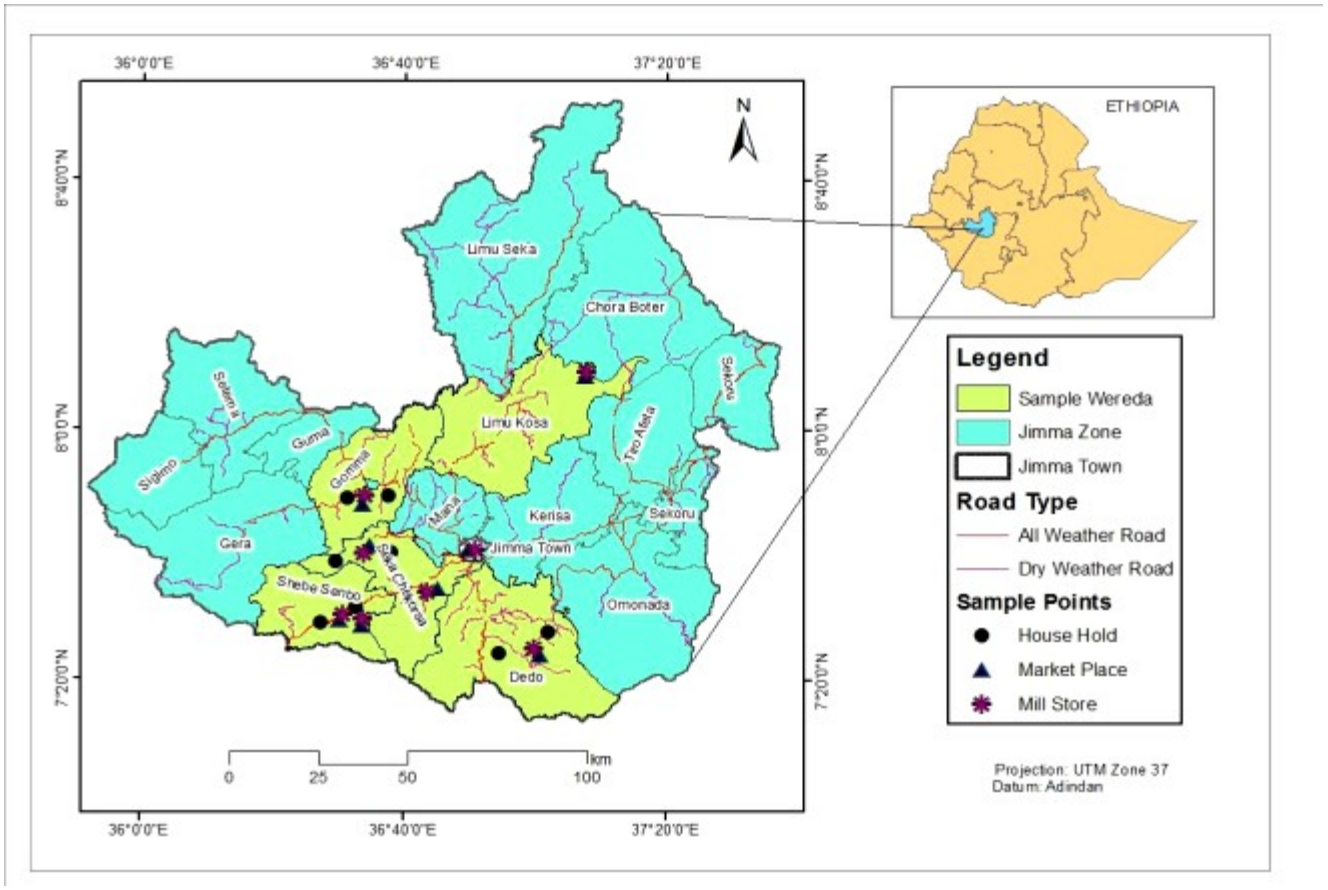

Figure 3.1. Map of sampling sites.

N.B. Map of Ethiopia depicting sampling points in the districts (Woreda) of Jimma zone in the Oromiya Regional States of Ethiopia. Sampling points are indicated by solid bullet points, triangle and asterisk for households, market place (shops) and coffee mill stores respectively.

A total of 74 *Coffea arabica* samples of 1 kg each were collected from different sampling sites over two years in Jimma from households, marketplaces or mill stores. The samples comprised coffee from two processing methods (washed or dry processed), different conditions of storage (mill houses, resident, and shops), different storage packaging (sisal sacks, fertilizer bags and plastic bags), and different coffee type (cherry, parchments or green bean) (Table 3.2). Moisture contents of the samples were measured before packing using an electronic moisture tester (HOH-Express-HE-50, Germany) at the collection sites.

3.3.2. Fungal isolation and morphological characterization

From each sample, beans were randomly selected and surface sterilized by treating with 1.3% sodium hypochlorite for 2 minutes. Beans were rinsed three times with sterile distilled water. Malt extract agar medium amended with 0.01% chloramphenicol was used for plating experiments to avoid bacterial outgrowth. Five surface sterilized beans were aseptically placed on a Petri dish and incubated upright at 25°C for 10 days. Every microbial colony that appeared on the beans were counted as black *Aspergilli*, yellow *Aspergilli*, *Penicillium* spp., *Fusarium* spp., and others. Fungal colonies were picked up with a sterile needle and purified by transferring to Czapek's Dox agar medium. Pure isolates were preserved on Czapek's Dox agar slants in the refrigerator at 4°C for further study. The frequency of isolation of fungal genera and group and total microbial load were computed based on the following formula.

$$\text{Frequency (\%)} = \frac{number\ of\ beans\ from\ which\ the\ genus\ isolated}{Total\ number\ of\ beans\ analyzed}\ X\ 100$$

$$\text{Percent of fungal infection (\%)} = \frac{number\ of\ beans\ from which\ a\ fungus\ isolated}{Total\ number\ of\ beans\ analyzed}\ \text{X}\ 100$$

Isolates were visually analyzed based on colony growth rates, texture, degree of sporulation, color of mycelia, shape of conidial heads, vesicles, the number of branching points between vesicle and phialides (i.e. uniseriate or biseriate), phialides and conidia were observed for

the primary screening of isolates to genus level according to Navi *et al.* (1999). Representative isolates were maintained in Czapek's Dox agar slant and 10% glyceron in Addis Ababa University and BCCM, Belgium respectively.

3.3.3. Molecular identification of fungi

Representative isolates from the toxigenic genera including *Asperillus*, *Penicilliu*, and *Fusarium* were selected for molecular study and mycotoxin analysis.

3.3.3.1. DNA extraction

DNA was extracted from 100 mg of freeze-dried mycelium using cetyl-trimethyl-ammonium bromide (CTAB; Sigma-Aldrich, St. Louis, MO) protocol as described by Stewart and Via (1993) with some modification. Spores from five day old cultures were inoculated in to yeast extract sucurose (YES) broth medium on 2mL plastic wells and grown for five days. The mycelia were separated from the broth and transferred to 2mL epindrhof tubes and maintained at -80^{o}C for one hour. After one hour, the mycelia were freeze dried for an overnight in frieze dryer. The following day the mycelia were pulverized in eppendorf tubes using Micro Pestle. To about 50 mg pulverized mycelia, 500 µL solution containing 1:3(m/v) protinase K in CTAB buffer (2% cetyl trimethylammonium bromide, 1% polyvinyl pyrrolidone,100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA) was added and vortexed for 30s in a vortex mixer. Then, it was incubated at 65^{o}C for 30 min in a water bath. After incubation the DNA was rested on ice for 15 min. To the cooled NDA 500 µL chloroform isoamyl alcohol (24:1) was added and mixed by rotating the tubes by hand and centrifuged at 13200 rpm for 10 min. The tubes were removed from the centrifuge and 300 µL upper aqueous phases was taken to new tubes. An equal volume (300 µL) of isopropanol was

added and mixed gently. Then it was centrifuged at 13200 rpm for 10 min. The supernatant was discarded with micropipette and the pellet was washed with 400 mL of 70% ethanol by centrifugation at 13200 rpm for 1 minute (to remove the isopropanol). After centrifugation, the supernatant was discarded and the pellet was left to dry in the tubes for 15 min in fume hood. After drying, the DNA was resuspended in 50 µL Tris EDTA and maintained at -20 ^{o}C. DNA concentrations of all samples were determined using Quantus fluorometery, Promega USA according to the machines user manual. Different concentrations ranges were taken and tested for suitability of PCR. Master mix consisting of 14.5 µL H_2O (Sigma), buffer 5 µL, dNTPs 1.25 µL, primers 1 µL each (ITS11 and ITS4) and Taq polymerase 0.125 µL was prepared per samples. The master mix (22.85 µL) was dispensed in reaction plates and 2 µL templet DNA from each sample was added to respective reaction plates. The reaction plates were sealed by a parafilm and all the above procedures were done on ice. PCR amplification condition described in section 3.3.3.2 was followed. The PCR products were subjected to gel electrophoresis on 1.5% agarose gel with 1-2 µL of loading dye (6X) at 120V for 45 minutes with DNA mass ruler (Thermo Scientific Gene Ruler, 100 bp DNA Ladder). At the end of 45 minutes the gel was submerged in ethidium bromide (0.5 µg/mL) for 30 minutes. DNA bands on the gel were captured using Gel Doc XR+ system integrated with Image Lab^{TM} (BioRad Molecular Imag Gel DecxR, USA).

3.3.3.2. DNA sequencing

Fungal DNA was extracted as described in section 3.3.3.1 and amplified using ITS1 and ITS4 primers indicated in Table 3.1. PCR thermal protocol was as follows: initial 5 min denaturation at 95 °C, 32 amplification cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 60 s, and a final extension step of 72 °C for 10 min in a total volume of 50 µL reaction mix and

2µL temple DNA. PCR products of 5µL were loaded to 1.5% agarose gel with 2µL of 6x DNA loading dye (Life Technologies) and with 100 bp DNA mass ruler (Thermo Scientific) to assess suitability for sequencing. The gel was ran for 45 minutes at 120V and socked in ethidium bromide tank (0.5 µg/mL) for 30 minutes. Images of DNA bands were captured using Bio Rad Molecular Image Gel Dec XR^+ (USA) with Image Lab Version 5.0 build 18 software. The samples were purified with the help of E.Z.N.A. Cycle Pure Kit, OMEGA Bio Tek based on the manual provided by the manufacturer. After cold storage for an hour at -80^0C the DNA samples were freeze dried for an overnight and sent to sequencing company (Macrogen, Korea). PCR reactions were performed in GeneAmpR PCR system 9700 (Applied Biosystem, Singapore). The identity of each isolate was determined by comparing the rDNA sequences with the data in GenBank (NCBI) Standard Nucleotide BLAST (https://blast.ncbi.nlm.nih.gov) based on the best score.

3.3.3.3. Identification of *Aspergillus* isolates using species specific primers

Aspergillus isolates were identified using the primer pairs indicated in Table 3.1. The PCR conditions for *A. ochraceus* and *A. westerdijkiae* were according to Gil-Sema *et al.* (2009) with some modification. For *A. ochraceus*, the PCR cycle comprised 5 min at 95 °C, 35 cycles for 30s at 95°C, 30s at 62°C, 40s at 72°C and finally 3 min at 72°C. For *A. westerdijkiae* it was 5 min at 95°C, 30 cycles for 30 s at 95°C, 30s at 63°C, 40s at 72°C and finally 5 min at 72°C. Amplification conditions for *A. niger* was 95°C for 5 min, 35 cycles at 95°C for 30s, 62 to 72°C gradient for 30s, 72°C for 30s and 72°C for 5 min in BioRad T100 Thermal Cycler 2013, Singapore. PCR products were ran on agarose gel and visualized as described in section 3.3.3.2.

Table 3.1. Species specific primers use in PCR

Species	Primers	Size	Sequence	References
A. ochraceus	OCHRAF	430 bp	5′ CTTTTTCTTTTAGGGGGCACAG 3′	Gil-Serna *et al.*,
	OCHRAR		5'CAACCTGGAAAAATAGTTGGTTG 3′	2009
A. westerdijkiae	WESTF	430 bp	5′ CTTCCTTAGGGGTGGCACAG 3′	Gil-Serna *et al.*,
	WESTR		5'CAACCTGATGAAATAGATTGGTTG 3′	2009
A. niger	AnF	357 bp	5’GGATTTCGACAGCATTTTCCAGAACG’3	Palumbo and
	AnR		5’GATAAAACCATTGTTGTCGCGGTCG3’	O’Keeffe, 2014
Specific to fungi	ITS1F	600 bp	5’TCCGTAGGTGAACCTGCGG3’	Manter and
	ITS4		5’TCCTCCGCTTATTGATATGC3’	Vivanco, 2007

3.3.4. Determination of OTA and AFs production capacity using ELISA kit

To assess the mycotoxin production (OTA and AFs) of isolates 1.5mL yeast extract sucrose broth was used in 2mL plastic walls. Loop full spores from seven-day old cultures were used as inocula and grown for 5-7 days at 25 °C. On the seventh day the culture filtrates were transferred in to 2mL tubes using micropipette and stored at -20^{0}C for mycotoxins analysis as described below.

Fungal culture filtrates (100 µL) were diluted 10 fold with Sigma Aldrich reagent water and 150 µL of diluted filtrate was extracted with 350 µL of 100% methanol in 200 µL Eppendorf tubes by vortexing for 30 seconds. The resulting solution was tested for OTA and total AFs with Agra Quant Ochratoxin Assay and Agra Quant Total AFs Assay ELISA kit (Romer Labs) respectively. ELISA Method validations (Figure 3.6) for OTA and AFs were as follows: five levels (0, 2, 5, 20 and 40 ppb for OTA and 0, 4, 10, 20 and 40 ppb for AFs) were used to validate the method. All correlation coefficients were above 0.99 and 50% inhibitions for OTA and AFs were 6.98 and 9.7 ppb respectively. LOD and LOQ were 1.9

and 2 ppb respectively for OTA and average recovery for 10 and 30 ppb were 86±2 and 98±1%. LOD and LOQ for AFs were 3 and 4 ppb with recovery above 101±7%.

For OTA analysis in coffee, one hundred grams of coffee sample was ground till 50% pass through a 20 mesh screen (0.85 mm) and 10 grams were sub sampled for OTA analysis. The samples (10 gram) were vigorously mixed with 80% acetonitrile in distilled water for 5 minutes. The supernatant was filtered to clarify. The clarified extracts were dilute to 10:1 with 70 % methanol in distilled water and OTA analysis was conducted as described in the manual of Sigma Aldrich OTA Elisa Kit for coffee, all analyses were done in triplicate.

3.3.5. Data analysis

For statistical evaluation of the data R statistical software version 3.0.3. (2014.0306) was used. To assess correlations Spearman rank correlation or Pearson correlation analysis was performed. To test the influence of the environmental predictive factors on the response factors (percentage of fungal incidence and OTA content) a non-parametric Kruskal-Wallis test ($\alpha = 0.05$) was performed (since the assumptions for an ANOVA were violated). In case there were significant differences according to the Kruskal-Wallis test a post-hoc MWU test was done to depict significant impact of factor levels. Ochratoxin A and AF contents of culture filtrates and coffee samples were analyzed using Analytical calibration Log-logit linear curve template provided by the ELISA kit manufacturer.

3.4. Results

3.4.1. Fungal incidence and environmental factors influencing fungal load

Mycological analysis of 74 coffee samples indicated that 87% of the samples were infected with fungi (Figure 3.2). The genus *Aspergillus* was predominantly present (79%; Figure 3.3), followed by *Fusarium* (8%) and *Penicillium* (5%). Eight percent of the samples contained with other fungal genera such as *Trichoderma* spp., and *Rhizopus* spp.

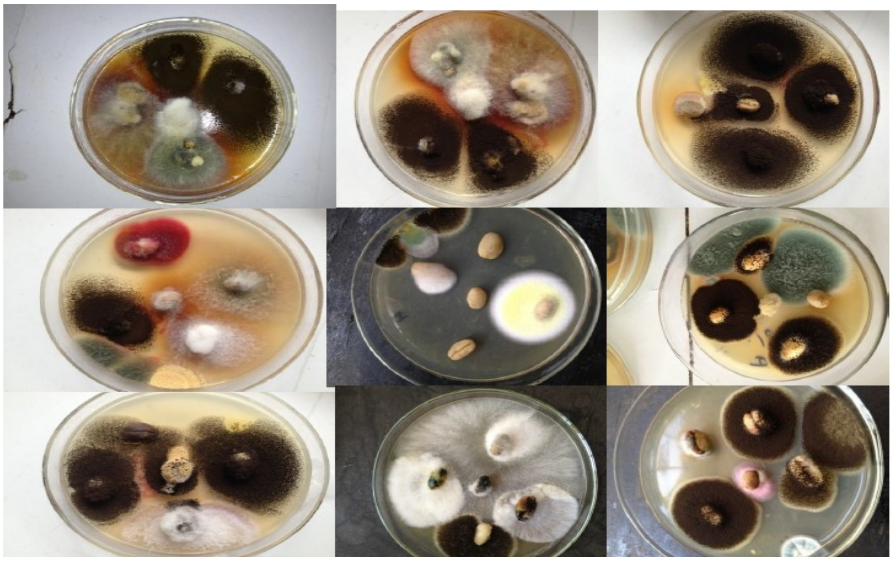

Figure 3.2. Fungal contamination on coffee samples

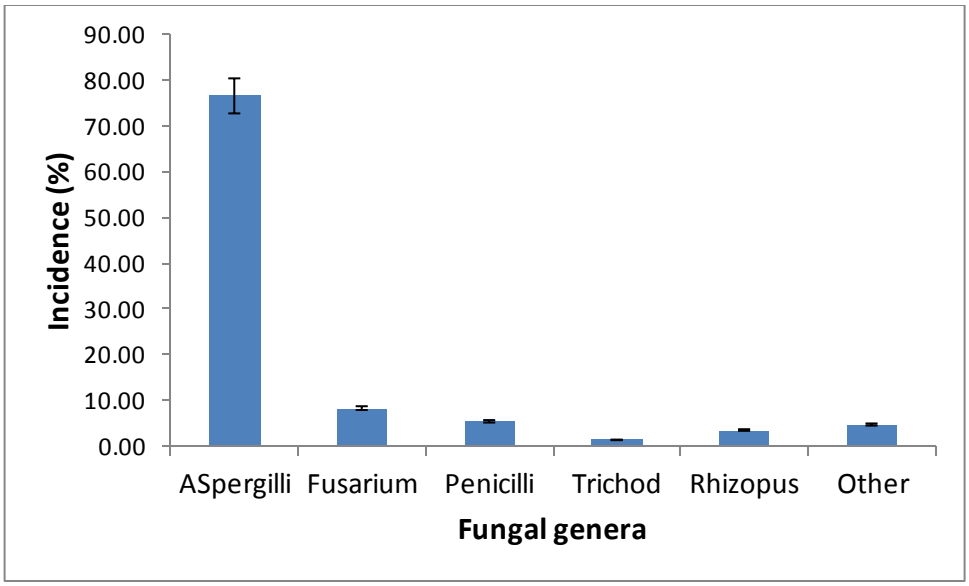

Figure 3.3. Percentage of fungal incidences on coffee bean samples. Error bars with 5% of the values.

All environmental predictive variables considered during sample collection are indicated in Table 3.2. Statistical analysis based on the data represented in Table 3.2 revealed that there was a clear correlation between fungal incidence and moisture content. The Spearman rank correlation between fungal incidence and moisture content was 0.524 ($p < 0.001$). The correlation between moisture content and OTA concentration was not significant (Spearman's rank correlation coefficient = 0.004; $p < 0.897$).

Via a non-parametric Kruskal-Wallis rank sum test (Table 3.5)the effects of processing method, coffee sample type, storage material and storage time on the fungal incidence was assessed. Based on this analysis there was a statistical effect of respectively coffee sample type ($p < 0.005$), processing method ($p < 0.002$), the storage packaging ($p < 0.035$) and the storage time ($p < 0.002$) on fungal incidence. Post-hoc analyses (Table 3.5) on the processing methods showed that fungal incidence was highest for coffee samples dried on soil ground, samples dried on concrete structures or samples dried on traditional spreading

materials. Coffee samples obtained from wet processing were significantly less contaminated by fungi (Table 3.4). Although samples from wet processing resulted in low median levels of OTA (Table 3.4), they were not significantly different from other processing types ($p < 0.306$). With regard to sample type, the parchment coffees samples had significantly lower fungal incidence (59%) compared with cherry (94%) and green bean (88%) but there were no differences in OTA levels between the three sample types. Evaluation of storage packaging (sisal sacks, fertilizer bags and plastic bags) revealed that there was a significant impact on fungal incidence ($p < 0.035$). Mean incidence obtained for fertilizer bag (91%), sisal sacks (87%) was significantly different from the fungal incidence in plastic bags (73%). Finally, the highest fungal incidence was observed on samples stored for four months and a gradual decline of fungal load was observed with a prolonging storage.

Table 3.2. Distribution of fungal genera and their incidence (%) on coffee samples obtained from various sources

Location	Sample ID	Process type	Matrix	Period (months)	Moisture (%)	Packing material	Incidence (%)	OTA (µg/kg)	Toxigenic fungal species isolated from the commodities											
									Aspergillus								*Fusarium*		*Penicillium*	
									A. fl	*A. fum*	*A. mel*	*A. nig*	*A. ochr*	*A. steyn*	*A. tub*	*A. west*	*F. ver*	*F. gr*	*P. bre*	*P. ver*
Dedo	ETDE1	DC	Beans	4	12	FB	100	ND					1							
Dedo	ETDE2	DC	Beans	4	13	FB	100	1.2												
Dedo	ETDE3	DC	Beans	4	13	FB	100	0.72												
Dedo	ETDE4	DC	Beans	4	14	FB	100	2.56												
Dedo	ETDE5	DC	Beans	4	13	FB	100	3.05												
Dedo	ETDE6	DC	Beans	6	12	FB	80	2.98												
Dedo	ETDE7	DSG	Cherry	6	10.1	FB	88	ND												
Dedo	ETDE8	DSG	Cherry	6	12.7	FB	92	2.54				1	1					1		
Dedo	ETDE9	DSG	Cherry	6	14	FB	100	1.2												
Dedo	ETDE10	DSG	Cherry	6	11	FB	88	1.78					4	1		3				
Gomma	ETGO1	DC	Beans	4	14	SS	100	ND												
Gomma	ETGO2	DC	Beans	4	14	SS	100	ND												
Gomma	ETGO3	DC	Beans	6	13	FB	100	ND												
Gomma	ETGO4	DC	Beans	4	13	FB	100	ND												
Gomma	ETGO5	DC	Beans	4	13	PB	100	ND								1				
Gomma	ETGO6	WP	Beans	4	12.4	PB	48	ND												
Gomma	ETGO7	WP	Parchment	6	13.4	PB	48	0.12												
Gomma	ETGO8	WP	Parchment	6	13.4	PB	52	22.64												
Gomma	ETGO9	DC	Beans	3	10.2	SS	60	ND												
Gomma	ETGO10	DC	Beans	4	13	FB	92	ND				1								
Shebe	ETSH1	DT	Beans	6	10	FB	80	0.03												
Shebe	ETSH2	DT	Beans	6	9.8	FB	24	0.56						1						
Shebe	ETSH3	DT	Beans	6	10.5	FB	72	1.66												

Shebe	ETSH4	DT	Beans	6	10.4	FB	88	ND						
Shebe	ETSH5	DT	Beans	6	13	FB	96	ND						
Shebe	ETSH6	DT	Beans	6	12.8	FB	96	1.07						
Shebe	ETSH7	DT	Cherry	3	10	FB	92	ND						
Shebe	ETSH8	DT	Beans	3	13	SS	100	5.94						
Shebe	ETSH9	DT	Beans	3	12	FB	100	1.76						
Shebe	ETSH10	DT	Beans	3	13	FB	100	0.69						
Seka	ETSE1	DC	Beans	6	13.5	FB	68	ND						
Seka	ETSE2	DC	Beans	6	13	FB	100	ND						
Seka	ETSE3	DC	Beans	6	14.6	FB	88	0.64						
Seka	ETSE4	DC	Beans	6	16.2	FB	88	0.95						
Seka	ETSE5	DC	Beans	6	13.8	FB	100	1.46						
Seka	ETSE6	DC	Beans	6	16.3	FB	88	ND						
Seka	ETSE7	DC	Beans	6	14.8	FB	100	2.81						
Seka	ETSE8	DSG	Cherry	6	11.3	FB	100	22.9						
Seka	ETSE9	DSG	Cherry	6	14	FB	100	1.39						
Seka	ETSE10	DSG	Cherry	6	13.7	FB	100	0.1						
Limmu	ETLI1	DC	Beans	6	12	PB	100	ND						
Limmu	ETLI2	DC	Beans	6	14	PB	100	0.42						
Limmu	ETLI3	DC	Beans	4	13	SS	92	ND	1		1			
Limmu	ETLI4	DC	Beans	4	9.4	SS	56	1.02	2					
Limmu	ETLI5	DC	Beans	4	13	SS	100	ND	1	1	1			
Limmu	ETLI6	DC	Beans	4	13	SS	96	ND						
Limmu	ETLI7	DSG	Cherry	4	12	SS	96	ND	1				1	
Mana	ETMA1	DT	Beans	6	12	SS	92	1.61	1					1
Mana	ETMA2	DT	Beans	6	13	SS	92	2.54						
Mana	ETMA3	DT	Beans	6	11.4	SS	76	1.98	1		2			
Mana	ETMA4	DT	Beans	6	12	SS	92	1.87						
Mana	ETMA5	DT	Beans	6	12	SS	92	ND						

Mana	ETMA6	DT	Beans	6	13	SS	96	0.95										
Mana	ETMA7	DT	Beans	6	13	SS	92	0.51										
Mana	ETMA8	DT	Beans	4	12	SS	96	12.29										
Mana	ETMA9	DT	Cherry	4	13	SS	100	3.42										
Mana	ETMA10	DT	Cherry	6	12.2	SS	88	3.6										
Mana	ETMA11	DT	Beans	6	13	SS	100	3.02										
Jtown	ETJT1	DC	Beans	4	13.4	PB	100	3.27										
Jtown	ETJT2	WP	Parchment	6	7	PB	76	1.72								1		
Jtown	ETJT3	WP	Parchment	6	11	PB	88	0.44	2	1	1		5					1
Jtown	ETJT4	WP	Parchment	6	7.1	PB	32	ND					1					
Jtown	ETJT5	WP	Parchment	6	7.2	PB	60	0.87					2					
Addis	ETM1	DC	Beans	13	13.3	SS	84	2.16	1				3					
Addis	ETM2	DC	Beans	13	9	SS	72	2.26					1					
Addis	ETM3	DC	Beans	3	12	SS	80	3.1					3			1	1	
Addis	ETM4	DC	Beans	3	11	SS	80	2.41					3					
Addis	ETM5	DC	Beans	13	11	SS	80	1.53										
Addis	ETM6	DC	Beans	13	12	SS	80	0.6										
Addis	ETM7	DC	Beans	13	12	SS	80	ND										
Addis	ETM8	DC	Beans	13	11	SS	80	ND										
Addis	ETM9	DC	Beans	13	13	SS	88	ND					1			2		
Addis	ETM10	DC	Beans	13	13	SS	72	ND										
Addis	ETM11	DC	Beans	13	12	SS	80	ND										

Jtown = Jimma town, DC=samples dried on concrete structures, DT= samples dried by traditional means (on "selen"), DSG= sample dried on soil ground, WP < wet processed (washed coffee samples), FB= fertilizer bags, PB < plastic bags, SS= sisal sacks, ND = no detectable OTA levels. A. fl: *A. flavus*; A. fum: *A. fumigatus;* A. mel: *A. mellus*; A. nig : *A. niger* ; A. ochr: *A. ochraceus*; A. ste: *A. steynii*; A. tub: *A. tubigenesis*; A. wes: *A. westerdijkiae*; F. ver: *F. verticilioides*; F. gr: *F. graminearum*; P. bre: *P. brevicompactum*; P. ver: *P. veruculosum*

3.4.2. Morphological and molecular characterization of the isolates

In total 63 isolates were obtained in this study are depicted in Table 3.3 (the others belong to the genera *Trichoderma, Rhizopus,* ...and are not mentioned in the Table). Morphological characteristics of pure culture isolates were used as a primary screening for fungal identification. Pure culture *Aspergilli* isolates were grouped to black *Aspergilli* (Section *Niger*), yellow *Aspergilli* (*Circumdati*), blue to green (*Flavi*) based on their cultural and microscopic characteristics. The genus *Penicillium* and *Fusarium* were identified by observing the conidial bearing structures, macro and micro-conidia characteristics.

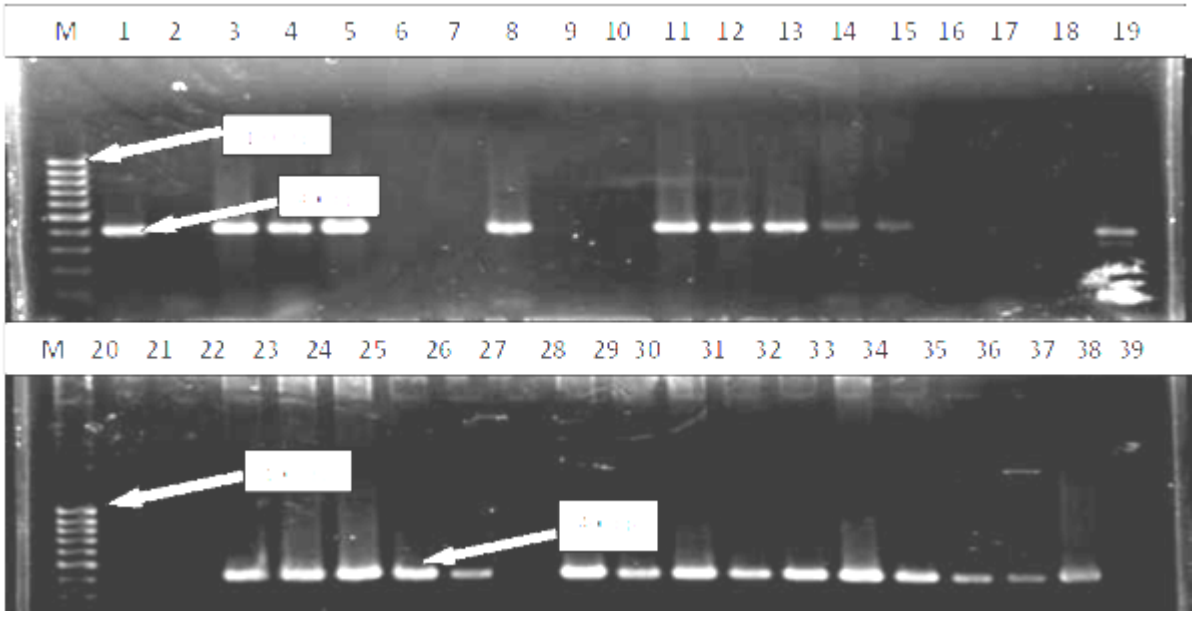

Figure 3.4. PCR assay with primers OCHRA1 and OCHRA2 specific to *A. ochraceus*

N.B. Lane M: 1000 pb GeneRuler, Lanes 1-38 samples DNA. Lanes 1, 3-5, 8, 11-15, 19, 22-26, 28-37 indicates DNA of 400 bp identified as *A. ochraceus*.

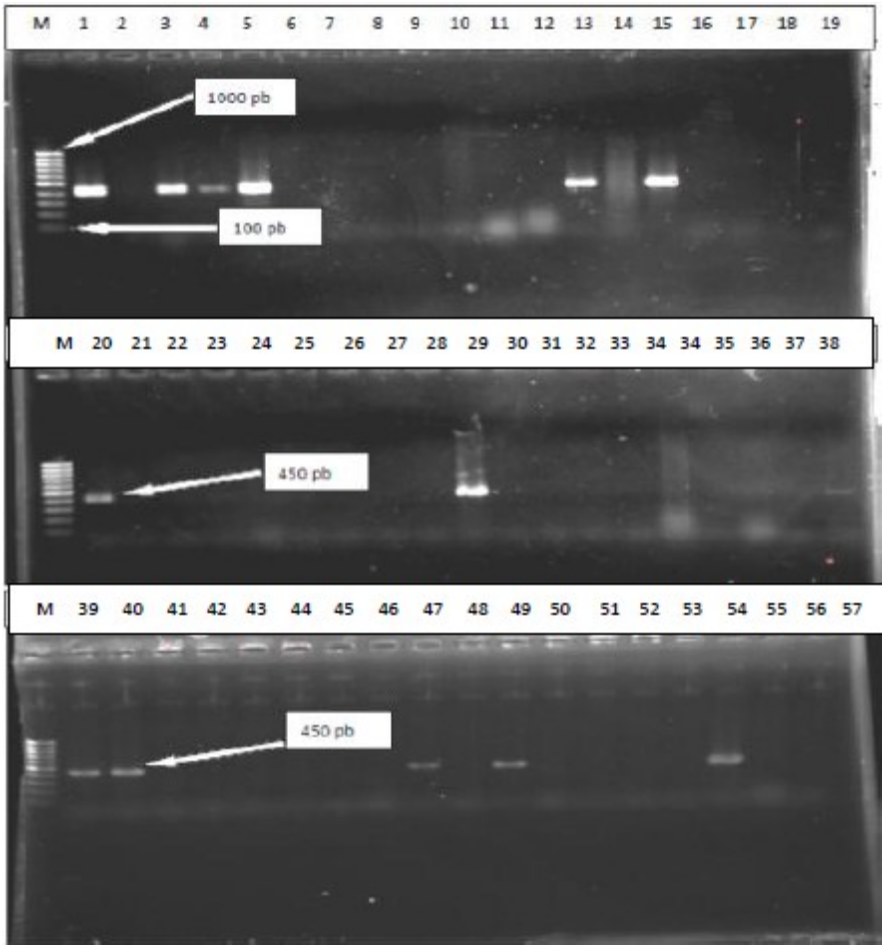

Figure 3.5. PCR assay with primers WESTF and WESTR specific to *A. westerdijkiae*

Lane M: DNA molecular size marker, Lanes 1-56 samples DNA. Lanes no. 1, 3, 4, 5, 13, 15, 20, 29, 39, 40, 47, 49 and 54 indicate DNA of 450 pb, identified as *A. westerdijkiae*.

Using a combined approach of sequencing the ITS1-ITS4 region and specific PCR assays all fungal isolates listed in Table 3.3 were identified to the species level. The presence of *A. flavus*, *A. niger*, *A. fumigatus*, *A. melleus*, *A. westerdijkiae*, *A. ochraceus A. steynii*, *A. tubigenesis*, *F. verticilioides, F. graminearum P. brevicompactum* and *P. verruculosu* was shown in Ethiopian coffee. The identification of the isolates showed convincingly that *A. ochraceus* (Figure 3.4) and *A. westerdijkiae* (Figure 3.5) were the predominant species present in *Arabica* coffee samples.

3.4.3. Ochratoxin A and AFs production potential of isolates

The OTA and AFs production potential of all isolates were assessed based on standardized method (Figure 3.6) when grown in YES medium at 25°C. The results presented in Table 3.3 indicated that 65% of the isolates were capable of producing OTA (10 µg/L to 482 µg/L). Two *A. flavus* isolate ETJT35 and ETM131 were capable of AFs production (19 and 14 µg/L respectively) in YES medium under the tested conditions. As indicated in Table 3.3, six of 12 *A. westerdijkiae* isolates produced very high amounts of OTA (284.19 – 482.14 µg/L), one *A. westerdijkiae* isolate produced relatively low OTA amounts (42 µg/L). The remaining five *A. westerdijkiae* isolates did not produce detectable amounts of OTA in this study. Of the 32 *A. ochraceus* isolates only one isolate produced high concentrations of OTA (134 µg/L OTA) while 26 isolates produced OTA from 10 -58 µg/L in YES medium. The remaining 5 isolates of *A. ochraceus* did not show detectable OTA production in YES medium. There is a huge discrepancy between the levels of OTA production by *A. westerdijkiae* and *A. ochraceus*. Hardly, *A. ochraceus* isolate displayed OTA levels in the range of the OTA levels observed for *A. westerdijkiae* (Table 3.3).

Table 3.3. Mycotoxin production by various fungal species in Yeast Extract Sucrose medium

S.No	Isolate ID	Identity	Mycotoxin production (µg/L)		S.No	Isolate ID	Identity	Mycotoxin Production (µg/L)	
			OTA	AF				OTA	AF
1	ETM91	*A. westerdijkiae*	456	ND	33	ETM36	*F. graminearum*	ND	ND
2	ETJT31	*A. ochraceus*	58	ND	34	ETJT35	*A. flavus*	ND	19
3	ETMA31	*A. westerdijkiae*	473	ND	35	ETDE10R	*F. graminearum*	ND	ND
4	ETDE101P	*A. westerdijkiae*	456	ND	36	ETM131	*A. flavus*	ND	14
5	ETDE102P	*A. westerdijkiae*	433	ND	37	ETM96	*A. ochraceus*	10	ND
6	ETM31	*A. ochraceus*	ND	ND	38	ETLI41	*A. ochraceus*	13	ND
7	ETM32	*A. ochraceus*	ND	ND	39	ETJT36	*P. brevicompactum*	17	ND
8	ETDE1010	*A. steynii*	465	ND	40	ETM111	*A. ochraceus*	21	ND
9	ETLI51	*A. ochraceus*	27	ND	41	ETM41	*A. ochraceus*	19	ND
10	ETM33	*A. ochraceus*	38	ND	42	ETM97	*A. westerdijkiae*	ND	ND
11	ETJT51	*A. ochraceus*	32	ND	43	ETLI52	*A. westerdijkiae*	ND	ND
12	ETJT52	*F. graminearum*	ND	ND	44	ETLI74	*A. ochraceus*	10	ND
13	ETM34	*A. westerdijkiae*	284	ND	45	ETM42	*A. ochraceus*	11	ND
14	ETDE102	*A. westerdijkiae*	42	ND	46	ETM37	*A. ochraceus*	15	ND
15	ETM92	*A. westerdijkiae*	482	ND	47	ETLI43	*A. ochraceus*	15	ND
16	ETDE100	*A. ochraceus*	ND	ND	48	ETM18	*A. ochraceus*	15	ND
17	ETJT33	*A. fumigatus*	ND	ND	49	ETMA11	*P. verruculosum*	14	ND
18	ETDE103	*A. ochraceus*	ND	ND	50	ETGO55	*A. westerdijkiae*	ND	ND
19	ETDE105	*A. ochraceus*	10	ND	51	ETM21	*A. ochraceus*	18	ND
20	ETMA32	*A. westerdijkiae*	ND	ND	52	ETLI33	*A. westerdijkiae*	ND	ND
21	ETDE104	*Aspergillus sp.*	ND	6	53	ETLI45	*A. ochraceus*	10	ND
22	ETM35	*A. ochraceus*	20	ND	54	ETM38	*A. ochraceus*	12	ND
23	ETDE106	*A. ochraceus*	18	ND	55	ETSH23	*A. steynii*	15	ND
24	ETLI73	*P. brevicompactum*	15	ND	56	ETDE109	*A. ochraceus*	19	ND
25	ETLI53	*A. tubigenesis*	ND	ND	57	ETM22	*P. verruculosum*	ND	ND
26	ETDE81	*A. niger*	11	ND	58	ETDE82	*G. moniliformis*	ND	ND
27	ETDE83	*A. ochraceus*	14	ND	59	ETJT34	*A. ochraceus*	11	ND
28	ETM12	*A. ochraceus*	13	ND	60	ETM43	*A. ochraceus*	11	ND
29	ETJT53	*A. ochraceus*	134	ND	61	ETJT32	*A. ochraceus*	12	ND
30	ETM121	*A. ochraceus*	14	ND	62	ETGO10	*A. niger*	420	ND
31	ETLI32	*A. ochraceus*	ND	ND	63	ETJT37	*A. melleus*	ND	ND
32	ETJT54	Fungal sp.	ND	ND					

ND = no detectable amounts of ochratoxin A (OTA) or aflatoxin (AT).

One *A. steynii* isolate ETDE1010 and one *A. niger* isolate ETGO10 produced 465 and 420 µg/L OTA (Table 3.3). Two isolates of *P. brevicompacutum*, and an isolate of *A. steynii* produced OTA in the range of 15-17 µg/L. *A. westerdijkiae* species produced significantly higher OTA as compared to *A. ochraceus* isolates ($p < 0.001$) under the conditions tested. However, *A. ochraceus* species were more abundantly present than *A. westerdijkiae* species in the coffee samples.

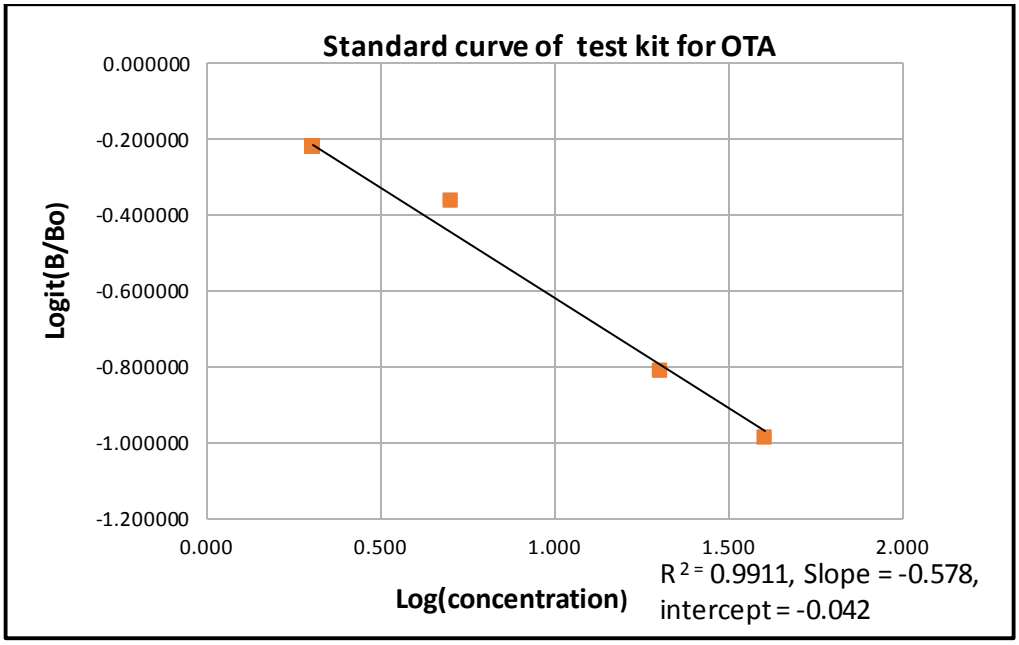

Figure 3.6. Calibration curve at o, 2, 5, 20, and 40 ppb for ELISA method validity.

Log (concentration) = log of standard concentrations (0, 2,5,20 and 40 ppb). Logit (B/B_0) = method of linearizing dose (concentration) response (absorbance) curve. (inverse of A = Transmitance).

Table 3.4. processing methods vs fungal incidence with OTA level

Processing methods	Fungal incidence (%)		OTA level (µg/kg)
Dry on concrete	Max	100	3.27
	Min	56	0
	Mean	89.05	2.66
	Median	92**a**	1.498**a**
	SD	12.466056	1.097621
Dry on soil ground	Max	100	22.9
	Min	88	0
	Mean	95.5	4.4
	Median	98**a**	1.391**a**
	SD	5.424811	8.196432
Dry traditional means	Max	100	12.88
	Min	24	0
	Mean	88.77	2.55
	Median	92**a**	1.758**a**
	SD	16.762771	2.8963
Wet/washed/	Max	88	22.64
	Min	32	0
	Mean	57.7	0.53
	Median	52**b**	0.443**a**
	SD	18.883099	8.382132

N.B. Values with different characters are different at 0.05 levels

Table 3.4 showed that median OTA level for the differenct processing methods was not significantly different. However, median OTA level was relatively higher for samples obtained from dry processed (dryed by traditional meanses)

3.4.4. Ochratoxin A content of coffee samples

As indicated in Table 3.2, OTA was detected in 64 % of the coffee samples. The OTA level in the contaminated samples ranges from 0.03-22.9 µg/kg with a median of 1.5 µg/kg (Table 3.2). Four samples (8.7%) were contaminated above the limit of 5 µg/kg (EC, 2010) set for roasted coffee (Table 3.2).

Table 3.5. Kruskal-Wallis rank sum test

Predictive factor vs. Response	chi-squared	df	p-value
percentage infected vs. processing method	15.461	3	0.00146
percentage infected vs. storage type	9.7924	2	0.00748
percentage infected vs. storage material	6.7223	2	0.03469
percentage infected vs. coffee sample type	12.6353	3	0.00549

Multiple comparison test after Kruskal-Wallis

Comparisons	obs.dif	critical.dif	difference
Dry on concrete -Dry on soil ground	8.8717105	22.07065	FALSE
Dry on concrete - Dry traditional	0.5181704	15.42757	FALSE
Dry on concrete –Washed parchment	30.422932	23.33665	TRUE
Dry on soil ground-Dry traditional	9.389881	23.57314	FALSE
Dry soil ground-Washed parchment	39.294643	29.36464	TRUE
Dry traditional-Washed parchment	29.904762	24.76244	TRUE
Mill stores -Resident	17.170027	13.99817	TRUE
Mill stores -Shop	2.747878	15.00043	FALSE
Resident-Shop	14.422149	15.80986	FALSE
Fertilizer bags – Plastic bags	16.255682	17.99448	FALSE
Fertilizer bags – Sisal sacks	10.792339	12.9745	FALSE
Plastic bags-Sisal sacks	5.463343	18.06857	FALSE
Beans-Cherry	6.679825	40.81752	FALSE
Beans-Parchment	28.486842	24.35175	TRUE
Cherry -Parchment	37.194444	29.90348	TRUE

NB. obs.dif = observed difference, critical.diff = critical difference, df = degrees of freedom, TRUE = significant difference in percentage of fungal incidence exist between the paired factors, FALSE = no significance difference existed between the paired factors.

3.5. Discussion

In the present study, the presence of fungi and OTA in coffee samples for the local market in Ethiopia has been assessed. The results of this study indicated that the overall fungal incidence was 87 %. This result was nearly similar to results by Fikre *et al.* (2015) (80%) and El Aaraj *et al.* (2015) (87 %) who evaluated fungal incidence from coffee samples obtained from Limmu (Ethiopia) and from Morocco respectively. However, 100 % incidence was reported by Urbano *et al.* (2001) on Robusta coffee samples by the direct plating technique.

In search for an explanation for this high incidence, the present study assessed the influence of several environmental storage parameters. A statistically positive correlation was obtained between fungal incidence and moisture content of coffee samples but this could not be confirmed for OTA. Nevertheless, the huge range of moisture contents in coffee samples (7% to 16.3%) illustrates eventual aberrancies during the drying process.

Significant variation in fungal incidence was observed between the different coffee processing types (Table 3.5). Wet processed coffee samples were less contaminated with fungi as compared to dry processed coffee samples. This effect might be attributed to the early removal of coffee pulp during the washing process, the extra drying steps on drying beds, and the better storage conditions that are used during this type of coffee processing (Batista *et al.*, 2003; Silva *et al.*, 2008; Kleinwachter and Selmar, 2010). Compared to wet processed coffee samples, coffee samples dried on soil ground were infected more frequently with fungi. This type of coffee is dried on soil ground and as such is prone to contamination with post-harvest spoilage fungi such as *Aspergillus* and *Penicillium* which

are omnipresent fungal genera. This result is concordant with Suarez *et al.* (2003) who studied coffee samples in Mexico and reported fungal incidence between 80 % for wet processed coffee compared to 92% for dry processed coffee.

Of all fungal genera *Aspergillus, Fusarium* and *Penicillium* were dominantly present with an incidence 79 %, 8 %, and 5 %, respectively. The presence of the genus A*spergillus*, *Penicillium* and *Fusarium* in association with coffee samples has been previously reported by other authors in Ethiopia (Silva *et al.,* 2008; Alemu, 2014; Fikre *et al*., 2015). In all of these studies the genus *Aspergillus* was by far predominantly present. However, the genus *Aspergillus* occurs in much higher percentage (79 %) in this study as compared to the findings of Alemu (2014) (49 %). A striking difference on the level of *Aspergillus* species present is the dominance of *A. ochraceus* and *A. westerdijkiae.* Alemu (2014) previously reported on the dominance of *A. niger* (section *Nigri*) on coffee beabs. In an analogous study in Thailand, Noonim *et al.* (2008) showed that *Aspergilli* from section *Circumdati*, including *A. westerdijkiae* and *A. melleus* predominated in the North of Thailand whereas section *Nigri* dominated (75 %) in coffee samples from Southern Thailand. A correlation between species and geographic origin was not encountered in current study although the sampling area was probably too small to draw conclusions on this issue.

The majority of the publications on ochratoxigenic fungi (Frisvad *et al*., 2004a; Noonim *et al*., 2008; Gil-Serna *et al*., 2009; Rezende *et al.,* 2013) revealed that the most potent OTA producing fungi belong to the section *Circumdati* (yellow *Aspergilli*) which includes the species of *A. westerdijkiae*, *A. ochraceus*, *A. melleus*, *A. steynii.* Current study revealed for the first time OTA presence in coffee consumed by the local people and the presence of ochratoxigenic fungi in *Arabica* coffee beans using molecular techniques. *A. westerdijkiae*,

A. ochraceus, *A. niger A. melleus*, and *A. steynii* were the predominant OTA producing species. Additionally this is the first investigation in Ethiopia to reveal the presence of potent toxigenic fungi *A. westerdijkiae*, *A. melleus* and *A. steynii* in Ethiopian coffee.

Using an *in vitro* approach, OTA and AFs producing potential of isolates were assessed. Seventy one percent of the *Aspergillus* isolates produced OTA (10 µg/L to 482 µg/L) on YES medium. *A. westerdijkiae* was the most potent producer of OTA under the tested conditions although not all isolates produced detectable amounts of OTA in YES. This result is in agreement with the findings of Frisvad *et al.* (2004a), Samson *et al.* (2006) and Gil-Serna *et al.* (2009) who showed that *A. ochraceus*, *A. westerdijkiae* and *A. steynii* were the predominant OTA producing species in coffee. The lower production potential of *A. ochraceus* as compared to *A. westerdijkiae* was remarkable. Of the 32 *A. ochraceus* isolates, only one isolate ETJT51 produced higher (134 µg/L) amounts of OTA. Almost all (99%) *A. ochraceus* isolates produced low (84 %) or no (15%) OTA (10 – 58 µg/L) in YES medium. The *Nigri* section within *Aspergillus* was predominantly present in this and other studies including Pardo *et al.* (2004), Noonim *et al.* (2008) and El Aaraj *et al.* (2015). Still, *A. carbonarius* which is the main OTA producer from section *Nigri* in the cool climate (Taniwaki *et al.*, 2003; Leong *et al.*, 2004) was not detected in this study.

Isolates ETLI73 and ETJT36 identified as *P. brevicompactum* and ETMA11 identified as *P. verruculosum* belong to the genus *Penicillium* and produced significant amount of OTA (14-17 µg/L) in YES medium implying that this genus is also important when considering OTA contamination in Ethiopian coffee. Vega *et al.* (2006a) reported the production of OTA by *P. brevicompactum* in Yeast Malt Agar media contrary to the report of Geisen *et al.* (2004). Secondary metabolite production depends on various factors including growth medium

composition. Since, YES medium supplemented with trace metal (Frisvad, 1981) as recommended by Visagie *et al.* (2014) and Samson *et al.* (2014), was used in this study, suitability of this media might explain discrepancies in reports on toxin producing capacity.

With respect to AFs, only two isolates ETJT35 and ETM131 identified as *A. flavus* showed AF production at 19 and 14 µg/L levels in YES medium respectively. The co-occurrence of ochratoxigenic and aflatoxigenic *Aspergilli* implies the possible co-existence of OTA and AFs in coffee samples. Although the effect of OTA and AFs on human health is well-documented, the combined toxic effect of these mycotoxins on human health is not clearly known and synergistic or additive effects might be possible.

As indicated on Table 3.2, despite the median OTA level is lower than the regulatory limit (5 µg/kg), 64% (46/72) of coffee samples were positive for OTA and this signifies the potential risk of this toxin on coffee consumers as OTA gradually accumulates in the blood plasma. Blood plasma OTA level study will be very important to better understand the exposure level of coffee consumers in the future.

3.6. Conclusion and recommendations

Coffee produced in Ethiopia and sold for local consumption is contaminated with various filamentous fungi. The dominant fungal genera associated with Ethiopian coffee were the genera *Aspergillus*, *Fusarium* and *Penicillium*. Among the genus *Aspergillus* the toxigenic species of *A. westerdijkiae*, *A. ochraceus, A. steynii, A. niger*, and *A. flavus* were present and this was reflected by toxin detection in the coffee samples. However, the dominant toxigenic species were *A. westerdijkiae* and *A. ochraceus.*

Coffee samples were also found contaminated with OTA and samples obtained from dry processing were found to be more contaminated with fungi and OTA. The median OTA level (1.5 µg/kg) in the locally sold Ethiopian coffee obtained in this study is less than half of the EU tolerable limit (5 µg/kg) set for roasted coffee although for some samples the threshold value was exceeded. The differences in median level of OTA contamination between dry and wet processed coffee were not significant. It is highly recommended that, coffee intended for local consumption should be appropriately dried, packed and stored to maintain the quality and safety of coffee. A shift towards wet coffee processing method is a better strategy to reduce fungal invasion and mycotoxins contamination to minimize the risks associated with OTA consumption by local people.

CHAPTER IV. The association of coffee bean defects with Ochratoxin A in the beans: A possible indicator of ochratoxin A contamination

Abstract

Consumption of low quality coffee beans expose consumers to ochratoxin A (OTA) associated health risks of which toxicity of the kidney (nephrotoxicity) is the major problem. A simple method of screening and ruling out of OTA contaminated coffee beans using visual and olfactory senses is necessary for local consumers. The purpose of this study was to examine the various types of coffee bean defects existed in the locally consumed coffee in relation to OTA contamination and search possible physical indicator to identify beans with OTA contamination. Coffee bean samples were evaluated visually for the presence of defective beans and the defects were given scores based on SCAA (Specialty Coffee Association of America) and Ethiopian Ministry of Agriculture and Rural Development Coffee and Tea Quality Control and Liquoring Center manuals. The samples were also assessed for fungal incidence and OTA contamination levels. Spearman's rank correlation and non-parametric Kruskal-Wallis rank sum tests were used for statistical analysis using R software version 3.0.3 (2014-03-06). Significant positive correlation ($r = 0.53$, $p < 0.00$) existed between coffee bean defect points and OTA contamination levels. Significant difference existed in percent of fungal incidence ($\chi^2 = 14.87$, $p < 0.01$) with in the processing method. No statistical significant difference existed in median bean defect point within the processing methods ($\chi^2 = 1.10$, $p < 0.78$), storage types ($\chi^2 = 2.58$, p-value = 0.28), and storage material ($\chi^2 = 3.71$, $p < 0.16$). The predominant coffee bean defects encountered in this study in descending order were black beans (47.5 %), cherry (13.5 %),

immature bean (8.1 %), sour bens (7.9%), insect infested (7.9 %), moldy beans (4.4 %), and broken beans (3.7%). Ochratoxin A contamination levels in coffee beans were positively correlated with black beans *($r = 0.33$, $p < 0.03$)*, visible mold overgrowth ($r = 0.80$, $p < 0.00$), beans damaged by insects ($r = 0.71$, $p < 0.00$), and presence of husk ($r = 0.67$, $p < 0.00$). Visual inspection of the incidence of these bean defects is a reliable indication of OTA contamination in the beans. Therefore, from this study it is possible to recommend to local coffee rosters and consumers to avoid coffee beans with sign of mold overgrowth (musty odor), black beans, contains husk and damaged by insects as these types of coffee beans are most likely contaminated with OTA and causes health risk.

Key words: Ochratoxin A, coffee bean, local consumption, bean defects.

4.1. Introduction

Coffee consumption in the world was estimated to nearly nine million tons in 2015 with an average global consumption growth rate of 2% (ICO, 2006). Coffee is produced by more than 60 developing countries and the earnings from coffee exports are of vital importance to these countries. Coffee is an important means of social development and a great source of rural employment, providing a livelihood for some 100 million people around the world (Nicolas, 2007; Pedegrast, 2010; Gray *et al*., 2013).

Ethiopian government is strongly seeking to boost coffee production in order to increase the country's exportable supplies of coffee, which is in turn expected to maximize the generation of foreign exchange. Undoubtedly, this may have negative impact on local coffee consumption. However, coffee drinking remains an essential part of the social and cultural habit of the country. In terms of per capita consumption, Ethiopia is the largest coffee consuming country in Africa and 50% of nationally produced coffee is consumed locally (Tadesse, 2015).

Most of the coffee consumed locally is of lower quality since some of the beans have been originally destined for export but often rejected from export. Interestingly, even though it may be a lower quality than what is exported, the price of coffee in the local marketplace is sometimes higher than the international price. This high price of substandard coffee on the local market has pushed some consumers, particularly those with low purchasing power to utilize even poorer quality beans with very high defects that are characterized by mix of black, sour, broken, discolored, insect damage, moldy, husk, cherry, soil, stick, stone, off odor, Wanza (seed of *Cordia africana*) and others. In worst case this segment of the local

community was even forced to make coffee beverage from the husk (skin) of coffee cherry as an alternative to coffee beans (Abu, 2015a).

Coffee bean defects are described as foreign materials of non-coffee (e.g., stones/sticks) and non-bean origin (e.g., husks/hulls), abnormal beans regarding shape and visual appearance, such as black beans or any defect that impairs brewed coffee taste and flavor (Leroy *et al*., 2006). Coffee bean defects can be divided into primary and secondary defects. Black beans and sour beans are usually identified as primary coffee bean defects. Secondary defects principally include broken beans, insect-damaged, faded, green, moldy and silver skinned beans. Bee *et al*. (2005) and Agresti *et al*. (2008) have demonstrated that coffee cup quality is strongly compromised by bean defects since they reduce significantly the characteristic quality of coffee beverage. Bay and large, several kinds of coffee bean defects are encountered in locally sold coffee beans compared to the export portion in coffee growing countries like Ethiopia and Brazil (Mendonca *et al*., 2008).

Insect infestation of coffee beans can severely reduces both the physical and organoleptic quality of the beans. Moreover, insects have been shown to serve as vectors for a wide array of fungi. In some cases, insect disseminated fungi produce potent toxins in the host plant, which might create sever problems to this agribusiness (Kulandaivelu *et al*., 2010). Studies indicate that the coffee berry borer (CBB), *Hypothenemus hampei* (Ferrari) (Coleoptera: Scolytidae) could disseminate toxigenic fungi in coffee plantations (Kulandaivelu *et al*., 2010). Once the fungi get their way through the cherry, the spores can remain within the holes created by the insect on the beans after harvest and processing. Inappropriate drying

and poor storage conditions aid the spores to germinate and increase mycotoxin concentration in the beans.

Beside insect damage, extrinsic coffee bean defects including presence of soil, stone, stick, and husk can serve as means for soil fungi to get into the beans. Based on this background information it can be hypothesize that coffee beans with higher defect points may contain higher fungal incidence and OTA contamination compared to coffee beans with standard quality. There might be coffee bean defects that can be used as indicator for OTA contamination in the beans and these defects may serve as possible visual/physical marker for screening OTA contaminated coffee beans during coffee purchase in the local markets.

4.2. Objectives

4.2.1. General objective

The main goal of this study was to search for possible visual/physical method of discriminating OTA contaminated coffee beans that can easily be used by local people to avoid health risks associated with consumption of OTA contaminated coffee beans.

4.2.2. The specific objectives of this study were to:

- examine coffee bean defect types and corresponding OTA contamination levels in the locally sold Ethiopian coffee beans destined for local consumption
- edentify the major types of coffee bean defects accountable to the OTA occurrence in coffee samples
- suggest visual means of judgment to avoid coffee beans with possible OTA contamination during coffee purchasing from the local market

4.3. Material and methods

4.3.1. Sampling site and coffee samples

As previously described in section 3.3.1, 74 *Coffee arabica* samples (ca. 1 kg) were collected from the sampling sites. The samples represented coffee from two processing methods (washed or dry processed), different conditions of storage (mill houses, resident and shops), different storage methods (sisal sacks, fertilizer bags and plastic bags) and different coffee type (cherry, parchments and green bean). Moisture contents of the entire samples were measured on the site using electronic moisture tester (HOH-Express-HE-50, Germany) at the collection sites. The samples were labeled with district name, sample number, date of collection and stored at 4^{o}C in refrigerator for further study.

4.3.2. Coffee defect count

Coffee defect count for all the 74 coffee samples were conducted by using the manuals of SCAA (2015) and Ethiopian Ministry of Agriculture and Rural Development Coffee and Tea Quality Control and Liquoring Center developed in 2009 as indicated in Table 4.1. From every sample 100 grams coffee beans were taken for defect count. All types of bean defects were counted and defect points were assigned as indicated in Table 4.1 based on their occurrence. If a coffee bean sample has more than one defect, the highest defect is counted. For example, beans with black and insect damaged defects were counts as one full defect due to its black attribute. Portion of beans judged as defective were also weighed and the weights were converted in to percentage.

Table 4.1. Green coffee defect count rating system

Type of defect	Number of occurrence	Defect point
Immature	5	1
insect damage	5	1
Foxy	5	1
Broken	10	1
Bad smell/moldy	5	1
Black	1	1
White/sour	1	1
Pod/cherry	1	1
Husk	1	1
Stick big	1	10
Stick medium	1	5
Stick small	1	3
Stone big	1	10
Stone medium	1	5
Stone small	1	3
Wanza seed (*Cordia africana*)	1	10
Soil big	1	10
Soil medium	1	5
Soil small	1	3
Soiled bean	5	1

Source: **Error! Hyperlink reference not valid.**SCAA, 2015

4.3.3. Fungal incidence on coffee samples

Percentage of fungal infection on coffee beans and frequencies of fungal occurrences were determined as previously described in section 3. 3. 2.

4.3.4. Ochratoxin A analysis

Ochratoxin A content of the coffee samples was analyzed as explained in section 3.3.4.

4.3.5. Data analysis

For statistical analysis R software version 3.0.3 (2014-03-06) was used. Spearman's rank correlation was used to examine the association of total bean defect points with OTA concentration levels. Kruskal-Wallis rank sum test was used to evaluate significance

differences among the independent variables in relation to the predictor (bean defect point). Multiple comparison tests after Kruskal-Wallis (kruskalmc) was used see observed values above critical at 0.05 levels.

4.4. Results

The result of coffee bean defect count for all samples indicated that more than 13 types of defects were encountered and none of the samples were without defect (Table 4.2). The predominant coffee bean defects encountered in this study in descending order were black beans (41.6 %), immature bean (27.0%), insect damaged (20.5%), moldy beans (20.4%), broken beans (15.5%), husk (11.4%) sour beans (7.3%), and cherry (5.9 %),(Figure 4.1).

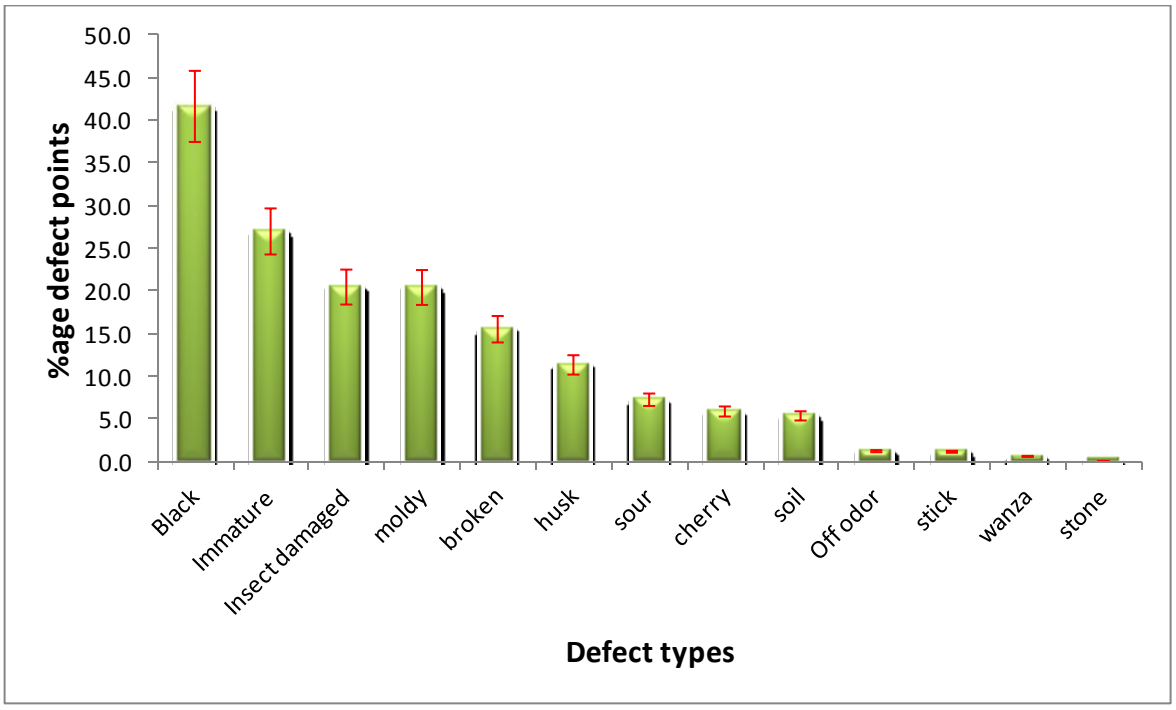

Figure 4 1. Percentage of defect types encountered in coffee samples collected from local market. Error bars are based on 95% confidence interval of defect proportion.

Table 4.2. Coffee bean defect encountered in the samples obtained from various processing methods and storage materials

Sample origin	Sample Code	Processing type	Storage type	Storage Material	%incidence	OTA (ug/kg)	Total defect points
Dedo	ETDE1	DC	Resident	FB	100	ND	38.2
Dedo	ETDE2	DC	Resident	FB	100	1.2	46
Dedo	ETDE3	DC	Resident	FB	100	0.72	39.2
Dedo	ETDE4	DC	Resident	FB	100	2.56	68
Dedo	ETDE5	DC	Resident	FB	100	3.05	171
Dedo	ETDE6	DC	Resident	FB	80	2.98	48
Dedo	ETDE7	DSG	Resident	FB	88	ND	24
Dedo	ETDE8	DSG	Resident	FB	92	2.54	57.3
Dedo	ETDE9	DSG	Resident	FB	100	1.2	63.6
Dedo	ETDE10	DSG	Resident	FB	88	1.78	55.4
Gomma	ETGO1	DC	Shop	SS	100	ND	45
Gomma	ETGO2	DC	Shop	SS	100	ND	28
Gomma	ETGO3	DC	Mill stores	FB	100	ND	37
Gomma	ETGO4	DC	Shop	FB	100	ND	26
Gomma	ETGO5	DC	Shop	PB	100	ND	22
Gomma	ETGO6	WP	Shop	PB	48	ND	15.4
Gomma	ETGO7	WP	Shop	PB	48	0.12	13.9
Gomma	ETGO8	WP	Shop	PB	52	22.64	150.7
Gomma	ETGO9	DC	Shop	SS	60	ND	32
Gomma	ETGO10	DC	Shop	FB	92	ND	10
Shebbe	ETSH1	DT	Mill stores	FB	80	0.03	17.2
Shebbe	ETSH2	DT	Mill stores	FB	24	0.56	92.8
Shebbe	ETSH3	DT	Mill stores	FB	72	1.66	53.6
Shebbe	ETSH4	DT	Mill stores	FB	88	ND	54
Shebbe	ETSH5	DT	Mill stores	FB	96	ND	59
Shebbe	ETSH6	DT	Resident	FB	96	1.07	44.6
Seka	ETSE8	DSG	Resident	FB	100	22.9	196.3
Seka	ETSE9	DSG	Resident	FB	100	1.39	69
Seka	ETSE10	DSG	Resident	FB	100	0.98	56.7
Limmu	ETLI1	DSG	Shop	PB	100	ND	29
Limmu	ETLI2	DC	Shop	PB	100	0.42	98
Limmu	ETLI3	DC	Shop	SS	92	ND	99
Limmu	ETLI4	DC	Shop	SS	56	1.02	43.4
Limmu	ETLI5	DC	Shop	SS	100	ND	110
Limmu	ETLI6	DC	Mill stores	SS	96	ND	72
Limmu	ETLI7	DSG	Resident	SS	96	ND	31.2
Mana	ETMA1	DT	Mill stores	SS	92	1.61	95.2
Mana	ETMA2	DT	Mill stores	SS	92	2.45	54.1
Mana	ETMA3	DT	Mill stores	SS	76	1.97	122
Mana	ETMA4	DT	Mill stores	SS	92	1.87	93.3
Mana	ETMA5	DT	Resident	SS	92	ND	66
Mana	ETMA6	DT	Resident	SS	96	0.95	81.6
Mana	ETMA7	DT	Resident	SS	92	0.51	37
Mana	ETMA8	DT	Resident	SS	96	12.29	188.7
Mana	ETMA9	DT	Resident	SS	100	3.42	83.8
Mana	ETMA10	DT	Resident	SS	88	3.59	87.7
Mana	ETMA11	DT	Resident	SS	100	3.02	77.8
Jtown	ETJT1	DC	Shop	PB	100	3.27	10.2
Jtown	ETJT2	WP	Shop	PB	76	1.72	83.4
Jtown	ETJT3	WP	Shop	PB	88	0.44	69.6
Jtown	ETJT4	WP	Shop	PB	32	ND	24.6
Jtown	ETJT5	WP	Shop	PB	60	0.87	37.8

Shebbe	ETSH7	DT	Resident	FB	92	ND	54
Shebbe	ETSH8	DT	Resident	SS	100	5.94	187.8
Shebbe	ETSH9	DT	Mill stores	FB	100	1.76	125.8
Shebbe	ETSH10	DT	Mill stores	FB	100	0.69	22.7
Seka	ETSE1	DC	Mill stores	FB	68	ND	51
Seka	ETSE2	DC	Mill stores	FB	100	ND	87.2
Seka	ETSE3	DC	Mill stores	FB	88	0.64	98.2
Seka	ETSE4	DC	Mill stores	FB	88	0.95	78.6
Seka	ETSE5	DC	Resident	FB	100	1.46	124.6
Seka	ETSE6	DC	Resident	FB	88	ND	44
Seka	ETSE7	DC	Resident	FB	100	2.81	154.3
Addis	ETM1	DC	Mill stores	SS	84	2.16	189.6
Addis	ETM2	DC	Mill stores	SS	72	2.26	185.2
Addis	ETM3	DC	Mill stores	SS	80	3.1	127.6
Addis	ETM4	DC	Mill stores	SS	80	2.41	91.4
Addis	ETM5	DC	Mill stores	SS	80	1.53	140
Addis	ETM6	DC	Mill stores	SS	80	0.6	37
Addis	ETM7	DC	Mill stores	SS	80	ND	25.4
Addis	ETM8	DC	Mill stores	SS	80	ND	65.2
Addis	ETM9	DC	Mill stores	SS	88	ND	67.4
Addis	ETM10	DC	Mill stores	SS	72	ND	75
Addis	ETM11	DC	Mill stores	SS	80	ND	112.6

N.B. DC=dried on concrete drying floor, DSG=dried on soil ground, WP < washed parchment, DT=dried by traditional means, FB=fertilizer bags, SS=sisal sacks, PB= plastic bags.

Correlation analysis of coffee bean defect point and OTA concentration in coffee bean samples indicated significant correlation between bean defect point and OTA concentration ($r = 0.56$, $p < 0.00$). Among the primary and secondary defect types listed in Table 4.1, OTA contamination levels in coffee beans were significantly correlated with black beans ($r = 0.33$, $p < 0.03$), visible mold ($r = 0.80$, $p < 0.00$), insect damaged beans ($r = 0.71$, $p < 0.00$), husk ($r = 0.67$, $p < 0.00$). However, no significance correlations were observed between coffee beans OTA concentration levels and beans with defects including sour ($r = 0.16$, $p < 0.29$), presence of cherries ($r = 0.15$, $p < 0.32$), sticks ($r = 0.00$, $p < 0.97$), soils ($r = 0.04$, $p < 0.81$), stones ($r = 0.08$, $p < 0.61$), immature beans ($r = 0.08$, $p < 0.59$), Wanza seed ($r = 0.09$, $p < 0.55$), broken beans ($r = 0.01$, $p < 0.95$) and with beans off odor ($r = 0.13$, $p < 0.38$).

Table 4.3. Coffee bean defect points, fungal incidence and OTA contamination levels on coffee samples from various

Sample Source	Beans defect points (%)	Median fungal incidence (%)	Median OTA level(µg/kg)
Dedo	11.16	100	1.77
Gomma	6.90	48	0.12
Shebbe	12.99	96	1.07
Seka	17.54	100	1.18
Limmu	8.82	96	0.42
Mana	18.04	92	2.21
Jimma town	4.12	76	0.87
Adds Ababa	20.40	80	2.21

No significant difference in median bean defect point were observed among the samples collected from different sources ($x^2 = 9.40$, $p < 0.23$). However, percentage of bean defect point was highest for samples obtained from Addis Ababa (20.40%), followed by Mana (18.04%),

Seka (17.54%) and Shebbe (12.99%). The lowest percentage of bean defect was observed for samples obtained from Jimma town (4.12%) Table 4.3.

Statistically, no significant difference existed in median coffee bean defect point (x^2= 2.78, p < 0.43) with in the processing types. However, median bean defect point obtained for DC coffee samples was highest (94.70), followed by DT (83.80) and WP (69.60) Figure 4.2.

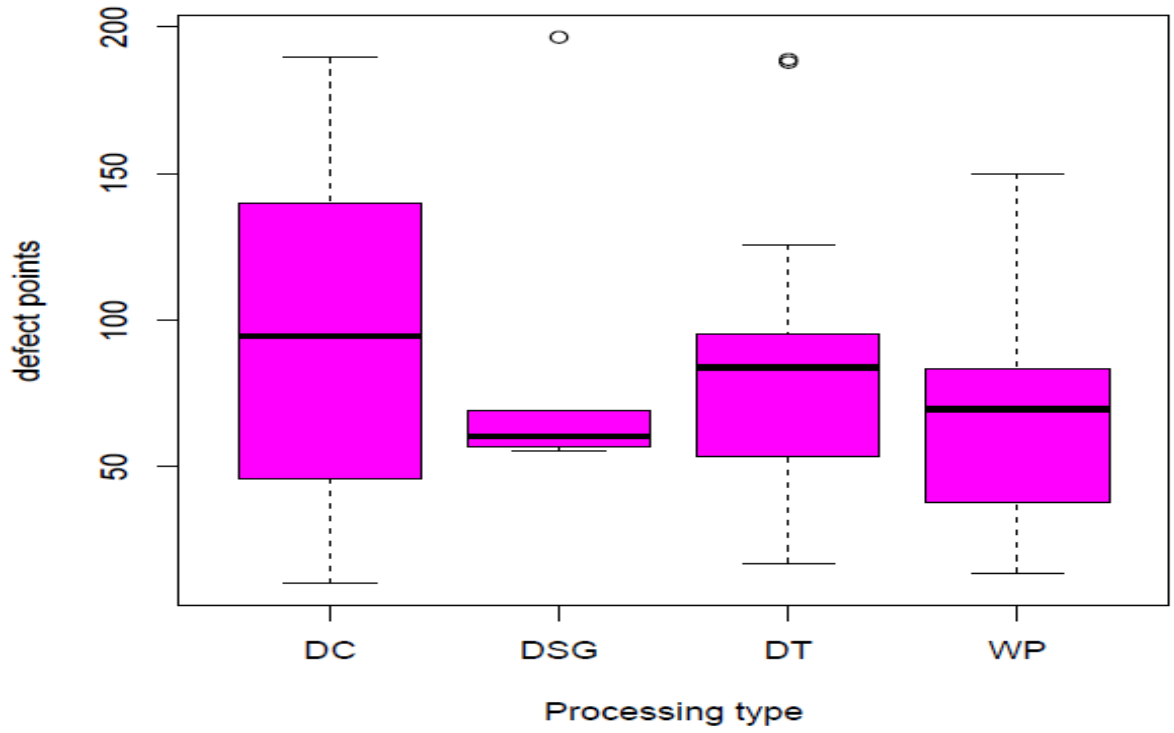

Figure 4.2. Coffee bean defect points in relation to processing type (DC = samples dried on concrete DSG = samples dried on soil ground, DT = samples dried by traditional methods like on animal skin, clothes etc, WP < wet processed sample. Box = inter quartile range, bold lines = median values, lower and upper hinges = lower and higher values, circles = outliers).

Significance difference in median bean defect was also observed among the different storage types (p < 0.00). Median coffee bean defect point (93) for beans stored in mill store was highest followed by residence (69) and least (56) for bean samples obtained from shop (Figure 4.3).

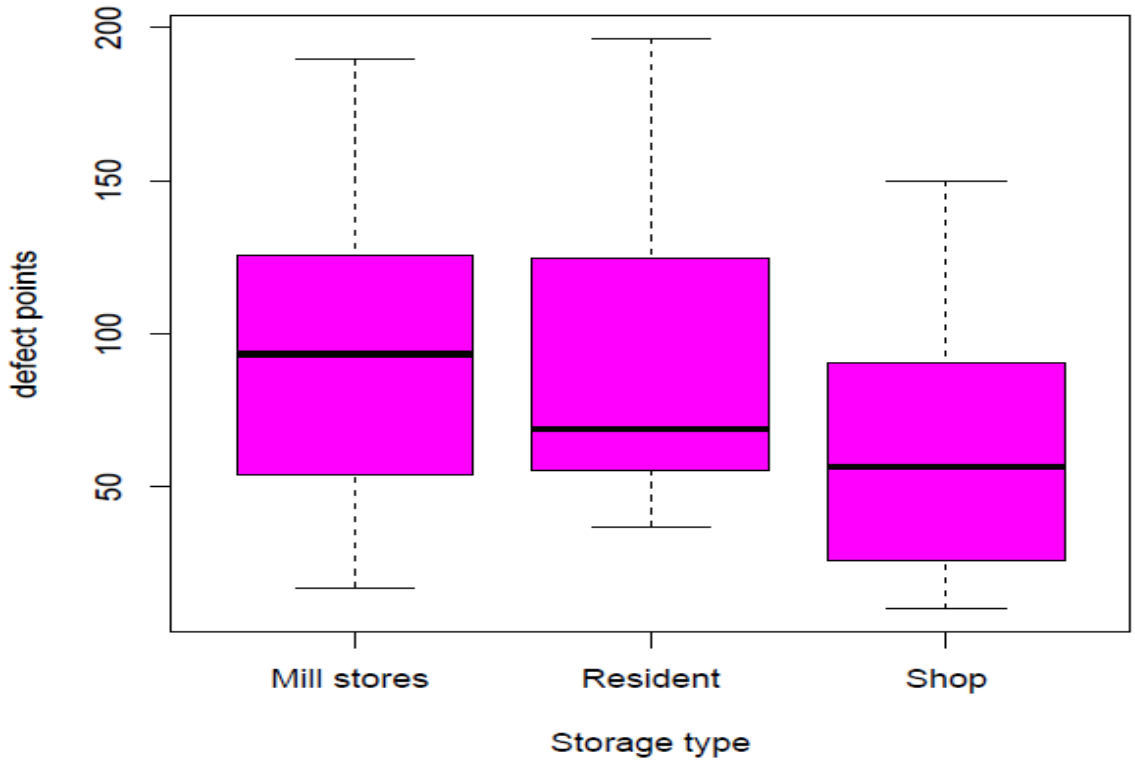

Figure 4.3. Coffee bean defect points in relation to storage types (Box = inter quartile range, bold lines = median values, lower and upper hinges = lower and higher values).

No significance difference exists between bean defect points and duration and storage material.

There was no correlation between bean defect points and fungal incidences ($r = 0.23$, $p < 0.06$).

4.5. Discussion

The purpose of this study was to search for possible visual screaning method of coffee beans contaminated with OTA based on physical defects so that the local community can easily avoid OTA contaminated beans based on the physical appearance of the beans. Coffee beans delivered to local market and consumed by the local community were examined for bean defect levels, bean defect types, fungal incidence and OTA contamination level. The results of the investigation revealed that the average bean defect level was as high as 219 (11 %) in the locally consumed coffee in relation to 86 defects (4%) maximum allowed defect level (SCAA, 2015). Thirteen types of defects were observed in the samples of which four types of defects (beans damaged by insects, moldy beans, beans mixed with husk, and black beans) were showed significant association with of OTA contamination levels. These defects were identified as the major contributors to higher OTA level in the locally consumed coffee beans. This finding is in line with the study of Taniwaki, *et al.* (2014) who have investigated that ochratoxigenic fungi and OTA in defective coffee beans and found out the sour and black defective beans had the highest OTA concentration. Similarly, the occurrences of black beans in higher percentage coincide with the finding of Toci and Farah (2008) who have revealed the presence of considerable proportion of black and sour beans in Brazilian Arabica coffee. Several studies (Franca *et al.*, 2005; Pamela *et al.*, 2008) elucidate the difference between defective and non defective beans in terms of physical and chemical attributes including OTA contamination levels. In search for physical means of discrimination between defective and non defective beans, Abebe *et al.* (2014) have reported the presence of higher coffee bean defects in Ethiopian coffee samples that originated from Sidamo (58 % defective) and lower for samples obtained from Harar (24.86 % defective).

There were two major reasons for the higher proportion of defective beans in the locally sold coffee and portion consumed by the producing farmers. One of the major reasons, as evidently observed during sample collection, was the sorting of sound beans (Appendix A2) for international market and defective beans for local market (AppendixA1 and A2). Sound beans were appropriately dried, sorted manually, packed in sisal sacks, and supplied to ECX (Ethiopian Commodity Exchange) for grading. Based on physical defect count and sensorial (organoleptic) analysis grades were given to the beans by trained panel of expertise. Coffee beans fail to meet the export standard quality redirected to the local market. Defective beans removed from quality beans by manual picking were also pooled and mixed with some sound beans and supplied to local markets for the local community. This phenomenon is true in the developing countries in the world including the world's top coffee producing country Brazil (Franca *et al.*, 2005).

Secondly, coffee farmers harvest sound red cherries on time and supply to coffee processing firms. Alternatively they can also dry the cherries and supply sound dried cherries to coffee mill houses whose target were foreign market. Any inappropriately harvested, poorly dried, deteriorated cherries collected from soil ground ended up in the nearby open local market at a relatively cheaper price (Appendix A2). Even more deteriorated cherries that could not be sold at the local market can be consumed by the families of the producing farmers implying the risk of OTA to the producing farmers.

As explained above, both the coffee processing industries and the coffee farmers contribute to the supply of low quality defective beans to the local market. Unlike cereal grains which can alternatively be used as animal feed, there is no any other option to utilize coffee beans other than beverage making in Ethiopia. As a result, both defective beans rejected by exporters and deteriorated portion supplied to the local market by the producing farmers synergistically

contribute to the higher percentage of defects in the locally available coffee. This leads to health risks of local coffee consumers due to consumption of OTA. The relatively lower price of highly defective beans attract very poor portion of the community as this group of community cannot afford to pay good quality beans at a higher price. This indicates the vulnerability of the poor population group to OTA associated risks in connection with coffee consumption.

Detail explanation of the root causes of all bean defects encountered in this study is beyond the scope of this study. However, in search for the variables accounted for each defect types various processing types, storage types and storage material were evaluated. The statistical analysis of the data did not support our previous assumption that processing types, storage types and storage materials might have effect on bean defect points. As previously explained by Oliveira *et al*. (2008) and Taniwaki *et al*. (2014) bean defects like immature beans, black beans, and sour beans can originate from coffee plantation field related to inappropriate harvesting, and poor processing practices. Therefore, a comprehensive study of coffee processing from field to cup is needed to appropriately point out the root causes of all type of bean defects.

Several authors explained the effect of poor pre and post harvest practices on the quality of coffee and OTA contamination (Toci and Farah, 2008; Batista *et al*., 2009; Birhan *et al.,* 2014). The findings of these authors indicated that bean defects are the result of poor agricultural practices and poor post harvest handling practices. These poor agricultural practices include inappropriate processing and post harvest handling practices with wrong storage materials and rooms that contribute to the elevated bean defects and OTA contamination levels.

One of the entrusting finding in this study was the statistically significant association of bean defects including moldy beans, beans infested with insects, beans having husk, and black beans with high OTA contamination level. This finding pointed out the order of significance of these defects to OTA contamination and can be potential indicator of toxin presence in the coffee samples.

The result of OTA analysis indicated the highest toxin level in cherry samples with visible mold overgrowth. As previously hypothesized in this study, visual observation of mold overgrowth on coffee samples implicated with the highest OTA content. This finding is substantiated visually by observable mold overgrowth on coffee samples that can be used as primary evidence for possible OTA contamination in the local market. Since these kinds of coffee are relatively cheaper in price, they attract the poorest communities in the market and this community makes coffee beverage by using both the beans and husk of the cherry. On a previously published paper by Abu and Teddy (2013), the use of coffee husk for coffee beverage making was reported due to the higher price of beans in non coffee growing areas. The husk of the cherry which is the external cover of the beans is more exposed to fungal and insect attack. Local people who make beverage of coffee by including the husk are at the highest risk of exposure to OTA. During sample collection for this study it has been observed that the local people were selling and buying the husk. The presence of OTA in coffee husk at a higher proportion was already explained (Taniwaki *et al*., 2014).

The strong positive correlation of OTA with insect infested beans is another potential indicator of OTA contamination. Insects associated with coffee cherries are known to disseminate toxigenic fungi (Vega *et al*., 2006b) among the cherries and indication of insect puncture on the

bean could be one condition for OTA contamination. This finding substantiated the possibility of insect infestation as one clue for OTA contamination.

The other potential clue identified in this study for OTA contamination was the presence of husk or the incomplete removal of the husk from the beans. Positive correlation of OTA with husk containing beans implies one of the potential sources of OTA can be the husk. The study of Viani (2002) indicated that coffee husks were significant source of OTA contamination and cleaning of green coffee effectively reduced OTA levels in coffee beans.

The black beans were also found to be associated with OTA in this study. In line with this finding, Taniwaki *et al*. (2014) have revealed the association of OTA with black and sour defective beans in samples obtained from two coffee growing regions of Brazil. During the sample collection it has been observed that fully black beans were sold at half the price of sound beans (nearly 45 Birr equal to 2 USD per kg) in Addis Ababa. People purchase these kinds of black beans and according to the sellers these black beans provide bitter taste to coffee beverages and some proportions of the black beans are mixed with quality beans. The commercialization and adulteration of defective beans with sound beans was reported from Brazil (Taniwaki, *et al*., 2014). However, the supply of defective beans like black, insect damaged, moldy and husk were not reported from Ethiopia. The act of commercializing black defective beans for beverages making in Ethiopian local market apparently contributes to OTA related health risks. This kind of act should be discouraged by setting mycotoxin regulation on the locally sold coffee in order to protect public health. At the same time an alternative way of utilizing the bulk of defective coffee beans that can be removed from export beans should be explored out instead of damping in the local market for human consumption. Very few reports (Barragan and Rodriguez, 2010)

are available on the alternatives use of defective coffee beans as a medium for the growth of pesticide degrading bacteria.

In Ethiopia, local people usually wash the beans or cherry to removes impurities and the off odor from beans before roasting to make coffee beverage. However, washing and roasting cannot remove the toxin and the only option is to avoid beans with an indication of mold overgrowth (Appendix A2), insect bored, contains husk and having off odor to scent as these type of beans are associated with the toxin.

4.6. Conclusion and recommendations

This study attempted to search for potential method used by the local community in discriminating OTA contaminated coffee at local market. The result of the study revealed the presence of higher percentage of defective beans in coffee portion supplied to the local market in Ethiopia. The reasons for this higher proportion of defective beans in the locally sold coffee were the supply of off grade coffee failed to pass to international market, mixing of manually sorted defective beans to sound beans, and the preference of farmers to sell good quality coffee to mill stores for export and poor quality to local market.

Presences of higher percentage of defective beans including insect damaged beans, moldy beans, beans with husk, and black beans were significantly associated with OTA contamination. These types of coffee bean defects can be used as indicators for possible OTA contamination.

Since defective beans were found to be the potential sources for OTA, coffee processing firms and coffee farmers should avoid mixing defective beans with sound beans to supply to the local market. The defective beans should be rejected as they are hazardous to the health of the

consumers. Researchers should explore for an alternative way of utilizing defective coffee beans that are unfit for human consumption.

The local community should make visual and olfactory assessment on the coffee beans and ascertain that the beans should not contains mold overgrowth, infested with insects, mix of husk, musty smell, black beans and mixed cherries.

Ethiopian coffee farmers should avoid the drying of cherries on soil ground and picking of cherries fallen on the soil ground for extended period of time in order to avoid or minimize mold colonization. Above ground drying beds and the use sisal sack for storing the cherries are highly recommended.

CHAPTER V. HPLC-VWD method to quantify Ochratoxin A in teff (*Eragrostis tef*) and wheat (*Triticum spp.*) destined for the local Ethiopian market

Abstract

Teff (*Eragrostis tef),* and Wheat (*Triticum spp.*) are among the most important cereal grains in terms of production and consumption in Ethiopia. No information is available on the presence of OTA in teff and wheat intended for local consumption in Ethiopia. Therefore, the purpose of this study was to develop a high performance liquid chromatography variable wavelength detector (HPLC-VWD) method for OTA analysis in teff and wheat flour. Method validation experiments indicated that OTA retention time (RT) was 5.356 minute.The linear regression line equations $y = 0.472697x + 0.517197$ was obtained using these standard concentration versus peak area of chromatographs with correlation coefficient (r) = 0.99884. The limit of ditection (LOD) and limit of quantification (LOQ) obtained in this study were 0.68 and 2.07 µg/kg for teff and 0.65 and 1.98 µg/kg for wheat respectively. With-in-a day relative standard deviation (RSD%) were 10.83 to 17.84 % for teff and 2.91 to 9.47 % for wheat. Between days RSD% were from 7.32% to 20.53 % for teff and 10.63 to 19.53 % for wheat. A total of 60 flour samples were examined for OTA contamination based on the validated method. Ochratoxin A was detected in 20% of teff and 50 % of wheat flour samples with median contamination levels of 1.18 and 6.76 µg/kg respectively. This validated method for OTA detection and quantification in teff and wheat using a HPLC-VWD detector meets the performance criteria for ochratoxin A.. An insight into the occurrence of OTA in teff is very valuable because the cereal might provide a low OTA risk alternative for wheat in Ethiopia. In addition, for the world market, an insight into the occurrence of OTA in teff is important in the light of an increasing interest in teff as niche market gluten free cereal.

Keywords: Ochratoxin, HPLC-VWD, cereal, teff, wheat

5.1. Introduction

In Ethiopia, teff (*Eragrostis tef*), wheat, barley, corn, sorghum, oats, and millet are the major cereal crops and these cereals represent 85% of the cultivated land and 90% of the field crop yield (CSA, 2000; Abu and Teddy, 2014). Teff and wheat alone contribute to 40% of the total calorific requirement per capita in urban Ethiopia (Demeke and Di Marcantonio, 2013). Teff is a cereal originally domesticated in Ethiopia and its neighboring Eritrea (D'Andrea, 2008). It is the main ingredient of injera, a sourdough-risen flatbread of native Ethiopian people. In addition, worldwide interest in this crop is increasing because teff is an alternative cereal for customers on a gluten-restricted diet (Motuma *et al*., 2015).

Large state owned farms produce 8% percent of the Ethiopian wheat representing roughly 124,000 ha of land. The remaining 92% comprises small farmers growing both wheat and teff (Abu, 2014; Minto and Sawyer, 2013). These small farms often use traditional harvesting, threshing, and storage practices. Threshing of the grains is typically done by animal feet overriding (Refera, 2001). During this threshing process, the grains make contact with the soil and can also be contaminated with animal feces. This traditional technique can result in contamination with toxigenic fungal spores and various soil microorganisms. A similar problem occurs during storage. Insects are an important source of fungal infection during storage (Upadhyay and Ahmad, 2011). Therefore, traditionally, Ethiopian farmers' mix and store cereal grains like wheat, maize or sorghum with teff to avoid insect infestation of the grains. Teff is not attacked by beetles and weevils due to its small size and due to the formation of compact stacking by the seeds during storage which resulted in lower chance of invasion by insect mediated fungi. In some part of the country, farmers store wheat grains mixing with teff or put a bag full of wheat inside teff storage structure to avoid insect damage. This might imply that the

chance of teff contaminated by insect pest and the associated fungal infection is lowered. Cereals harvested and stored via these traditional practices are typically consumed by the producing farmers themselves or supplied to the local market by local traders. Grains produced by small farmers can be stored for some time before delivered to the local market in several ways including using fertilizer bags and other traditional storage structures made of mud and animal dung (Dubale *et al.*, 2014). These traditional storage structures can be easily breached by insects and consequently the grains can be infested with weevils (beetles) which can promote colonization of toxigenic fungi amongst others.

Ochratoxin A is an important mycotoxin present in various agricultural products such as cereals. The infection of cereals with OTA producing fungi occurs during harvest, transport, storage and processing stages and is strengthened by conducive environmental conditions and the poor agricultural and storage practices prevailing in Ethiopia (Amare, 2006; Monbaliu *et al*., 2010; Warth *et al*., 2012; Peng, *et al*., 2015). Ochratoxin A exhibits toxicity in animals and human, including nephrotoxic, hepatotoxic, immunotoxic, teratogenic and carcinogenic effects and represents therefore a serious health risk to livestock and humans (Peraica *et al*., 2008, Pfohl-Leszkowicz, 2009, Khoury and Atoui, 2010). Ochratoxin A inhibits protein synthesis (Creppy *et al*., 1984), disturbs mitochondrial respiration (Aleo *et al*., 1991) and causes increased lipid peroxidation (Omar *et al*. 1990). These adverse cellular events may represent important precursors to the development of more serious kidney disease. In order to limit these effects, European Regulation (EC) 1881/2006 sets maximum levels for Ochratoxin A 5µg/kg in raw cereal grains, 3 µg/kg in cereal based food products, and 0.5 µg/kg in foods for infant and young children (EC, 2007). One of the important characteristics of OTA is its heat stability which makes it persistent during cooking process (Zinedine and Manes, 2009). Consumers are unaware

of the exposure risk to OTA because of inadequate information on the locally sold teff or wheat and their derivatives. Moreover, due to the widespread nature of fungi in the environment, mycotoxins are considered unavoidable contaminants in foods and feeds. One of the most effective measures to protect the public health is to establish regulatory levels of toxins on the basis of toxicological data. There is no such OTA regulatory limit in Ethiopia for the locally sold agricultural products due to lack of sufficient data. This current study aims to develop valid HPLC-VWD method for OTA analysis in teff and wheat and generated occurrence data in order to insist regulatory limit for the locally consumed grains.

Hypothesis

In some part of Ethiopia, farmers mix and store cereal grains like wheat, maize or sorghum with teff to avoid insect infestation of the grains. Farmers store wheat grains mixing with teff or put a bag full of wheat inside teff storage structure to avoid insect damage. On the other hand cereal grains like wheat, maize, sorghum, and barely can be attacked by insects and this attack leads to fungal invasion and contamination by mycotoxin producing fungi. Based on this information, the following two hypotheses were developed;

1. Ochratoxin A contamination level in teff flour samples might be lower than OTA level in wheat.
2. Wheat flour sold for local consumption in Addis Ababa market might have higher OTA contamination level than the maximum tolerable limit set by EU (5 µg/kg) for raw cereals.

5.2. Objectives

5.2.1. General objective

The general objective of this study was to validate HPLC-VWD method for OTA analysis and quantify this toxin in teff and wheat grain flour samples.

5.2.2. The specific objectives of the current project

- Validate specific and sensitive HPLC-VWD methods for the determination of OTA in teff and wheat flour samples destined for local consumption.
- Determine OTA contamination in teff and wheat flours samples.

5.3. Material and methods

5.3.1. Sample materials and sampling site

A total of 60 flour samples (30 teff and 30 wheat) were collected from Addis Ababa (Figure 5.1) local retail markets in plastic bags from 10 sub-cities Form each sub-city six flour samples as indicated in Table 5.1 were purchased from most frequently visited retailers, milling houses and shops/supermarket. Representative samples, one kilogram each were taken assuming the flours are homogenized during milling. A portion of 50 g flour was sub sampled from every sample and kept in refrigerator at 4°C for OTA analysis after homogenization.

Table 5.1. Sampling sites (sub-cities), sample types, coding and samples sources

sample site /sub-cities/	sample type	no. of samples	sample coding	source
Addis Ketema	teff flour	3	AKTS1 - AKTS3	mill house 3
	wheat flour	3	AKWS4 - AKWS6	shop 2, mill house 1
Akaki Kaliti	teff flour	3	KATS4 -KATS6	mill house 3
	wheat flour	3	KAWS4 -KAWS6	shop 2, Mill house 1
Arada	teff flour	3	ARTS7 - ARTS9	mill house 3
	wheat flour	3	ARWS7 - ARWS9	shop 2, mill house 1
Bole	teff flour	3	BOTS10 - BOTS12	mill house 3
	wheat flour	3	BOWS10 - BOWS12	shop 3
Gullelle	teff flour	3	GUTS13 - GUTS15	mill house 3
	wheat flour	3	GUWS13 - GUWS15	shop 2, mill house 1
Kirkos	teff flour	3	KITS16 - KITS18	mill house 3
	wheat flour	3	KIWS16 - KIWS18	shop 1, mill house 2
Kolfe Keranyo	teff flour	3	KKTS19 - KKTS21	mill house 3
	wheat flour	3	KKWS19 - KKWS21	Shop 3
Lideta	teff flour	3	LITS22 -LITS24	mill house 3
	wheat flour	3	LIWS22 - LIWS24	shop 2, mill house 1
Nifas Silk-Lafto	teff flour	3	NLTS25 - NLTS27	mill house 3
	wheat flour	3	NLWS25 - NLWS27	Shop 3
Yekka	teff flour	3	YKTS28 - YKTS30	mill house 3
	wheat flour	3	YKWS28 - YKWS30	Shop 3
Total samples		60		

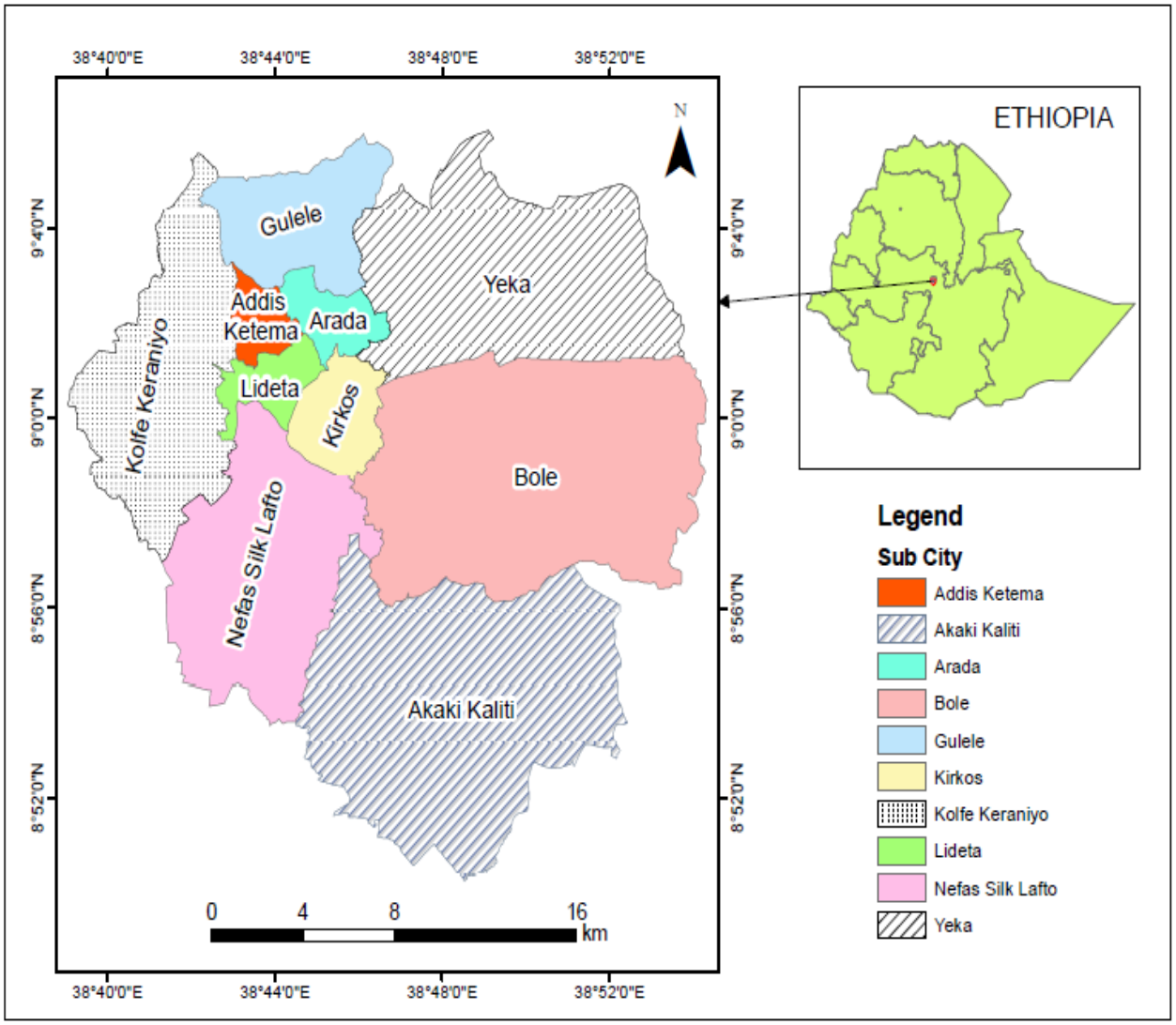

Figure 5.1. Map of Addis Ababa indicating ten sub-cities from where samples were collected

5.3.2. Ochratoxin A Standard preparation and reagents

The reagents used were HPLC grade water, HPLC grade methanol and HPLC grade acetonitrile were from Scharlab S. L. chemical supplier, Spain. Toluene, chloroform, acetic acid, phosphoric acid was analytical grade. Ochratoxin A standard solutions were prepared according to Vargas *et al*. (2005) AOAC Official Method 2004.10 as follows. Ochratoxin A standard solution of 1mg/mL was prepared by dissolving 1 mg OTA crystal powder (99.5% purity purchased from

Sigma Aldrich) in 1 mL toluene-acetic acid (99 +1, v/v). Ochratoxin A stalk solution (40µg/mL) was prepared and using a spectrophotometer (JENWAY Model 6405, U.K) following AOAC method (Vargas *et al*., 2005) as follows;

OTA (µg/mL) = (A x MW x 1000)/Ɛ

Where A = absorbance of the OTA stock standard solution at the wavelength of maximum absorption (λmax = 333 nm), MW = molecular weight of OTA (403.8); Ɛ (5440) = molar absorptivity of OTA in toluene-acetic acid (99 + 1, v/v). Ochratoxin A working standard solution (10 µg/mL) was prepared by transferring 500 µL of 40µL/mL OTA stalk solution to a 5 mL amber flask and diluted to 2000 µL with toluene-acetic acid (99+1, v/v). Two hundred micro liters of OTA working standard solution (10 µg/mL) were transferred into a 5 mL amber volumetric flask, and diluted to 2000 µL with toluene-acetic acid (99 + 1, v/v) to obtain a concentration of 1 µg/mL. Two hundred micro liters of this diluted working standard solution (1 µg/mL) were transferred into a 5 mL amber volumetric flask and evaporated to dryness under a steam bath. The resulting residue was re-dissolved with 5000 µL HPLC mobile phase to obtain a concentration of 40 ng/mL (standard one). Then this standard was used to prepare additional calibration solutions (2-40 ng/mL) as indicated in Table 5.1.

Table 5.2. Ochratoxin A standard calibration solutions

Standard solution	Volume of Standard 1(µL)	Volume of LC mobile phase(µL)	final OTA (ng/mL)
Standard 1	-	-	40
Standard 2	1500µL	500	30
Standard 3	1000µL	1000	20
Standard 4	500µL	1500	10
Standard 5	250µL	1750	5
Standard 6	100µL	1900	2

5.3.3. Ochratoxin A Extraction

Ochratoxin A extraction from teff and wheat flour samples was carried out based on alkaline extraction method developed Vega *et al*. (2009) with some modification as follows. Twenty five grams of flour samples (teff or wheat) were weighed accurately in a 250-ml flask and extracted with 100 mL methanol 1% sodium bicarbonate (50:50, v/v) by shaking for 30 min in an orbital shaker at 120 rpm. The solution was rested for 10 min and filtered through filter paper Whatman No. 1. Ten milliliters of the filtrate were mixed with 10 mL acetate buffer and the filtrate was further cleared by centrifugation at 12,000 rpm (eppendorf centrifuge 5418R, Germany) for five minutes. Then 10mL supernatant was passed through SPE column (*ISOLUTE* NH_2 aminopropyl) previously conditioned with 5 mL 15% methanol in acetate buffer (N-ethylmorpholine / acetate) pH 7.4. The columns were washed with 10 ml 15% methanol in acetate buffer and the samples were eluted with 3 mL 3% formic acid in methanol into 4 ml amber vials. The eluates were evaporated to dryness under steam bath at 50 ^{o}C and the residue was re-dissolved in 500 µL of HPLC mobile phase and centrifuged at 12,000 rpm for 5 min. The supernatant was used for OTA quantification.

5.3.4. HPLC conditions

The purified extracts were analyzed using a Agilent 1260 infinity equipped with quaternary pump (G1311C), Column Agilent Zorbax Eclipse plus C18 (4.6 mm ID× 100 mm length, 3.5 µm particle size.), VWD detector (DEAAU 03203) and manual injector (G1328C) integrated with Agilent Chemstation Rev.B.04.03.16 data acquisition and handling software(Agilent Technologies, Palo Alto, CA,USA). Twenty micro liters standard and samples were injected to HPLC with a 50 µL Agilent precisions Syringe. Both the standards used for calibration and

samples were run for 10 min at a flow rate of 0.8 mL/min and OTA chromatograms were generated at 333 nm excitation wavelength and 460 nm emission wavelength under isocratic pump condition. Ochratoxin A chromatograms generated by the equipment were identified by comparing the retention time (RT) of the samples with RT of external OTA reference standards. The concentration of the toxin in the sample extract was determined based on the area of the chromatogram of the samples and the linear calibration curve equation developed using OTA standards (Figure 5.2) and the equation generated from the calibration curve.

5.3.5. Preparation of standard calibration curve

Standard calibration solutions prepared in section 5.3.2 (Table 5.2) were used to produce calibration curve (Figure 5.2). A calibration curve was constructed by plotting the peak area for each standard concentration against the amount of OTA (ng/mL) injected in to HPLC equipment. The gradient (slope) and intercept of the calibration curve were used to compute the quantity of the OTA in the sample extracts. Ochratoxin A was identified by comparison of retention time of a corresponding peak with peak of the standard and concentration of OTA in the samples was quantified based on the area of the chromatogram using external standard method for the quantitative evaluation.

5.3.6. HPLC Method validation

5.3.6.1. Limit of detection (LOD), Limit of quantification (LOQ) and resolution

LOD and LOQ were determined in teff and wheat flours following the method of Kumar *et al.* (2012) with minor change using the formula LOD = (3)STD/S and LOQ = (10)STD/S, where STD is the mean standard deviation of chromatographic responses of recovery experiment obtained from spiked samples and S is the slope of the calibration curve.

Resolution, Rs, the absolute separation distance expressed as retention times (minutes) of two peaks, t_1 and t_2, and the baseline widths, W_1 and W_2, of the analyte and nearest peak expressed in terms of times calculated as $Rs = 2 (t_2 – t_1) / (W_1 + W_2)$.

5.3.6.2. Recovery tests

The recovery tests were performed by using repeated spike of OTA on non-contaminated teff and wheat samples at 4, 16, and 50 µg/kg concentration levels on 12.5g flour samples. The spiked samples were left at room temperature for the solvent to evaporate for a period of 24 hrs and extracted based on the method described in section 5. 3. 4. The average recoveries and relative standard deviation on each level were calculated and compared with AOAC (2012) Guide Line target values and EURACHEM Guide, The Fitness for Purpose of Analytical Methods (Magnusson and Ornemark, 2014).

Recovery (%) = (C1-C2)/C3 X 100 where

C1 = concentration determined in spiked sample

C2 = concentration determined in unspiked sample

C3 = concentration spiked on samples

5.3.6.3. Method precision /repeatability/

Repeatability conditions were independent test results obtained with the same method on identical test items with the same equipment within short intervals of time in the same day. This experiment was conducted using spiking in six replicates. Both the spiked and unspiked samples were extracted, cleaned-up and subjected to the HPLC analysis according to the previously described method (section 5.3.4). The precision of method was calculated in terms of intra-day

(within-run) and inter-day (between days) repeatability with relative standard deviation (RSD) by three replicated analysis of the samples. For the determination of intraday repeatability, three replicated samples spiked with OTA at three concentration levels (4, 16 50 µg/kg) were analyzed on the same day in triplicate, while the inter-day repeatability was estimated repeating the analyses on three consecutive days according to Kabak (2012). The two tailed student t-value and repeatability limit (r) were calculated by the formula $r = \sqrt{2} \times t \times Sr$ where $\sqrt{2}$ factor to reflect difference of two measurements, Sr is is repeatability standard deviations and *t* is the two tailed Students *t*-value.

5.3.7. Detection and Quantification of OTA in teff and wheat flour samples

The mass of OTA in the test sample was calculated according to Lin *et al.* (2005) using the following equation: Wm = Wa × (Vf/Vi) × (1/Vs) Where Wm = OTA concentration in the test sample in ppb (ng/g or ng/mL); Wa = amount of OTA corresponding to the area of OTA peak of the sample extract (ng); Vf = final volume of re-dissolved eluate (µL); Vi = volume of injected eluate (µL); Vs = volume test portion passing through the column (mL or g).

5.3.8. Data analysis

For statistical evaluation of the data R statistical software version 3.0.3. (2014.0306) was used. To assess correlations Spearman rank correlation analysis was performed. Ochratoxin A contents of samples were analyzed using Analytical calibration curve generated using the Agilent Chemstation software provided by the equipment manufacturer. A non-parametric Kruskal-Wallis Rank Sum Test was used to test significance followed by kruskalmc multiple comparison test.

5.4. Results

5.4.1. Method validation

Calibration curve, LOD and LOQ

Ochratoxin A retention time (RT) was 5.356 minute using HPLC VWD detector from standard OTA. No peak overlap observed at OTA RT (Figure 5.3). The linear regression line equations $y = 0.472697x + 0.517197$ was obtained using these standard concentration versus peak area of chromatographs with correlation coefficient (r) = 0.99884 (Figure 5.2). A linear HPLC response to OTA standards was observed for a concentration range 2 to 40 ng/mL manually injected in to the equipment where the lowest point of calibration curves was 2 ng/mL (Table 5.3 and Figure 5.2).

Table 5.3. Ochratoxin A standard concentrations used for calibration curve

Retention Time (min)	Level	Conc. (ng/µL)	Area	Conc./Area
5.356	1	2	1.4770	1.3541
	2	5	2.9551	1.6920
	3	10	5.7349	1.7437
	4	30	14.9801	2.0027
	5	40	19.0811	2.0963

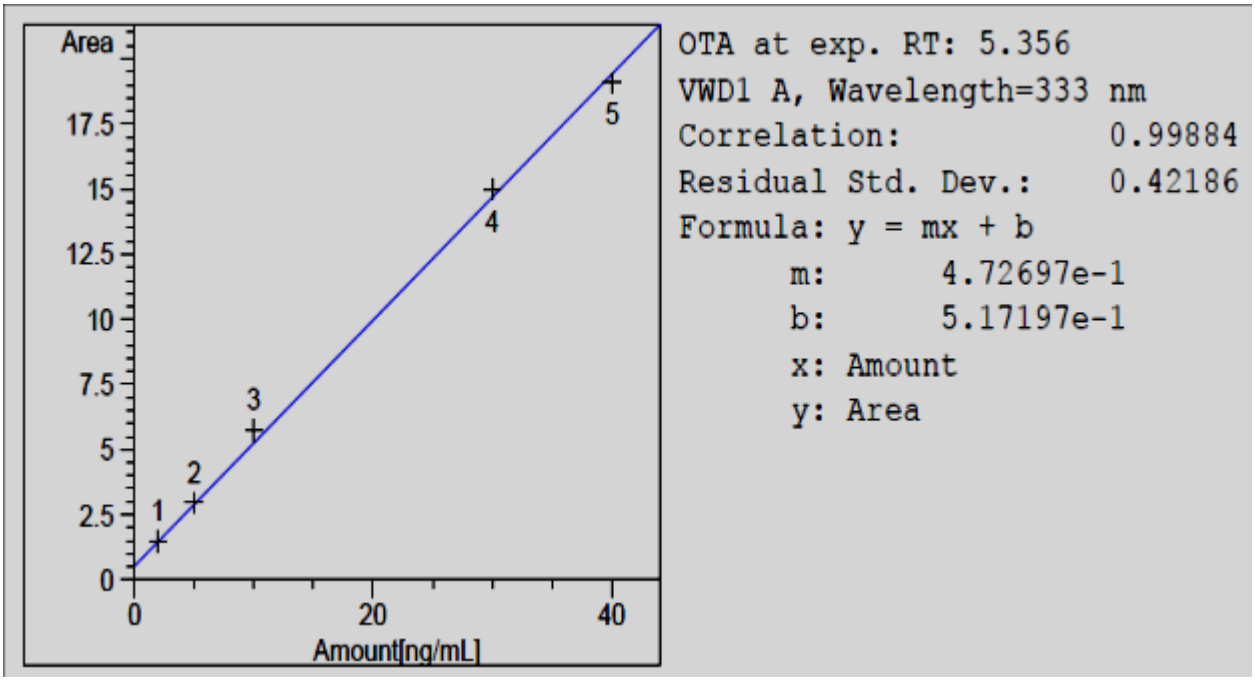

Figure 5.2. Ochratoxin A HPLC calibration curve for concentration range from 2 -40 ng/mL

Limit of detection (LOD) and limit of quantification (LOQ) were determined for both matrices based on recovery standard devotion (STD) obtained from the spiking at three levels (4, 16, and 50 ng/mL; Table 5.4) and slope of the calibration curve (S) using the formula LOD = (3) STD/S and LOQ = (10) STD/S (Table 5.4). The LOD and LOQ of OTA obtained in this study were 0.68 and 2.07 µg/kg for teff and 0.65 and 1.98 µg/kg for wheat (Table 5.4).

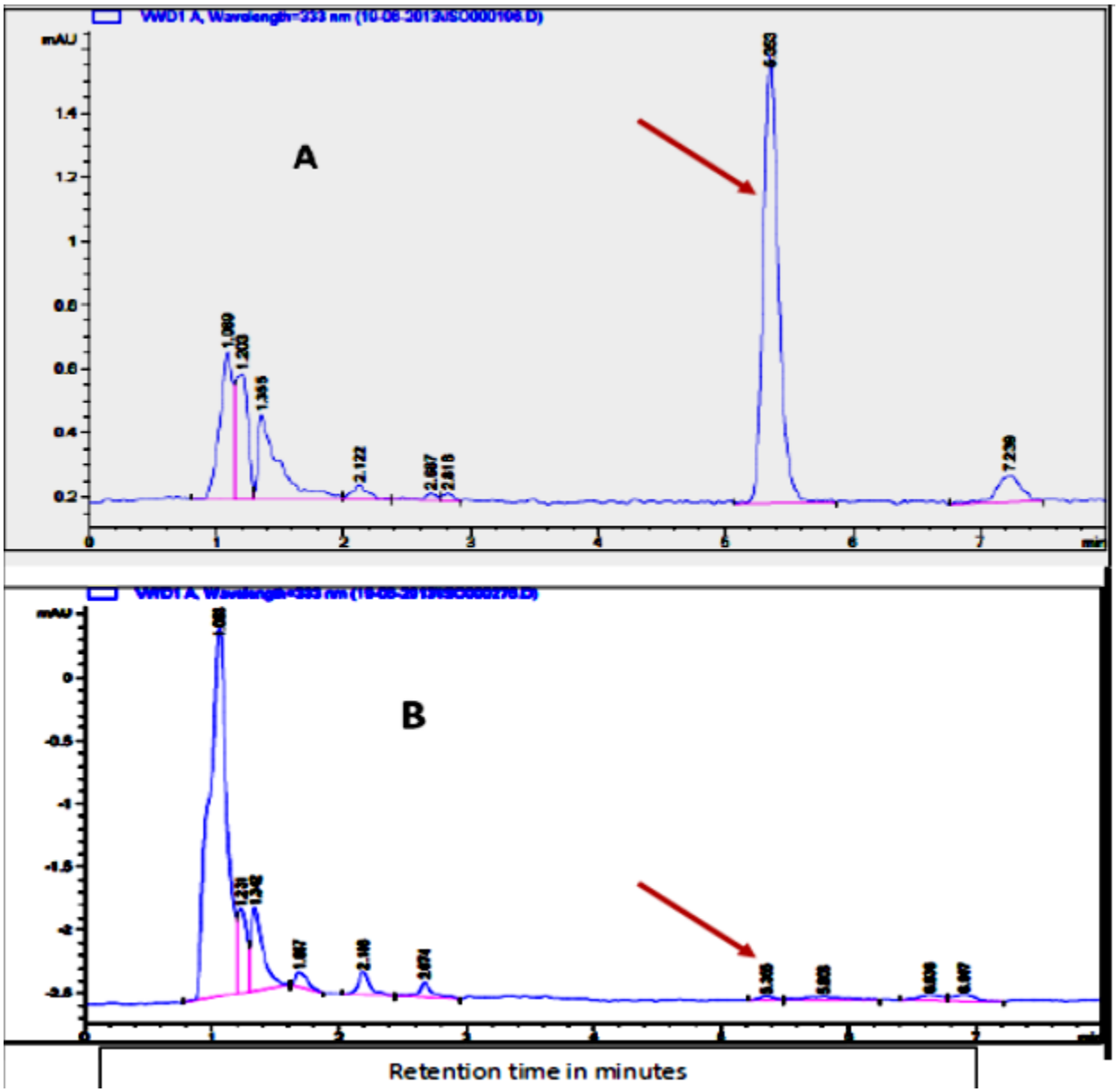

Figure 5.3. HPLC chromatogram of OTA standard and OTA in a contaminated sample

N. B. Ochratoxin A standard 40 ng/mL (A) and OTA chromatogram in a positive sample (B) at 5.356 min retention time

Table 5.4. Determination of LOD and LOQ in teff and wheat flour samples.

	teff			wheat		
injections	**4ng/mL**	**16ng/mL**	**50ng/mL**	**4ng/mL**	**16ng/mL**	**50ng/mL**
Injection 1	2.90	11.11	19.01	3.16	13.00	46.58
Injection 2	2.92	11.05	19.13	3.23	12.99	46.51
Injection 3	2.99	11.00	19.00	3.16	12.99	46.31
Injection 4	2.79	11.11	19.26	3.04	13.10	46.58
Injection 5	3.00	11.10	19.05	2.99	13.10	46.51
Injection 6	2.71	11.05	19.13	3.16	13.02	46.49
Injection 7	2.80	11.00	19.00	3.00	12.90	46.40
Injection 8	2.86	11.20	18.90	2.99	12.99	46.60
Injection 9	2.90	11.23	19.11	3.13	13.12	46.56
Injection 10	2.88	10.90	19.20	3.21	13.21	46.60
mean	2.87	11.08	19.08	3.11	13.04	46.52
STD	0.09	0.10	0.11	0.09	0.09	0.09
Average STD		0.10			0.09	
Slope (S)from cal.curv		0.47			0.47	
LOD = (3)STD/S		0.68			0.65	
LOQ = (10)STD/S		2.07			1.97	

N.B. STD = standard deviation, S = slope of calibration curve.

Recovery test

The results of recovery experiments to confirm precision are presented in Table 5.5. Within a day average recovery of OTA from spiked triplicate samples ranged from 71.43 % to 78.05 % (RSD% 10.83 to 17.84 %) for teff and from 82.31 % to 92.62 % (RSD% 2.91 to 9.47 %) for wheat flour samples. Between days average recovery of OTA ranged from 73.72 % to 78.49 % (RSD% 7.32% to 20.53 %) for teff and 74.50 % to 89.18 % (%RSD 10.63 to 19.53 %). Both within day and between days recoveries and corresponding RSD% values are in accordance with performance criteria for ochratoxin A EC 401/2006 (EC, 2006b).

Table 5.5. Within a day and between days recoveries at different concentration levels from teff and wheat flour samples

Sample type	Spiking level (µg/kg)	No. of repeats	within a day				between days			
			Mean recovered conc. (µg/kg)	Mean recovery (%)	STD	RSD%	Mean recovered conc. (µg/kg)	Mean recovery (%)	STD	RSD%
teff	4	3	3.12	78.05	13.02	16.68	3.12	74.63	8.68	11.64
	16	3	11.97	74.80	8.10	10.83	11.97	73.72	15.13	20.53
	50	3	35.72	71.43	12.75	17.84	35.72	78.49	5.74	7.32
wheat	4	3	3.29	82.31	2.60	3.16	3.57	89.18	17.41	19.53
	16	3	14.82	92.62	8.77	9.47	11.92	74.50	10.48	14.06
	50	3	45.84	91.67	2.67	2.92	38.17	76.33	8.12	10.63

N.B. STD = standard deviation of % recovery, RSD% = Relative standard deviation of repeatability= STD/meanx100= equivalent with CV/coefficient of variance/.

5.4.2. Determination of OTA in teff and wheat

The results of OTA HPLC quantitative analysis in teff and wheat flour samples collected from different retailers, milling houses and supermarket in Addis Ababa are indicated in Table 5.6. Of the 30 teff flour samples, 20% were found to be contaminated with OTA within the range of 0.48 – 7.12 µg/kg with median contamination level of 1.17 µg/kg (Figure 5.4), while the remaining 80% had no detectable contamination (LOD = 0.68 µg/kg). The highest OTA content (7.12 µg/kg) was found in a teff flour sample collected from millhouse. On the other hand, the result of OTA analysis in wheat indicated that 50% of the samples were found to be contaminated with OTA in the range of 0.45 – 17.35 µg/kg with median contamination level of 6.76 µg/kg (Figure 5.4). The highest OTA content (17.35 µg/kg) in wheat flour was observed in samples collected from local retailer shop. The median OTA level in wheat flour samples was fivefold higher than the median level obtained for teff (Figure 5.4).

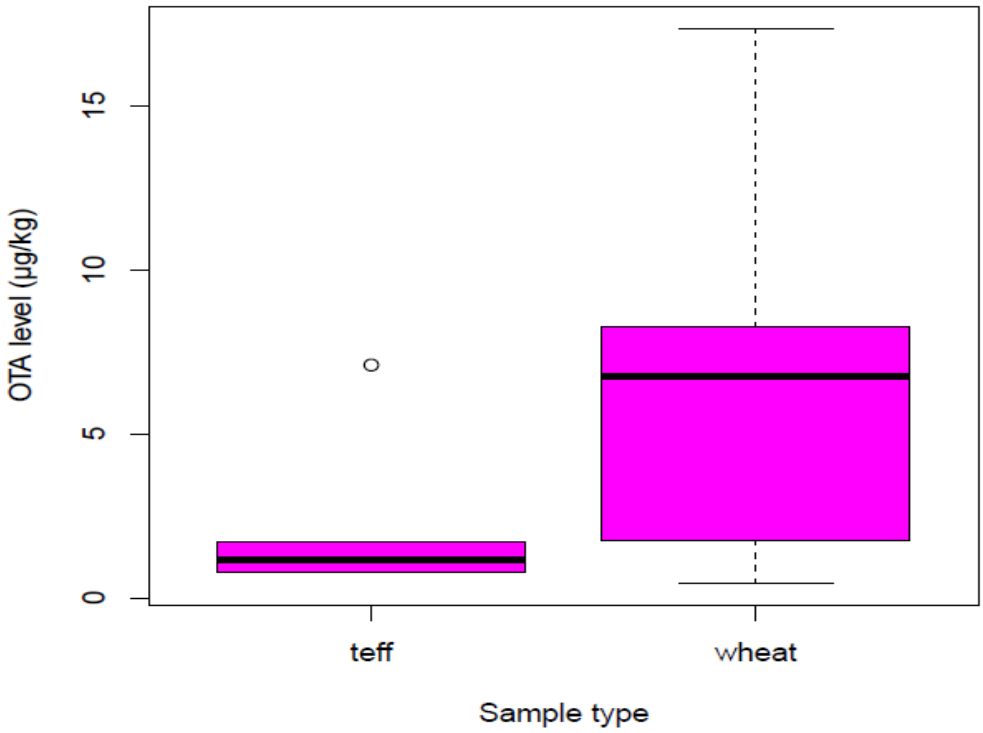

Figure 5.4. Ochratoxin A level in teff and wheat flour samples

(Box = inter quartile range, bold lines = mean values, lower and upper hinges = lower and higher values, circle = outlier).

Table 5.6. Ochratoxin A in teff and wheat flour samples quantified by HPLC-VWD

sample Origen	sample type	source	sample ID	y(response)	b(y-intercept)	m(slope)	concentration(ppb)	Vf(µL)	Vi (µL)	Vs (mL)	Vf/Vi	1/Vs (mL)	Wm (ppb)
Addis Ketema	Teff flour	mill house	AKTS1	0	0.52	0.47	0.14	600	20	10	30	0.1	0
Addis Ketema	Teff flour	mill house	AKTS2	0	0.52	0.47	0.14	600	20	10	30	0.1	0
Addis Ketema	Teff flour	mill house	AKTS3	0.06	0.52	0.47	0.26	600	20	10	30	0.1	0.77
Akaki Kalite	Teff flour	mill house	KATS4	0.06	0.52	0.47	0.26	600	20	10	30	0.1	0.78
Akaki Kalite	Teff flour	mill house	KATS5	0	0.52	0.47	0.14	600	20	10	30	0.1	0
Akaki Kalite	Teff flour	mill house	KATS6	0	0.52	0.47	0.14	600	20	10	30	0.1	0
Arada	Teff flour	mill house	ARTS7	0	0.52	0.47	0.14	600	20	10	30	0.1	0
Arada	Teff flour	mill house	ARTS8	0.13	0.52	0.47	0.42	600	20	10	30	0.1	1.25
Arada	Teff flour	mill house	ARTS9	0	0.52	0.47	0.14	600	20	10	30	0.1	0
Bole	Teff flour	mill house	BOTS10	0	0.52	0.47	0.14	600	20	10	30	0.1	0
Bole	Teff flour	mill house	BOTS11	0	0.52	0.47	0.14	600	20	10	30	0.1	0
Bole	Teff flour	mill house	BOTS12	0.11	0.52	0.47	0.37	600	20	10	30	0.1	1.1
Gullelle	Teff flour	mill house	GUTS13	0	0.52	0.47	0.14	600	20	10	30	0.1	0
Gullelle	Teff flour	mill house	GUTS14	0	0.52	0.47	0.14	600	20	10	30	0.1	0
Gullelle	Teff flour	mill house	GUTS15	0	0.52	0.47	0.14	600	20	10	30	0.1	0
Kirkos	Teff flour	mill house	KITS16	0	0.52	0.47	0.14	600	20	10	30	0.1	0
Kirkos	Teff flour	mill house	KITS17	0	0.52	0.47	0.14	600	20	10	30	0.1	0
Kirkos	Teff flour	mill house	KITS18	0	0.52	0.47	0.14	600	20	10	30	0.1	0
Kolfe Keranyo	Teff flour	mill house	KKTS19	0	0.52	0.47	0.14	600	20	10	30	0.1	0
Kolfe Keranyo	Teff flour	mill house	KKTS20	0	0.52	0.47	0.14	600	20	10	30	0.1	0
Kolfe Keranyo	Teff flour	mill house	KKTS21	0	0.52	0.47	0.14	600	20	10	30	0.1	0
Lideta	Teff flour	mill house	LITS22	0.21	0.52	0.47	0.57	600	20	10	30	0.1	1.72
Lideta	Teff flour	mill house	LITS23	0	0.52	0.47	0.14	600	20	10	30	0.1	0
Lideta	Teff flour	mill house	LITS24	0	0.52	0.47	0.14	600	20	10	30	0.1	0
Nifas Silk Lafto	Teff flour	mill house	NLTS25	1.06	0.52	0.47	2.37	600	20	10	30	0.1	7.12
Nifas Silk Lafto	Teff flour	mill house	NLTS26	0	0.52	0.47	0.14	600	20	10	30	0.1	0

Nifas Silk Lafto	Teff flour	mill house	NLTS27	0	0.52	0.47	0.14	600	20	10	30	0.1	0
Yekka	Teff flour	mill house	YKTS28	0	0.52	0.47	0.14	600	20	10	30	0.1	0
Yekka	Teff flour	mill house	YKTS29	0	0.52	0.47	0.14	600	20	10	30	0.1	0
Yekka	Teff flour	mill house	YKTS30	0	0.52	0.47	0.14	600	20	10	30	0.1	0
Addis Ketema	wheat flour	shop	AKWS1	0.35	0.52	0.47	0.88	600	20	10	30	0.1	2.63
Addis Ketema	wheat flour	shop	AKWS2	0	0.52	0.47	0.14	600	20	10	30	0.1	0
Addis Ketema	wheat flour	mill house	AKWS3	0	0.52	0.47	0.14	600	20	10	30	0.1	0
Akaki Kalite	wheat flour	shop	KAWS4	1.37	0.52	0.47	3.03	600	20	10	30	0.1	9.08
Akaki Kalite	wheat flour	shop	KAWS5	1	0.52	0.47	2.25	600	20	10	30	0.1	6.76
Akaki Kalite	wheat flour	mill house	KAWS6	1.4	0.52	0.47	3.1	600	20	10	30	0.1	9.29
Arada	wheat flour	shop	ARWS7	0	0.52	0.47	0.14	600	20	10	30	0.1	0
Arada	wheat flour	shop	ARWS8	0.01	0.52	0.47	0.15	600	20	10	30	0.1	0.45
Arada	wheat flour	mill house	ARWS9	0	0.52	0.47	0.14	600	20	10	30	0.1	0
Bole	wheat flour	shop	BOWS10	0	0.52	0.47	0.14	600	20	10	30	0.1	0
Bole	wheat flour	shop	BOWS11	1.09	0.52	0.47	2.44	600	20	10	30	0.1	7.31
Bole	wheat flour	shop	BOWS12	0	0.52	0.47	0.14	600	20	10	30	0.1	0
Gullelle	wheat flour	shop	GUWS13	0	0.52	0.47	0.14	600	20	10	30	0.1	0
Gullelle	wheat flour	shop	GUWS14	0.59	0.52	0.47	1.38	600	20	10	30	0.1	4.15
Gullelle	wheat flour	mill house	GUWS15	0	0.52	0.47	0.14	600	20	10	30	0.1	0
Kirkos	wheat flour	shop	KIWS16	0	0.52	0.47	0.14	600	20	10	30	0.1	0
Kirkos	wheat flour	mill house	KIWS17	0	0.52	0.47	0.14	600	20	10	30	0.1	0
Kirkos	wheat flour	mill house	KIWS18	1.06	0.52	0.47	2.37	600	20	10	30	0.1	7.12
Kolfe Keranyo	wheat flour	shop	KKWS19	0	0.52	0.47	0.14	600	20	10	30	0.1	0
Kolfe Keranyo	wheat flour	shop	KKWS20	0.01	0.52	0.47	0.15	600	20	10	30	0.1	0.45
Kolfe Keranyo	wheat flour	shop	KKWS21	0	0.52	0.47	0.14	600	20	10	30	0.1	0
Lideta	wheat flour	shop	LIWS22	2.67	0.52	0.47	5.78	600	20	10	30	0.1	17.35
Lideta	wheat flour	shop	LIWS23	1.11	0.52	0.47	2.49	600	20	10	30	0.1	7.47
Lideta	wheat flour	mill house	LIWS24	1.81	0.52	0.47	3.96	600	20	10	30	0.1	11.88
Nifas Silk Lafto	wheat flour	shop	NLWS25	0.56	0.52	0.47	1.32	600	20	10	30	0.1	3.96

Nifas Silk Lafto	wheat flour	shop	NLWS26	0	0.52	0.47	0.14	600	20	10	30	0.1	0
Nifas Silk Lafto	wheat flour	shop	NLWS27	0.08	0.52	0.47	0.3	600	20	10	30	0.1	0.89
Yekka	wheat flour	shop	YKWS28	0.01	0.52	0.47	0.15	600	20	10	30	0.1	0.46
Yekka	wheat flour	shop	YKWS29	0	0.52	0.47	0.14	600	20	10	30	0.1	0
Yekka	wheat flour	shop	YKWS30	0	0.52	0.47	0.14	600	20	10	30	0.1	0

N.B. y = area of OTA chromatograph, Wa = amount of OTA corresponding to the area of OTA peak injected to HPLC (ng), Wm = OTA concentration in the test sample in ppb (ng/g or ng/mL); Vf = the final volume of re-dissolved eluate (µL); Vi = the final volume of injected eluate (µL); Vs = the volume of test portion cleaned up by passing through the SPE column (mL).

5.5. Discussion

5.5.1. Method validation

Method validation is crucial in analytical work because the type of matrix affect the extraction, purification, recovery and quantification of the toxin. Methanol 1% sodium bicarbonate (50:50, v/v) extraction, aminopropyl clean up with acetonitrile-water-acetic acid (49:49:2, v/v/v) as mobile phase provided acceptable performance characters including selectivity, LOD and LOQ, working range, trueness (recovery) and precision (repeatability). OTA chromatogram obtained at RT = 5.356 in this study indicate good OTA peak resolution (Rs > 1.88) which is one of the important system suitability test (SST) criteria in method development and validation.

The profiles of calibration curves developed form six levels of the reference material (Figure 5.2) indicate that the chromatographic conditions were adequate under the frame of Eurachem Guide, The Fitness for Purpose of Analytical Methods. The conditions of the calibration curve were adequate for the OTA analysis in terms of linearity. The chromatograms show a clean base line, without interference close to OTA chromatogram (Figure 5.3A). Correlation coefficient (r = 0.99884) obtained for the regression lines demonstrate good relationship between peak area and concentration of OTA in the range of 2-40 ng/mL recovered from both matrices.

Solvents used for extraction, dissolution, and elution were analyzed in order to determine whether they contribute to the measurement signal and found to be negative for the OTA chromatogram at 5.356 minutes RT. Teff and wheat flour samples were also judged as blank

and used for spiking experiment based on previous analysis using the method validated in this study.

In this study the retention time (RT) of OTA using acetonitrile-water-acetic acid, v/v/v/ as mobile phase and 0.8 mL flow rate was identified as 5.356 minutes after repeated injection (X6) of OTA standards of various concentrations.

The recovery values obtained for teff and wheat ranging between 71.43 to 78.53% and 82.85 to 92.63%, respectively are acceptable with the requirements of the Commission Regulation (EC) No. 401/2006 (EC, 2006b) and Eurachem Guide for OTA that set performance criteria for OTA recovery rate from 70 -110%. Ochratoxin A recovery range for wheat obtained in this study was also in agreement with the findings of Ibanez-Vea, *et al*. (2011) and Vega *et al*. (2009), (50-120%) and (85-89% respectively in breakfast cereals from Spanish markets and cereals from Chilean market.

5.5.2. Ochratoxin A in teff and wheat flour samples

This study reveals OTA contamination level in teff and wheat flours destined for human consumption in Addis Ababa in the range of 0.78 – 7.12 µg/kg and 0.45 – 17.35 µg/kg with median level of 1.17 µg/kg and 6.76 µg /kg, respectively. Of the 30 wheat flour samples investigated 26.7% were found contaminated with OTA above the EU limit (5 µg/kg). However, none of the teff flour samples exceeded this EU limit. Although, the variation in median OTA concentration levels between teff and wheat flour samples obtained in this study are statistically insignificant ($p < 0.48$), the median OTA contamination in wheat (6.76 µg /kg) was nearly six fold higher that the median OTA (1.17 µg/kg) in teff. The median OTA level obtained in this current work for both teff and wheat was lower compared to the

previous report from Ethiopia (Amare *et al*., 2006). Amare *et al*. (2006) analyzed 33 teff and 107 wheat grain samples and obtained 27.3% teff and 23.4% wheat samples contamination by OTA with median of 31.9 µg/kg for teff and 14.9 µg/kg for wheat. One possible explanation for this lower level of OTA in teff and wheat flour samples in this current analysis could be the cleaning and sorting of teff and wheat impurities before milling. In the case of teff, this small grain undergoes sieving and cleaning from varieties of impurities, immature grains and straw that possibly reduce OTA. Removal of wheat bran also contributes to the reduction of OTA as demonstrated by the study of Peng *et al*. (2015). Similarly, Scudamore *et al*. (2003) indicated that scouring could remove up to 44% of OTA from wheat and their finding was in agreement with Cabanas *et al*. (2008) who have shown that white flour contains much lower concentrations of OTA than whole meal flour. On the other hand Amare *et al*. (2006) analyzed whole grains without either sorting or polishing this might contribute to the higher level of the toxin.

The occurrence of OTA in wheat was reported by several authors from different countries (Ibanez-Vea *et al*., 2011; Zhang *et al*., 2011; Kumar *et al*., 2012; Kara *et al*., 2015). However, information on the existence of OTA in teff is negligible (Amare *et al*., 2006). Teff is only produced and consumed in Ethiopia and Eretria which might be the reason why this grain was not well studied and data on the level of mycotoxin in this grain was insufficient. Levels of OTA contamination in wheat and other cereal samples varies from county to country (Table 5.7). Both the mean (6.34 µg/kg) and median (6.76 µg/kg) OTA levels in wheat flour obtained in this study are slightly higher than the mean value of OTA (3.7 µg/kg) obtained by Toffa *et al*. (2013) from cereal samples in Niger. Further lower OTA median level (0.2 µg/kg) in wheat samples was reported by Ibanez-Vea, *et al*. (2011)

from Spanish market. Over all analysis indicated that the mean level of OTA in Ethiopian wheat (6.34 µg/kg) was higher than the results obtained in China (Zhang *et al*., 2011), Spain (Ibanez-Vea *et al*., 2011), and Turkey (Kara *et al*., 2015) (Table 5.7).

The occurrence of OTA in cereal grains including wheat, maize, sorghum, barely, millet and oat has been reported by several studies (Kumar *et al*., 2012; Ibanez-Vea *et al*., 2011; Vega *et al*., 2009). One of the causes of variation in OTA contamination level in cereal grains samples is the bran. Removal of the bran significantly reduces the level of the toxin. The mean, median and maximum values obtained from Ethiopia by Amare *et al*. (2006) and this study too, were higher than the rest of the world (Table 5.7). This higher OTA values might be attributed to the use of ancient grain threshing system using herds of animals on soil ground in Ethiopia (Figure 2.4) which enable fungi to get easy access to the grain. Additionally, during the threshing process animal droppings can also serve as source of inocula. The transport of grain using donkey back, and outdated storage structures can also contribute to higher OTA presence in Ethiopian flour samples. This study revealed the contamination of teff and wheat flour samples sold in Addis Ababa market and generated useful data using HPLC-VWD apparatus.

Table 5.7. Comparison of OTA obtained in this study with the study of other investigators

Samples	Number of samples	% Positives samples	OTA (µg/kg)			Reference
			mean	median	maximum	
teff	30	20	2.21	0.99	8.64	This study, Ethiopia
teff	33	27.3	32.7	31.9	80	Amare *et al*., 2006, Ethiopia
wheat	30	50	6.34	6.76	17.35	This study, Ethiopia
wheat	107	23.34	19.6	14.9	66	Amare *et al*., 2006, Ethiopia
wheat	22	36.36	4.25	4.06	9.1	Zhang *et al*., 2011, China
wheat	14	64	0.43	0.2	1.12	Ibanez-Vea *et al*., 2011, Spain
wheat	16	27.6	0.1	0.25	0.92	Kara *et al*., 2015, Turkey
wheat	50	58	5.76	4.13	21.17	Kumar *et al*., 2012, India

5.6. Conclusion and recommendations

In this study, OTA extraction, purification and quantification using HPLC-VWD method was validated for teff and wheat matrices. The validated method accurately quantifies OTA in teff and wheat matrices. Instrument response obtained indicated the accuracy of the method ascertained by low LOD and LOQ for OTA analysis in teff and wheat. The median and mean OTA contamination levels obtained in teff were far below EU legal limits implying the relative safety of teff consumption as compared to wheat. On the other hand both the mean and median OTA contamination levels in wheat are above EU legal limits and mean and median levels of other countries indicating the risk of OTA in wheat and wheat based food items in Addis Ababa. The median OTA level in wheat flour samples was five fold higher than the median level obtained for teff implying more teff consumers are less likely exposed to OTA associated risks

In light of this current finding, since data on OTA contamination in Ethiopian cereals grains in general is scarce, more studies on the locally consumed agricultural products is necessary to fully understand the level of risk associated with this mycotoxin.

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APPENDICES

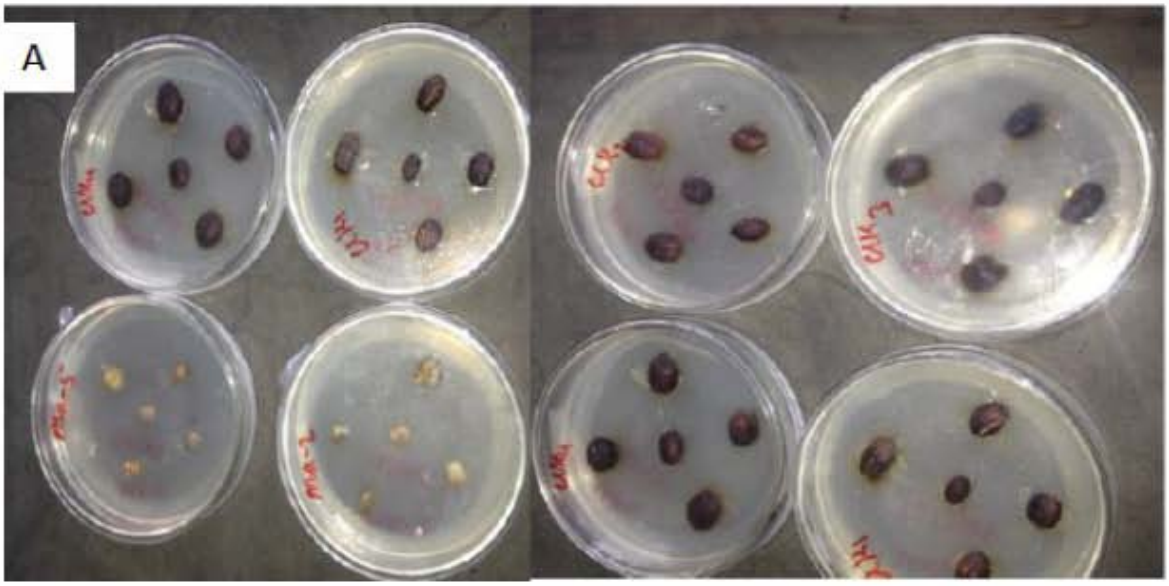
Appendix A1. Coffee sampling from mill store and concrete drying site.

A = coffee beans stored in sisal bags, B= cherries dried on concrete sun drying structure, C and D = bean sample taking.

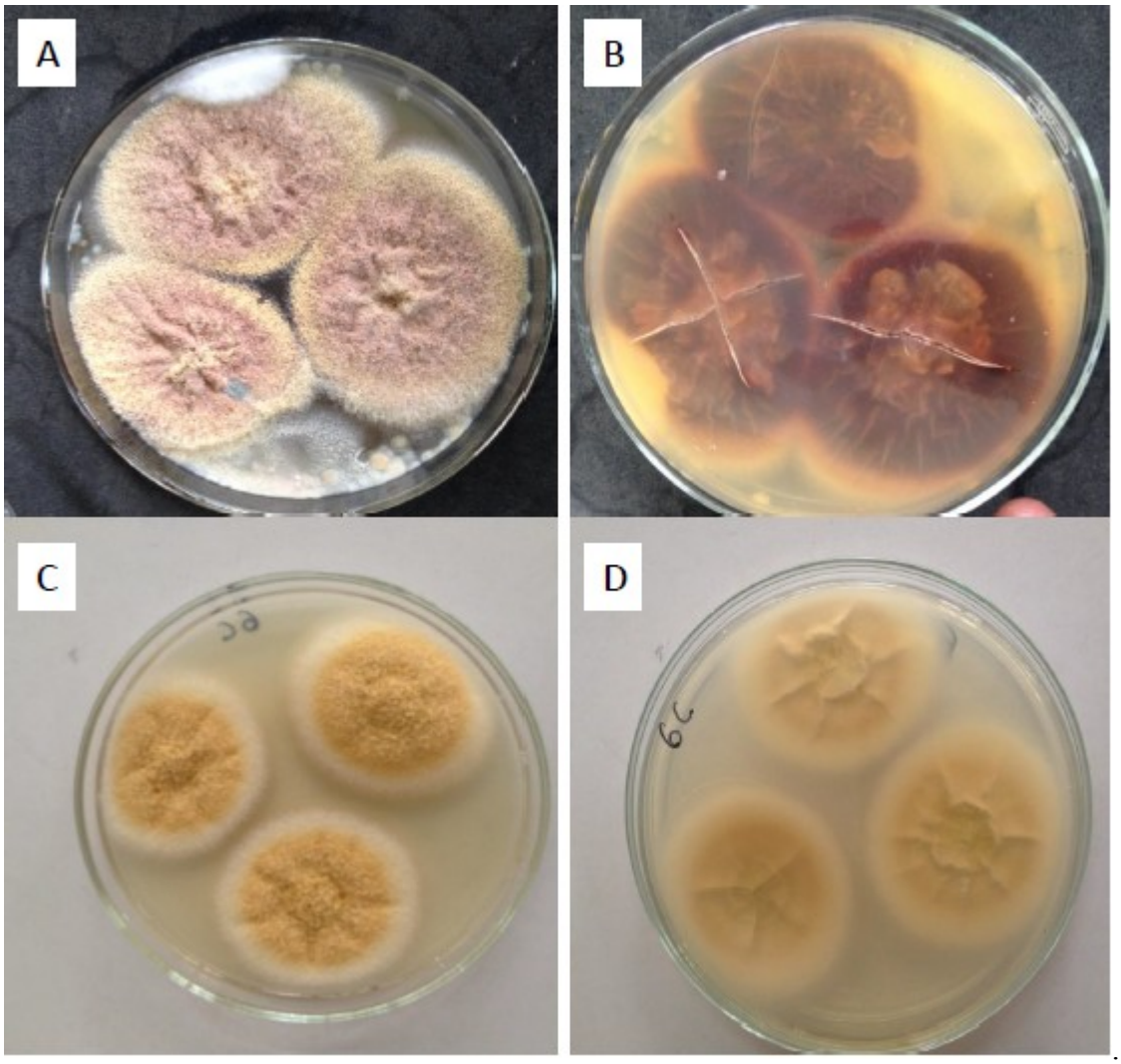
Appendix A2. Coffee cherries with visible mold overgrowth sold in local open market

Local open market in Seka ,Jimma (A, B and C). Manual sorting of black beans from sound beans at mill store (D).

Appendix A3. Isolation (A) and enumeration (B) of fungi from coffee samples.

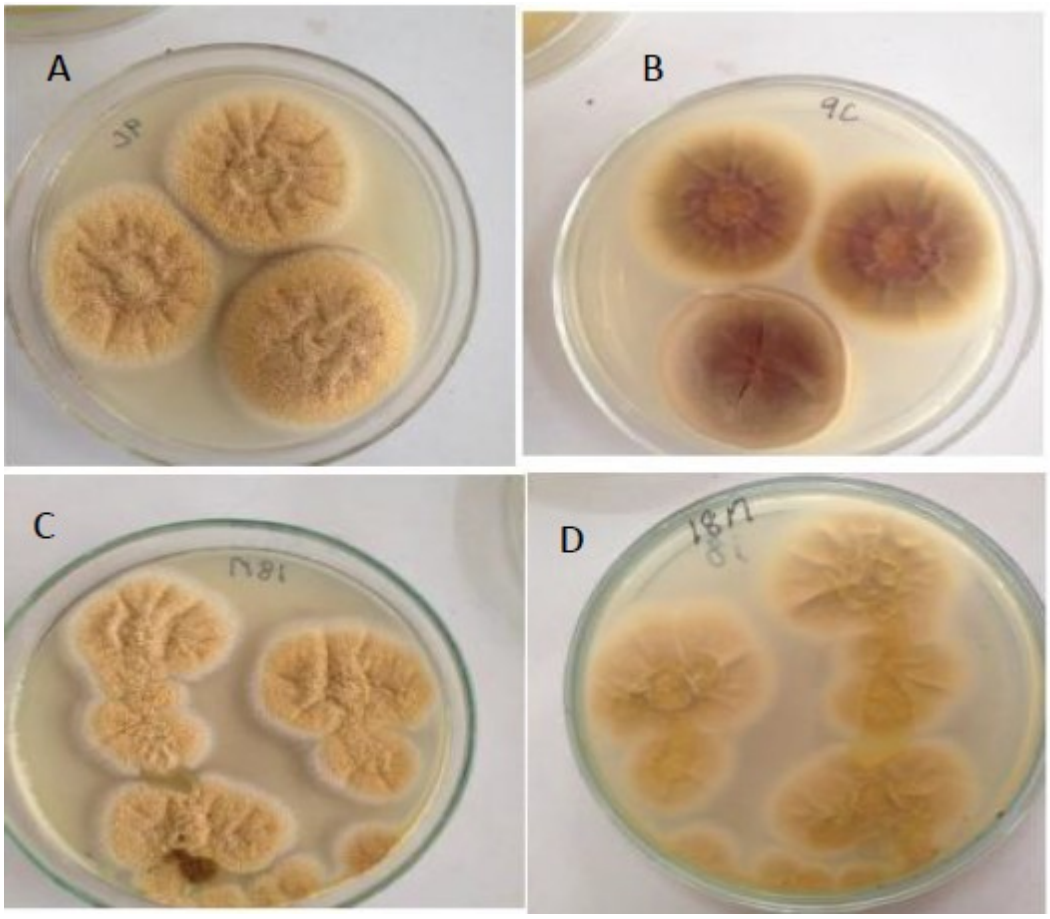


Appendix A4. Seven day old culture of *A. westerdijkiae* and *A. ochraceus* isolates on Czapek dox medium at 25 ^{0}C.



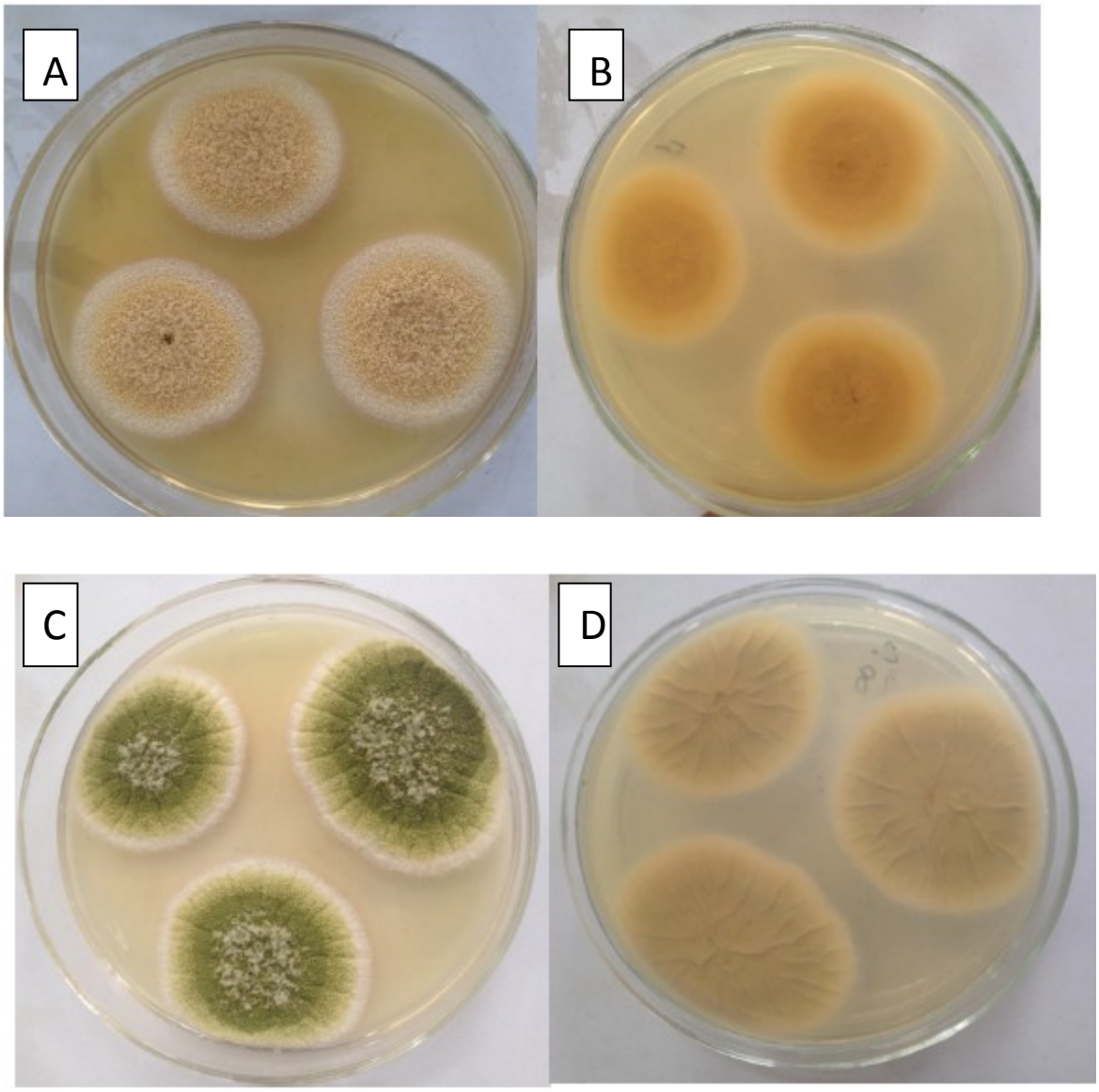
N.B. *A. westerdijkiae* (A = obverse side, B = reverse side), *A. ochraceus* (C = obverse side, D = reverse side)

Appendix A5. Seven day old culture of *A. westerdijkiae* and *A. ochraceus* on malt extract medium at 25 ^{0}C.



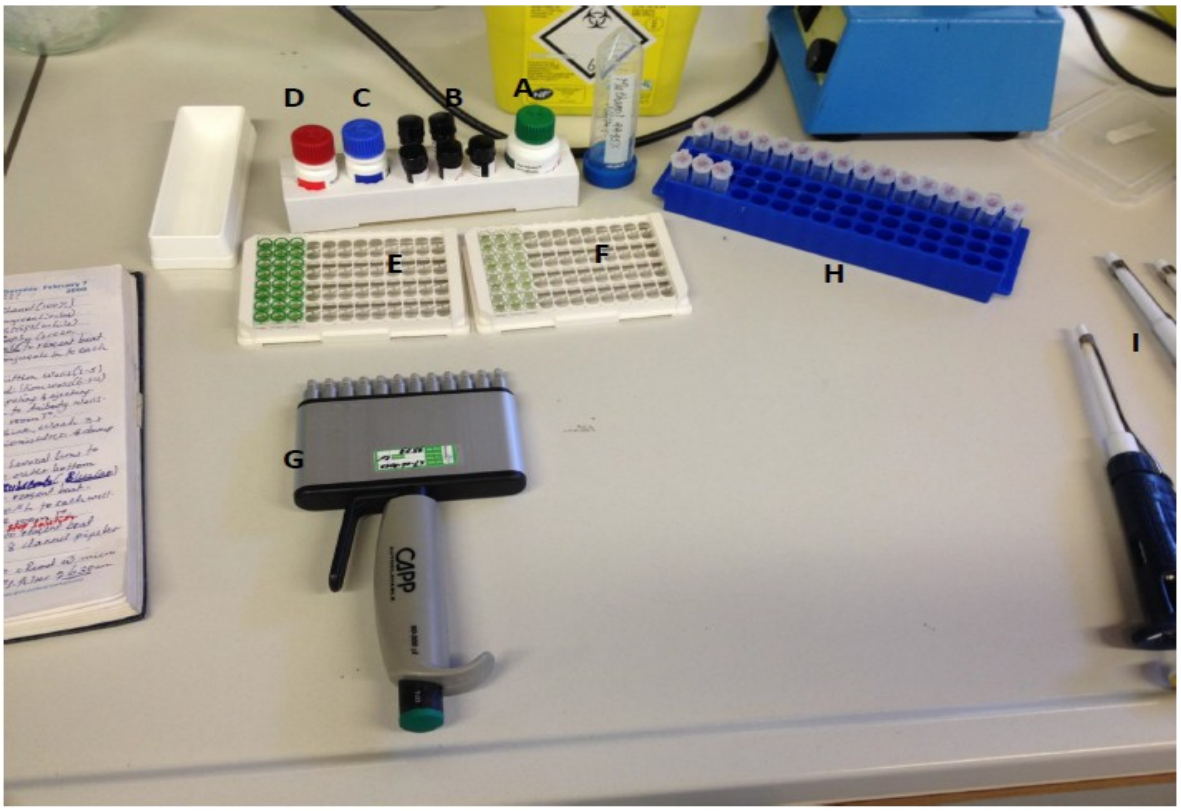
N. B. *A. westerdijkiae* (A = obverse side, B = reverse side) and *A. ochraceus* (C = obverse side, D = reverse side)

Appendix A6. Seven day old culture of *A. steynii* and *A. flavus* isolates on Czapek dox medium at 25 ^{0}C



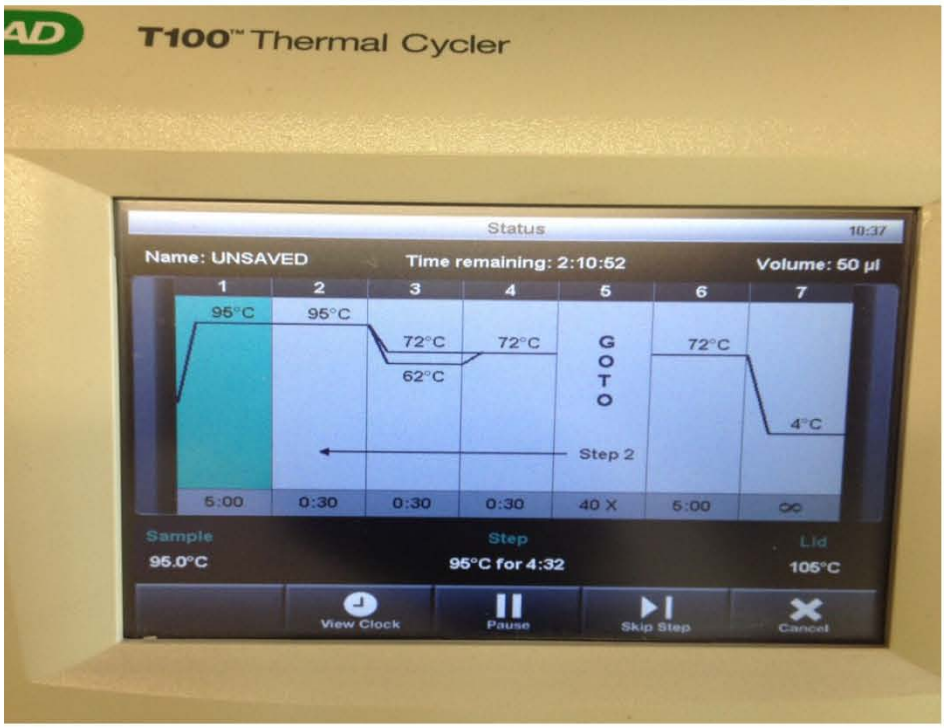
N. B. *A. steynii* (A = obverse side, B = reverse side) and *A. flavus* (C = obverse side D = reverse side) on Czapek dox medium at 25 ^{0}C.

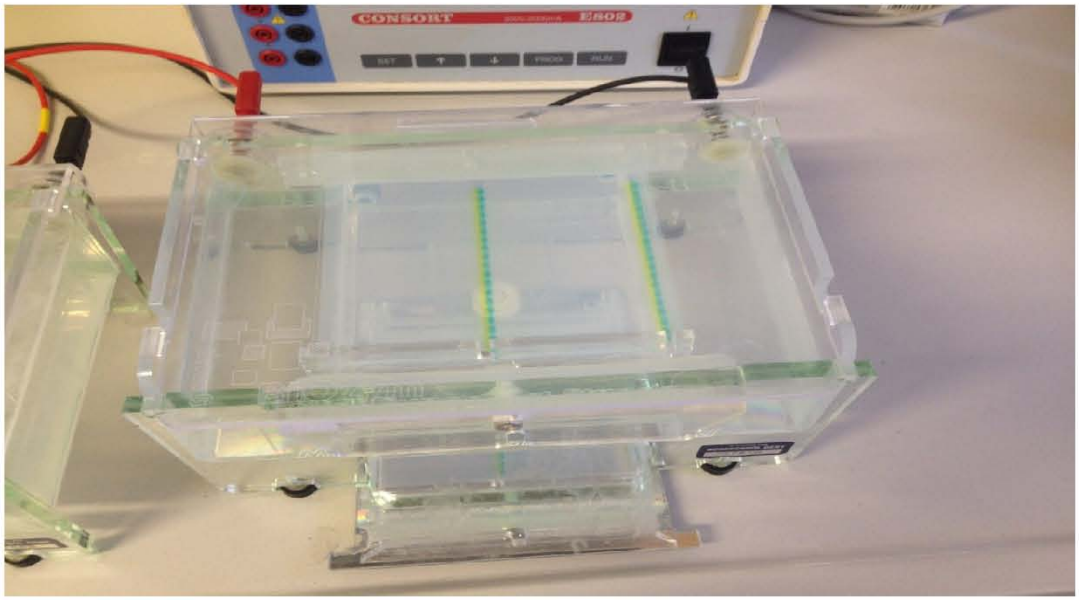
Appendix A7. ELISA Kit for OTA assay



N.B. A = conjugate (green-capped), B = five level OTA standards (0, 2, 5, 20 and 40 ppb), C = substrate (blue-capped), D = stop solution (red-capped), E = Dilution strips in microwell strip holder, F = antibody coated microwell strips in a microwell strip holder G = e ight channel micropipette, H = sample extract, I = single channel micropipette, J = ELISA reader machine.

Appedix A8. Gradient thermal cycler and gel electrophoresis chamber





Appendix B1. Meteorological data of sampling sites

Sampling Site	Altitude(m)	Average annual T(^{O}C)	Average annual rainfall(mmHg)
Gomma	1720	19.7	1764
Shebbe	1769	19	1686
Seka	1880	18.7	1700
Dedo	2220	16.5	1720
Limmu	2000	17.5	2000
Mana	2000	19.4	1754
Jimma towen	1780	19	1624
Addis AbabaM	2000	15.9	1089

Appendix B2. Summary of calculated tolerable human intake of OTA by international organizations

Organization	Tolerable intake	Limit	Reference
European Food Safety Authority (EFSA)	PTWI	120 ng/kg bw/week	EFSA, 2006
Health Canada	PTDI	3 ng/kg bw/day	Kuiper-Goodman *et al*., 2010
Health Canada	NCRI	4 ng/kg bw/day	Kuiper-Goodman *et al*., 2010
Joint FAO/WHO Expert Committee on FoodAdditives (JECFA) 2007	PTWI	100 ng/kg bw/week	JECFA, 2007
Scientific Committee of Food (SCF) of the European Union	PTDI	5 ng/kg bw/day	EFSA, 2006

N.B. TDI, tolerable daily intake; PTDI, provisional tolerable daily intake; PTWI, provisional tolerable weekly intake; NCRI, negligible cancer risk intake.

Appendix B3. Acute oral toxic levels (LD50) of various mycotoxins in different organisms (/kg/body weight)

Name of mycotoxins	Type of organism	LD50(/kg/body weight)
Ochratoxin A	pig	1 mg
	chicken	3.3 mg
	turkey	5.9 mg
Aflatoxin B1	pig	0.6 mg
	chicken	6.3mg

DON	turkey	9 mg
	Poultry	140 mg
	mice	46-78 mg
Zearalenone	Mice and rats	4000-20000 mg
T2-Toxin	pig	5 mg
	chicken	2-6 mg
	Rodent	5-10 mg
H2-Toxin	chicken	7.2 mg
	mice	6.5 mg mg
Fumonisins		unknown

(Source: Eeckhout *et al*., 2013).

Appendix B4. Coffee bean defect encountered in each samples (Detail)

Sample origin	Sample_Code	Processing_type	Storage_type	Storage_Material	%incidence	OTA (ug/kg)	Defect types													Total defect points
							black	sour	mold	cherry	insect damage	stick	immature	soil	husk	wanza	bad smell	Stone	broken	
Dedo	ETDE1	DC	Resident	FB	100	ND	26.0	0.0	0.0	0.0	0.0	0.0	4.0	0.0	2.0	0.0	3.0	0.0	3.2	38.2
Dedo	ETDE2	DC	Resident	FB	100	1.20	30.0	0.0	0.0	2.0	0.0	0.0	2.0	0.0	3.0	0.0	3.0	0.0	6.0	46.0
Dedo	ETDE3	DC	Resident	FB	100	0.72	28.0	0.0	0.0	0.0	0.0	0.0	5.6	0.0	0.6	0.0	3.0	2.0	0.0	39.2
Dedo	ETDE4	DC	Resident	FB	100	2.56	51.0	0.0	0.0	0.0	0.0	0.0	6.0	0.0	4.0	0.0	3.0	0.0	4.0	68.0
Dedo	ETDE5	DC	Resident	FB	100	3.05	100.0	0.0	0.0	0.0	46.7	1.3	4.8	0.4	6.0	0.0	3.0	0.0	8.8	171.0
Dedo	ETDE6	DC	Resident	FB	80	2.98	15.0	0.0	4.0	4.0	6.7	0.3	6.0	0.8	6.0	0.2	2.0	0.0	3.0	48.0
Dedo	ETDE7	DSG	Resident	FB	88	ND	5.0	2.0	0.0	0.0	3.0	0.0	6.0	0.0	5.0	0.0	3.0	0.0	0.0	24.0
Dedo	ETDE8	DSG	Resident	FB	92	2.54	0.0	0.0	0.0	50.0	2.3	0.0	0.0	2.0	0.0	0.0	3.0	0.0	0.0	57.3
Dedo	ETDE9	DSG	Resident	FB	100	1.20	0.0	0.0	10.6	50.0	0.0	0.0	0.0	0.0	0.0	0.0	3.0	0.0	0.0	63.6
Dedo	ETDE10	DSG	Resident	FB	88	1.78	0.0	0.0	2.4	50.0	0.0	0.0	0.0	0.0	0.0	0.0	3.0	0.0	0.0	55.4
Gomma	ETGO1	DC	Shop	SS	100	ND	8.0	3.0	0.0	5.0	6.0	1.0	7.0	7.0	0.0	0.0	2.0	0.0	6.0	45.0
Gomma	ETGO2	DC	Shop	SS	100	ND	8.0	0.0	0.0	0.0	0.0	0.0	9.0	4.0	0.0	0.0	2.0	1.0	4.0	28.0
Gomma	ETGO3	DC	Mill stores	FB	100	ND	5.0	0.0	0.0	0.0	0.0	0.0	30.0	0.0	0.0	0.0	2.0	0.0	0.0	37.0
Gomma	ETGO4	DC	Shop	FB	100	ND	4.0	0.0	0.0	0.0	0.0	0.0	20.0	0.0	0.0	0.0	2.0	0.0	0.0	26.0
Gomma	ETGO5	DC	Shop	PB	100	ND	8.0	0.0	0.0	0.0	0.0	0.0	12.0	0.0	0.0	0.0	2.0	0.0	0.0	22.0
Gomma	ETGO6	WP	Shop	PB	48	ND	4.0	0.0	0.0	8.0	0.0	0.0	1.0	0.0	0.4	0.0	2.0	0.0	0.0	15.4
Gomma	ETGO7	WP	Shop	PB	48	0.12	7.0	0.0	0.0	2.0	0.7	0.0	1.8	0.0	0.4	0.0	2.0	0.0	0.0	13.9
Gomma	ETGO8	WP	Shop	PB	52	22.64	60.0	40.0	20.0	4.0	16.7	0.0	1.2	2.0	4.0	0.0	2.0	0.0	0.8	150.7
Gomma	ETGO9	DC	Shop	SS	60	ND	4.0	0.0	0.0	0.0	0.0	0.0	22.0	0.0	4.0	0.0	2.0	0.0	0.0	32.0
Gomma	ETGO10	DC	Shop	FB	92	ND	8.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.0	0.0	0.0	10.0
Shebbe	ETSH1	DT	Mill stores	FB	80	0.03	4.0	7.0	0.0	0.0	0.0	0.0	1.8	0.4	2.0	0.0	0.0	2.0	0.0	17.2
Shebbe	ETSH2	DT	Mill stores	FB	24	0.56	38.0	44.0	0.0	0.0	2.0	0.0	7.4	0.4	0.0	0.0	0.0	0.0	1.0	92.8
Shebbe	ETSH3	DT	Mill stores	FB	72	1.66	20.0	12.0	0.0	0.0	0.0	0.0	5.2	2.6	3.0	0.0	0.0	10.0	0.8	53.6
Shebbe	ETSH4	DT	Mill stores	FB	88	ND	12.0	3.0	0.0	0.0	0.0	1.0	34.0	0.0	0.0	0.0	2.0	0.0	2.0	54.0

Shebbe	ETSH5	DT	Mill stores	FB	96	ND	20.0	0.0	0.0	0.0	0.0	0.0	32.0	0.0	0.0	0.0	2.0	0.0	5.0	59.0
Shebbe	ETSH6	DT	Resident	FB	96	1.07	27.0	5.0	1.2	0.0	0.0	0.0	6.6	0.6	4.0	0.2	0.0	0.0	0.0	44.6
Shebbe	ETSH7	DT	Resident	FB	92	ND	0.0	0.0	0.0	0.0	5.0	0.0	45.0	0.0	2.0	0.0	2.0	0.0	0.0	54.0
Shebbe	ETSH8	DT	Resident	SS	100	5.94	150.0	7.0	0.0	0.0	10.0	0.0	1.4	0.0	1.0	4.4	0.0	0.0	14.0	187.8
Shebbe	ETSH9	DT	Mill stores	FB	100	1.76	100.0	3.0	2.4	0.0	1.0	0.0	14.4	0.2	0.8	0.0	0.0	0.0	4.0	125.8
Shebbe	ETSH10	DT	Mill stores	FB	100	0.69	0.0	0.0	2.0	20.0	0.0	0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	22.7
Seka	ETSE1	DC	Mill stores	FB	68	ND	13.0	0.0	0.0	0.0	0.0	0.0	36.0	0.0	0.0	0.0	2.0	0.0	0.0	51.0
Seka	ETSE2	DC	Mill stores	FB	100	ND	50.0	25.0	0.4	0.0	0.0	0.0	4.6	0.6	3.0	0.4	0.0	0.0	3.2	87.2
Seka	ETSE3	DC	Mill stores	FB	88	0.64	50.0	35.0	2.8	0.0	0.0	0.0	2.0	2.8	0.0	0.0	0.0	4.0	1.6	98.2
Seka	ETSE4	DC	Mill stores	FB	88	0.95	35.0	35.0	0.0	0.0	0.0	0.0	2.8	0.2	1.6	0.0	0.0	0.0	4.0	78.6
Seka	ETSE5	DC	Resident	FB	100	1.46	70.0	37.0	0.0	6.0	0.0	0.0	5.2	0.0	3.0	0.4	0.0	0.0	3.0	124.6
Seka	ETSE6	DC	Resident	FB	88	ND	2.0	0.0	0.0	0.0	0.0	0.0	40.0	0.0	0.0	0.0	2.0	0.0	0.0	44.0
Seka	ETSE7	DC	Resident	FB	100	2.81	40.0	40.0	10.0	20.0	23.3	1.3	4.0	1.2	1.4	0.0	2.0	3.0	8.0	154.3
Seka	ETSE8	DSG	Resident	FB	100	22.90	100.0	0.0	20.0	40.0	13.3	0.0	0.0	0.0	20.0	0.0	3.0	0.0	0.0	196.3
Seka	ETSE9	DSG	Resident	FB	100	1.39	0.0	0.0	6.0	40.0	0.0	0.0	0.0	20.0	0.0	0.0	3.0	0.0	0.0	69.0
Seka	ETSE10	DSG	Resident	FB	100	0.98	0.0	0.0	10.0	40.0	0.0	0.7	0.0	1.0	2.0	0.0	3.0	0.0	0.0	56.7
Limmu	ETLI1	DSG	Shop	PB	100	ND	7.0	8.0	0.0	0.0	0.0	2.0	10.0	0.0	0.0	0.0	2.0	0.0	0.0	29.0
Limmu	ETLI2	DC	Shop	PB	100	0.42	80.0	0.0	2.0	0.0	0.0	0.0	6.0	0.0	1.0	0.0	3.0	0.0	6.0	98.0
Limmu	ETLI3	DC	Shop	SS	92	ND	77.0	0.0	0.0	0.0	0.0	0.0	20.0	0.0	0.0	0.0	2.0	0.0	0.0	99.0
Limmu	ETLI4	DC	Shop	SS	56	1.02	24.0	0.0	0.0	10.0	0.0	0.0	2.4	0.0	1.0	0.0	2.0	0.0	4.0	43.4
Limmu	ETLI5	DC	Shop	SS	100	ND	78.0	0.0	0.0	0.0	0.0	0.0	30.0	0.0	0.0	0.0	2.0	0.0	0.0	110.0
Limmu	ETLI6	DC	Mill stores	SS	96	ND	45.0	0.0	0.0	0.0	0.0	0.0	25.0	0.0	0.0	0.0	2.0	0.0	0.0	72.0
Limmu	ETLI7	DSG	Resident	SS	96	ND	0.0	0.0	1.2	30.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	31.2
Mana	ETMA1	DT	Mill stores	SS	92	1.61	65.0	0.0	0.0	0.0	0.0	0.0	18.8	1.2	0.0	0.0	3.0	3.0	4.2	95.2
Mana	ETMA2	DT	Mill stores	SS	92	2.45	38.0	0.0	0.0	0.0	3.3	0.3	2.8	0.0	1.8	0.0	3.0	0.0	4.8	54.1
Mana	ETMA3	DT	Mill stores	SS	76	1.97	100.0	0.0	0.6	0.0	0.0	0.0	6.0	0.2	0.2	0.0	3.0	0.0	12.0	122.0
Mana	ETMA4	DT	Mill stores	SS	92	1.87	76.0	0.0	2.0	0.0	0.3	0.0	6.0	0.0	2.0	0.0	3.0	0.0	4.0	93.3
Mana	ETMA5	DT	Resident	SS	92	ND	34.0	0.0	0.0	0.0	0.0	0.0	30.0	0.0	0.0	0.0	2.0	0.0	0.0	66.0
Mana	ETMA6	DT	Resident	SS	96	0.95	60.0	0.0	0.0	0.0	0.0	0.0	14.0	0.0	3.6	0.0	0.0	0.0	4.0	81.6

Mana	ETMA7	DT	Resident	SS	92	0.51	23.0	9.0	0.0	2.0	0.0	0.0	0.0	0.4	1.2	0.0	0.0	0.0	1.4	37.0
Mana	ETMA8	DT	Resident	SS	96	12.29	100.0	5.0	10.0	12.0	26.7	1.7	16.0	0.8	3.6	0.0	3.0	0.0	10.0	188.7
Mana	ETMA9	DT	Resident	SS	100	3.42	20.0	0.0	5.2	40.0	11.3	1.7	0.0	0.6	1.0	0.0	1.0	3.0	0.0	83.8
Mana	ETMA10	DT	Resident	SS	88	3.59	20.0	0.0	6.0	40.0	9.3	3.3	0.0	1.0	0.0	0.0	2.0	6.0	0.0	87.7
Mana	ETMA11	DT	Resident	SS	100	3.02	60.0	0.0	0.0	2.0	2.0	0.0	10.0	0.4	1.8	0.0	0.0	0.0	1.6	77.8
Jtown	ETJT1	DC	Shop	PB	100	3.27	6.0	0.0	0.0	0.0	0.0	0.0	0.6	0.0	0.0	0.0	3.0	0.0	0.6	10.2
Jtown	ETJT2	WP	Shop	PB	76	1.72	15.0	0.0	3.6	30.0	5.0	4.0	13.2	0.0	0.0	0.0	9.0	0.0	3.6	83.4
Jtown	ETJT3	WP	Shop	PB	88	0.44	18.0	12.0	1.8	18.0	5.0	1.0	3.6	6.0	1.8	0.0	0.0	0.0	2.4	69.6
Jtown	ETJT4	WP	Shop	PB	32	ND	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	18.0	0.0	0.0	0.0	6.6	24.6
Jtown	ETJT5	WP	Shop	PB	60	0.87	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	12.0	0.0	0.0	0.0	25.8	37.8
Addis	ETM1	DC	Mill stores	SS	84	2.16	165.0	0.0	3.6	0.0	6.0	0.0	12.0	0.0	0.0	0.0	3.0	0.0	0.0	189.6
Addis	ETM2	DC	Mill stores	SS	72	2.26	108.0	0.0	2.4	24.0	7.0	0.0	40.8	0.0	0.0	0.0	3.0	0.0	0.0	185.2
Addis	ETM3	DC	Mill stores	SS	80	3.10	15.0	21.0	3.6	36.0	8.0	2.0	30.0	1.2	1.8	0.0	6.0	0.0	3.0	127.6
Addis	ETM4	DC	Mill stores	SS	80	2.41	12.0	18.0	3.0	12.0	6.0	2.0	24.0	0.6	0.0	0.0	6.0	3.0	4.8	91.4
Addis	ETM5	DC	Mill stores	SS	80	1.53	60.0	0.0	9.0	18.0	15.0	2.0	30.0	0.0	0.0	0.0	0.0	0.0	6.0	140.0
Addis	ETM6	DC	Mill stores	SS	80	0.60	6.0	0.0	0.0	0.0	4.0	3.0	24.0	0.0	0.0	0.0	0.0	0.0	0.0	37.0
Addis	ETM7	DC	Mill stores	SS	80	ND	0.0	0.0	8.4	0.0	8.0	0.0	0.0	0.0	0.0	0.0	9.0	0.0	0.0	25.4
Addis	ETM8	DC	Mill stores	SS	80	ND	0.0	0.0	46.2	0.0	10.0	0.0	0.0	0.0	0.0	0.0	9.0	0.0	0.0	65.2
Addis	ETM9	DC	Mill stores	SS	88	ND	0.0	0.0	50.4	0.0	8.0	0.0	0.0	0.0	0.0	0.0	9.0	0.0	0.0	67.4
Addis	ETM10	DC	Mill stores	SS	72	ND	0.0	0.0	54.0	0.0	12.0	0.0	0.0	0.0	0.0	0.0	9.0	0.0	0.0	75.0
Addis	ETM11	DC	Mill stores	SS	80	ND	30.0	0.0	24.0	0.0	5.0	2.0	39.0	1.8	1.8	0.0	9.0	0.0	0.0	112.6

N.B. DC=dried on concrete drying floor, DSG=dried on soil ground, WP < washed parchment, DT=dried by traditional means, FB=fertilizer bags, SS=sisal sacks, PB= plastic bags

Appendix C1. Ingredients of mycological media used in this study

Czapek Dox agar medium

Ingredients	gram/Liter
Sucrose	30.000
Sodium nitrate	2.000
Dipotassium phosphate	1.000
Magnesium sulphate	0.500
Potassium chloride	0.500
Ferrous sulphate	0.010
Agar	15.000
Final pH (at 25°C)	7.3±0.

Malt extract agar medium (MEA)

Ingredients	grams / Litre
Malt extract	30.000
Mycological peptone	5.000
Agar	15.000
Final pH (at 25°C)	5.4±0.2

Yeast extract sucrose broth (YES)

Yeast extract	20.0g
$MgSO_4.5H_2O$	0.5g
Sucrose	150.0g
Agar	20.0g
Water	1000 mL
Trace metal solution ($ZnSO_4.7H_2O$ 1.0g, $CuSO_4.5H_2O$ 0.5g, Water 100 mL, pH 7.2)	1.0 mL

Declaration

I, the undersigned, declare that this PhD Dissertation is my own original work and has not been presented by me or any other person for a similar reason in any other university, and all sources of materials used for thise dissertation have been duly acknowledged.

PhD candidate: Teshome Geremew Signature  Date Feb. 28/2017

Supervisor: Dr. Dawit Abate

(PhD, Associate Professor) Signature _____Date__Fe. 28/2017

Addis Ababa, Ethiopia.