

Research Article

Determination of Ochratoxin A in Yemeni Green Coffee

Qais Abdullah Nogaim^{1*}, P. Mangala Gowri²

¹Food Science and Technology Department, Ibb University, Yemen

²I.I.C.T, Hyderabad, India

*Corresponding author

Dr. Qais Abdullah Nogaim

Email: qaisnogaim@yahoo.com

Abstract: The present study aims to determine the occurrence of ochratoxigenic fungi and ochratoxin A in Yemeni green coffee (*Coffea arabica* L.), by analyzing 70 samples which were collected from many local markets in some Yemeni Governorates during 2010/2011. The analysis carried out in Natural Products Chemistry Laboratories, Indian Institute of Chemical Technology (I.I.C.T), Hyderabad, India, and the results could be summarized as the following: by classification of *Aspergillus* genus we found these species *Aspergillus niger*, *Aspergillus ochraceus*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus parasiticus*, *Aspergillus acceleatus* and *Aspergillus candidus* by percentages were 27.5, 17.5, 15, 12.5, 12.5, 10 and 5%, respectively. And when we determine their (toxicity) ability to produced ochratoxin A in (YES) medium we found these percentages 30, 40, 20, 20, 0, 0 and 0 %, respectively. Meanwhile, the concentrations of ochratoxin A were ranged between 0.152 to 12.456 ng/ml of (YES) medium for 15 days growth. On the other hand, the positive samples of Yemeni green coffee beans which contaminated by ochratoxin A were 7 out of 70 samples by percentage of 10%, and the concentration of toxin ranged between 0.314 to 3.443 ng/g and the average was 1.211 ng ochratoxin A / per g of Yemeni green coffee beans.

Keywords: Yemeni coffee, Mycotoxins, Ochratoxin A.

INTRODUCTION

The term mycotoxin is derived from the Greek word 'mycos' meaning mould, and the Latin word 'toxicum', which means poison. Mycotoxins are relatively low-molecular weight secondary metabolites of fungal origin that are harmful to animals and humans. Mycotoxins are toxic secondary metabolites produced by various fungi which affect a wide range of agricultural products meant for human consumption and animal feed. Mycotoxins present in food products and animal feeds are an important problem concerning food and feed safety and significant economic losses are associated with their impact on human and animal health [1]. Ochratoxin A is a mycotoxin with nephrotoxic effects produced mainly by *Aspergillus* sections Nigri and Circumdati and *Penicillium verrucosum* [2]. Particularly *Aspergillus ochraceus*, *Aspergillus carbonarius*, *Aspergillus niger* and *Penicillium verrucosum* predominantly found in cereal grains, cocoa, spices, oilseeds, coffee beans and legumes resulting in human exposure. This mycotoxin has been classified as a possible human carcinogen (group 2B) by the International Agency for Research on Cancer (IARC). The Joint Committee FAO/WHO of Experts on Food Additives (JECFA) has established the provisional tolerable weekly intake (PTWI) of ochratoxin A at 100 ng/kg of body weight (bw) corresponding to approximately 14 ng/kg bw/day [3]. The genus *Coffea* is diverse comprising of about 103 species [4]. They are shrubs or small trees, native to subtropical Africa and southern Asia. Seeds of several species are the source of the popular beverage coffee.

After their outer hull is removed, the seeds are commonly called "beans". Coffee beans are widely cultivated in tropical and sub-tropical countries on plantations, for both local consumption and export to probably every other country in the world [5]. Coffee is one of the world's most popular beverages. It is also the most important consumed and traded food commodity worldwide and ranks second, after crude oil, among all commodities [6]. World coffee production grew by over 100% from 1950 to 1990. In 2005 coffee production reached 6.4 million tons worldwide and is projected to grow by 0.5–1.9%, annually. Global output is expected to reach 7.0 million tons in 2010. World consumption of coffee is projected to increase by 0.4% annually from 6.7 million tons in 1998–2000 to 6.9 million tons in 2010. Brazil remains the largest green coffee producer and exporter, accounting for approximately 35% of the world market [7]. Meanwhile Yemeni Mocha coffee is regarded as the most traditional coffee and still one of the world's greatest, uniquely delicious coffee. It takes its name from the Yemen port city called Mocha [8]. So, the present study aims to determine the occurrence of ochratoxigenic fungi and ochratoxin A in Yemeni green coffee (*Coffea arabica* L.), by analyzing 70 samples which were collected from many local markets in some Yemeni Governorates.

MATERIALS AND METHODS

Samples

A total of 70 samples of Yemeni green coffee beans (*Coffea arabica* L.), were collected by Yemen Standardization Metrology and Quality Control

Organization (YSMQCO) from many local markets in some Yemeni Governorates during 2010/2011.

Chemicals

All chemicals were analytical reagent grade, excepting chloroform, methanol and acetonitrile (HPLC grade). Ochratoxin A standard: was purchased from Sigma, Chemical Company, P. O. Box 4508, (St. Louis, MO, USA). The OchraStar™ Fit-Immunoaffinity Columns: Item No. COIAC2001, was purchased from Romer Labs Diagnostic GmbH, Technopark 1, 3430 Tulin, Austria.

PBS- buffer (0.05 M / 0.15 M NaCl, pH 7.4) : weight 8g NaCl ; 1.16g Na₂HPO₄*2H₂O ; 0.2g KH₂PO₄ ; 0.2g KCl (all P.A. Quality), dissolve in 990 mL of distilled or deionized water ; adjust pH to 7.4 using NaOH or HCl (both approximately 1 molar), fill up to 1000 mL with distilled or deionized water.

Isolation and Identification

Coffee beans were rinsed in distilled water to remove dust, then surface disinfected in 0.4% hypochlorite solution and again washed by distilled water after that, they dried by using tow layers of filter paper in a sterilized Petri dishes. One hundred beans per sample, five bean in each Petri dish were plated onto potato dextrose agar (PDA), a medium known to support growth of the target fungi. After incubation at 25 ± 2 °C for 5-7 days, all fungi isolates growing from the beans were enumerated. During the incubation period any emerged fungus was isolated on PDA slants and purified by using the single spore technique of Manandhar *et al.* [9]. The obtained isolates were identified using the microscopic and cultural characteristic according to [10-13]. Forty-five *Aspergillus* spp. isolates grown on Yeast Extract Sucrose (YES) at 25 ± 2 °C for 15 days to determine the toxicity of these isolates which were screened for OTA production by HPLC. The frequency occurrence of each fungal isolate was expressed as the percentage of sample a given organism. Predominant fungi were identified according to the identification key for common food-borne fungi [14].

Extraction and cleanup of OTA in coffee

The extraction and cleanup procedures of OTA in the coffee samples were performed according to the procedure of Romer Labs (OchraStar™ Fit-Immunoaffinity Columns: Item No.COIAC2001) pamphlet.

Extract

Twenty five grams of the finely blended samples were mixed with 100 mL of methanol/water (80/20). The suspension was blended for 3 hours at medium speed (100 RPM) on gyratory shaker, and then using a funnel, filter extract into a sample jar through Whatman filter paper. The extract was diluted with (PBS) until the content of methanol is not higher than 20 % (V/V).

Sample application

After that, the OchraStar™ Fit-Immunoaffinity Column was put on an adapter. The column and the extract must be at room temperature. The column does not require rinsing before application of the diluted extract. The OchraStar™ Fit-Immunoaffinity Column usually drips independently. The adapter with the syringe barrel was attached. The diluted extract was applied and allowed to pass the column. The flow rate should not exceed 1-3 mL/min.

Wash

After the diluted extract has completely passed through, the column should be washed with 2 x 10 mL distilled or deionized water. The first portion of the wash solution should be used to rinse the container. Any remaining liquid should be removed from the column by applying slight pressure on top of the column or by applying vacuum in the bottom. Take care that the column does not dry completely.

Elution

The syringe barrel was removed from the OchraStar™ Fit-Immunoaffinity Column and a suitable vial is placed under the column for the collection of the eluate. For elution of bound ochratoxin only water free methanol (HPLC Grade) was used. Then eluted with 1.5-3 mL methanol/acetic acid (98/2) (V/V), which should be applied to the column in several small portions (for example 3 x 0.5 mL). The methanol/acetic acid (98/2)(V/V), should be left on the column for a few seconds before starting elution to allow intensive contact with the gel. After the last portion was applied, the methanol still remaining in the column was removed, and added to the remaining eluate. This can be directly analyzed by HPLC. In case of low-level contamination, the eluate can be dried down and the residue can be dissolved in a small portion of mobile phase.

HPLC Conditions (Properties)

Column: Fortis. H₂O -Sum,

Size: 250 x 4.6 mm

Flow rate: 1 mL/min

Wavelength: 210 nm

Instrument: Shimadzu LC- 20 AT Pump (Quaternary), (Shimadzu Corporation, Japan)

SIL – 10 AD Auto samples

SPD – 10 A – UV – VIS detector

LC – Solutions Software

Injection Volume: 10 µL

Mobile phase: MeOH/Water,(80/20)

The OTA concentration in samples was determined by using peak area and then plotting it on a standard curve. OTA stock solution was prepared with toluene–acetic acid (99:1) (V/V). Working solution were prepared by diluting stock solution to 10 µg/mL. Concentration was determined according to AOAC, [15] by spectrophotometer at 333 nm.

RESULTS AND DISCUSSIONS

Coffee (*Coffea arabica* L.), a native of Africa, is grown in more than 50 countries throughout the tropics by more than 20 million coffee farming families [16]. The wide distribution of coffee throughout the tropics has exposed this plant to the fungal diversity within each region where it grows. Fungi associated with coffee have been extensively studied only from a plant pathogen perspective, including infection of the seed and berry. Our samples were 70 Yemeni green coffee beans, from the previous work we know that, the moisture content were $6.99 \pm 0.48\%$ as average. Meanwhile, the total fungi counts were 6.17×10^6 CFU as average. On the other hand the percentage of infection

was 41.85% as average, and these fungi genera were *Aspergillus*, *Penicillium*, Yeasts, *Mucor* and *Fusarium*, by percentages were 26.85, 7.29, 5.14, 1.43 and 1.14 %, respectively [17]. So, here we started by classification the species of *Aspergillus* genus isolates, and we found that, 27.5 % were *Aspergillus niger* which was the dominants, meanwhile the others species were *Aspergillus ochraceus*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus parasiticus*, *Aspergillus acceleatus* and *Aspergillus candidus* by percentages were 17.5, 15, 12.5, 12.5, 10 and 5%, respectively. After that, we assessed the toxicity of these fungi species in an in-vitro experiment by using (YES) medium for all these isolates for 15 days according to Davis *et al.* [18]. And the results were as below:

Table 1: The toxicity of *Aspergillus* spp. Isolates from Yemeni green coffee on YES Medium

Fungi species	No. of isolates	Positive OTA	Percentage %	Concentration OTA ng/mL YES
<i>Aspergillus niger</i>	10	3	30%	0.152- 0.329
<i>Aspergillus ochraceus</i>	10	4	40%	6.529- 12.456
<i>Aspergillus fumigatus</i>	5	1	20%	0.175
<i>Aspergillus flavus</i>	5	1	20%	0.221
<i>Aspergillus parasiticus</i>	5	0	0	0
<i>Aspergillus acceleatus</i>	5	0	0	0
<i>Aspergillus candidus</i>	5	0	0	0

Data in table (1) showed that, out of 45 isolates of *Aspergillus* spp. Which including *Aspergillus niger*, *Aspergillus ochraceus*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus parasiticus*, *Aspergillus acceleatus* and *Aspergillus candidus* when we determine their ability to produced ochratoxin A in (YES), we found that, the percentages of positive isolates were 30, 40, 20, 20, 0, 0 and 0 % , respectively. And the concentrations of ochratoxin A were ranged between 0.152 to 12.456 ng/ml of (YES) medium for 15 days growth. Our results were in harmony with many researchers in different countries who reported that, like other crops, coffee cherries and beans are subjected to contamination and consequent colonization by microorganisms during different phases of development, harvesting, preparation, transport and storage. Microbial action detrimental to the quality and safety of the final product will depend on environmental conditions as well as crop and product management. Studies on the microbiology of coffee cherries and beans have shown that the main toxigenic fungal genera (*Aspergillus*, *Penicillium* and *Fusarium*) are natural coffee contaminants, and are present from the field to the warehouse [19-21]. Fungi can infect agricultural crops during crop growth, harvest, storage or processing. The growth of fungi is not necessarily associated with the formation of mycotoxins and because of the stability of mycotoxins; they may be present in food when fungi are no longer present.

Furthermore, a fungus may produce different mycotoxins, and a mycotoxin may be produced by several different fungi. The mycotoxigenic potential depends on species and strains of fungus, composition of matrix and environmental factors (temperature and moisture). To determine the extent of coffee bean contamination by toxigenic fungi and Ochratoxin, forty eight of coffee samples were collected from local markets of the Saudi city, Jeddah by Bokhari [22] who was reported that, out of these samples, forty six toxigenic isolates related to the genera *Aspergillus* and *Penicillium* were isolated and tested for their production of Ochratoxin (OTA) using TLC chromatography. The finding was that, four of *A. ochraceus* produced OTA. Using HPLC, it was found that eight samples of coffee beans were naturally contaminated. The quantification of Ochratoxin A. ranged between 3.77-25.9 $\mu\text{g kg}$. This indicates that coffee, the most popular beverage in Saudi Arabia, has high level of toxin contamination when it is stored or displayed in less hygienic conditions. *A. carbonarius* and *A. niger* were described as sources of OTA within the last 10 years. Although it is commonly assumed that the major source of OTA in coffee is *A. ochraceus*, little solid evidence of this exists. The genus *Aspergillus* is one of the most important filamentous fungal genera. *Aspergillus* species are used in the fermentation industry, but they are also responsible of various plant and food secondary rot, with the consequence of possible accumulation of

mycotoxins. The ochratoxinogenic *A. niger*, *A. ochraceus* and *A. carbonarius* species are frequently encountered in agricultural products. Studies on the biodiversity of toxigenic *Aspergillus* species by Perrone *et al.* [23], they analyzed the biodiversity of ochratoxin producing species occurring on two important crops: grapes and coffee. Similar studies on the *Aspergillus* species occurring on coffee beans have evidenced in the last five years that *A. carbonarius* is an important source of ochratoxin A in coffee. Four new species within the black aspergilli were also identified in coffee beans: *A. sclerotiumniger*, *A. lacticoffeatus*, *A. sclerotiumcarbonarius*, and *A. aculeatinus*.

OTA is receiving increasing attention worldwide because of its wide distribution in food and feed and human exposure that most likely comes from low level of OTA contamination of a wide range of different foods. The OTA producing strains of *A. carbonarius* ranged between 70 and 100 % when grown *in vitro* and tested using HPLC, while the range of producing strains was around 2–20 % for *A. niger* and *A. tubingensis* [24]. Some reports claimed the production of OTA also by *A. japonicus* but it has not yet been confirmed [25 and 26]. Biodiversity of black aspergilli on Thai coffee beans Ochratoxin A contamination of coffee is a worldwide problem. The presence of OTA in green coffee bean has been reported by several authors in wide concentration ranging between 0.2 and 360 µg/kg [27]. Extensive sampling of green coffee beans of both Arabica and Robusta types worldwide indicated that although OTA contamination is more frequent in some areas including mainly African countries, no producing country was found to be free of contamination [27]. Although previously *A. ochraceus* was suggested to be sole source of OTA contamination on coffee, recent studies indicated that other species, including *A. steynii*, *A. westerdijkiae*, *A. carbonarius*, *A. lacticoffeatus*, *A. sclerotiumniger* and *A. niger* are also able to produce OTA on coffee [28]. Different types of black aspergilli were reported in coffee bean from different countries. *A. niger* and *A. carbonarius* occurred most frequently. Extensive studies have been carried out on the mycobiota of Brazilian coffee recently. From the study of arabica coffee beans by Taniwaki *et al.* [29], the results showed that *A. niger* was the species found most commonly (63 % of potential OTA producers), but only 3 % of them produced OTA. *A. ochraceus* also occurred commonly (31 % of isolates), and 75 % of those studied were capable of OTA production, a much higher percentage than reported elsewhere. *A. carbonarius* was found (6 % of isolates) only in the hottest region sampled, and only from beans in the drying yard or in storage. However, 77 % of the *A. carbonarius* isolates were capable of producing OTA. Other studies reported similar species distribution on Brazilian coffee beans. Martins *et al.* [30] used a conventional method to identify fungal flora in coffee bean. The predominant fungal genus was *Aspergillus*, including *A. niger* (83.3 %), *A. ochraceus* (53.3 %) and

A. flavus (25 %). The incidence of other genera was substantially lower than that of aspergilli. Magnani *et al.* [31] isolated and identified *Aspergillus* spp. that contaminate coffee beans by sequencing the ITS region of the isolates. The incidence of potentially ochratoxinogenic species was 82 % with *A. niger* being found most frequently, followed by *A. ochraceus* and *A. carbonarius*. However, the mycobiota of coffee beans in other countries can be significantly different, e.g. in Ilic *et al.* [32], Vietnamese Robusta coffee beans were studied, and *A. niger* was the only ochratoxinogenic species recovered. However, in another study carried out by Leong *et al.* [33], *A. carbonarius* isolates have also been recovered from Vietnamese Robusta and Arabica coffee bean samples. Significantly, more Robusta than Arabica beans were infected by fungi. As a result of the survey of ochratoxin-producing aspergilli in Thai coffee beans, they also identified 2 new black *Aspergillus* species. One of them (*A. aculeatinus*) is related to *A. aculeatus* and other uniseriate black aspergilli and could be recovered from both regions, while the other one (*A. sclerotiumcarbonarius*) is related to *A. carbonarius* and *A. ibericus*, and was found only in the Southern region of Thailand. The results confirmed former studies, only *A. carbonarius* and *A. niger* could produce ochratoxins. In their study, 100 % of the *A. carbonarius* isolates tested could produce large amounts of OTA but none of them produced ochratoxin B. This is in agreement with Joosten *et al.* [34], who reported that all *A. carbonarius* strains isolated from Thai coffee produced a significant amount of OTA. Similarly, Pardo *et al.* [35] found that all *A. carbonarius* isolates came from coffee beans from various countries produced OTA, and Leong *et al.* [36] also observed that almost all (110/113) of the examined *A. carbonarius* isolates came from Vietnamese coffee beans could produce OTA. However, Taniwaki *et al.* [29] observed that only 77 % of the *A. carbonarius* isolates came from Brazilian coffee beans produced OTA. Differences in the ratio of *A. carbonarius* isolates able to produce OTA could be due to misidentification of the non-OTA producer *A. ibericus* as *A. carbonarius* in previous studies. In contrast with previous reports, where 2–3 % of *A. niger* isolates isolated from coffee beans could produce ochratoxins [29], 13 % of the *A. niger* strains came from Thai coffee could produce both OTA and ochratoxin B but in rather small amounts compared to *A. carbonarius*. It is more likely that *A. carbonarius* is the source of OTA contamination in Thai coffee beans. OTA-producing fungi isolated from coffee beans have been identified as *Aspergillus ochraceus*, *A. carbonarius* and *A. niger* [37]. In a preliminary study of Vietnamese coffee beans, *A. niger* was the sole toxigenic species isolated, and 8.7% of isolates produced OTA [32]. In studies of Brazilian coffee beans, over 75% of *A. ochraceus* and *A. carbonarius* isolates produced OTA, whereas only 3% of *A. niger* isolates were toxigenic [29]. Within section Circumdati (yellow Aspergilli), two new ochratoxinogenic species, *A. westerdijkiae* and *A. steynii*, segregated from

A. ochraceus, have been described [28]. Ilic *et al.* [32], reported the incidence and toxigenicity of OTA-producing *Aspergillus* species infecting green coffee beans sourced from the main coffee-growing regions of southern and central Vietnam, and the OTA contamination of severely infected beans. The majority of coffee beans in all samples were infected with one or more fungi, with *A. niger* being the dominant species. Infection with total fungi, *A. carbonarius* and *A. niger*, was significantly less severe in Arabica than in Robusta beans. Infection with yellow Aspergilli also appeared to be less frequent in Arabica than in Robusta beans, however, differences were not significant. Other fungi commonly isolated included *Aspergillus flavus* and *A. tamarii*, *Rhizopus* spp. and, less commonly, *A. fumigatus* and *Penicillium citrinum*. The ability of *Aspergillus* spp. to produce large amounts of OTA on culture media is often, but not always, indicative of similar production on coffee. Sánchez-Hervás *et al.* [38] found that, with respect to OTA-producing fungi, a high percentage of black aspergilli strains (49.2%) were able to produce OTA. Additionally, most of the OTA-producing isolates were of moderate toxigenicity, producing amounts of OTA from 10 µg/g to 100 µg/g. Recently, black *Aspergillus* species (section Nigri), such as *Aspergillus carbonarius* and species belonging to the *Aspergillus niger* aggregate, have been described as the main source of OTA contamination in coffee, grapes and other agricultural products. Other authors [29, 32] have reported that 1–9 % of *Aspergillus niger* strains isolated from coffee beans produce the toxin. This difference could be attributed to a natural selection in the strain or to adverse environmental conditions. Also, Varga *et al.* [39] was reported that, all species in *Aspergillus* subgenus Circumdati section Circumdati produced at least one of the following important mycotoxins: ochratoxins (A and B), penicillic acids (penicillic acid, dehydropenicillic acid, orsellinic acid) and xanthomegnins (xanthomegnin, viomellein and vioxanthin), except one species: *A. robustus*. Ochratoxin production was tested in 172 strains representing species in sections *Fumigati*, *Circumdati*, *Candidi*, and *Wentii* of the genus *Aspergillus* by an immunochemical method using a monoclonal antibody preparation against ochratoxin A. Ochratoxin A was detected in *Aspergillus ochraceus*, *A. alliaceus*, *A. sclerotiorum*, *A. sulphureus*, *A. albertensis*, *A. auricomus*, and *A. wentii* strains. This was the first report of production of ochratoxins in the latter three species. Thirty samples of coffee beans were collected from different places of Jeddah, Saudi Arabia by Bokhari and Aly [40] to determine and identify fungal population. Twenty six species belonging to 7 genera were isolated using potato dextrose agar (PDA) and malt extract agar (MEA) media at 28°C. The most prevalent genera were *Aspergillus* and *Penicillium*. *Aspergillus* was present in 73 and 100% of the samples but *Penicillium* was present in 86.6 and 100% on the two mentioned media, respectively. Also, *Fusarium*, *Mucor*, *Rhizopus* and *Alternaria* were recovered in

moderate incidences on the two media. Out of the thirty samples of coffee beans collected, thirteen were contaminated with mycotoxin (43.3%). Mycotoxin profiles were also determined in these samples. It was found that aflatoxin G1 (Afl G1) showed the highest incidence rates of occurrence. It occurred in about 23.3% of all samples analyzed and in 54% of the mycotoxin contaminated samples. The other toxins detected were aflatoxins B1 (16.6%), B2 (10%), G2 (6.6%), ochratoxin (10%), patulin (16.6%) and sterigmatocystin (6.6%). A recent studies of coffee samples from a range of producing countries found Vietnamese coffee to have the highest level of OTA contamination, along with the highest percentage of defective beans. Batistaa *et al.* [41], found that, processed (green) coffee beans from *Coffea arabica* in Brazil were assessed for the presence of *Aspergillus* and *Penicillium* species both before and after surface sterilization, the ochratoxigenic potential of the isolates and ochratoxin A levels. Contamination by *Aspergillus* and *Penicillium* species was found on 96% and 42%, respectively, of 45 samples from 11 localities. After disinfection with 1% sodium hypochlorite, the levels fell to 47% and 24%, respectively. Of the 40 bean samples analyzed, 58% were infected with potentially ochratoxigenic fungi but only 22% of these were contaminated with ochratoxin A at levels that varied from 0.47 to 4.82 ng/g, with an average contamination level of 2.45 ng/g. Three coffee processes were evaluated (wet, mechanical and dry processes), by Suárez-Quiroz *et al.* [42] at different stages from harvesting to storage, and fungi producing OTA were enumerated and identified. The frequency of potential OTA producing fungi and their ability to produce OTA was also studied. By direct plating, the levels of contamination found in the coffee processes were 80, 72 and 92%, respectively, for parchment and dry cherry coffee and 20, 34 and 15% for green coffee. *Aspergillus ochraceus* isolated from the three processes accounted for 6.6, 8.3 and 3.3%, and *Aspergillus niger* for 15, 13 and 25% of the strains isolated, respectively. The toxigenic potential of five *A. ochraceus* and two *A. niger* strains was tested in FDA medium and coffee medium using the HPLC technique. There was no difference between the processes studied in terms of isolation and occurrence of ochratoxigenic fungi. In order to protect coffee from OTA formation, there is a real need to identify moulds able to produce this mycotoxin, and their relation with the processing method. In 2006 and 2007, 32 Thai dried coffee bean samples (*Coffea arabica*) from two growing sites of Chiang Mai Province, and 32 Thai dried coffee bean samples (*Coffea canephora* var. robusta) from two growing sites of Chumphon Province, Thailand, were collected by Noonim *et al.*[43] and assessed for the distribution of fungi with the potential to produce ochratoxin A (OTA). The overall percentage of fungal contamination in coffee was 98% and reduced to 60% after surface disinfection. There were remarkable ecological differences in the composition of

ochratoxigenic species present in these two regions. Arabica coffee bean samples from the North had an average of 78% incidence of colonization with *Aspergillus* of section *Circumdati* with *Aspergillus westerdijkiae* and *A. melleus* as the predominant species. *Aspergillus* spp. of section *Nigri* were found in 75% of the samples whereas *A. ochraceus* was not detected. Robusta coffee beans from the South were 98–100% contaminated with predominantly *A. carbonarius* and *A. niger*. *A. westerdijkiae* was only found in one sample. Of the 64 coffee bean samples analyzed, 98% were contaminated with OTA in levels of 0.6–5.5 µg/kg (Arabica) and 1–27 µg/kg (Robusta).

So, after we finished the classification of fungi genus and species on Yemeni green coffee and tested the toxicity of these fungi isolates on YES medium and detected the concentrations of OTA by HPLC, we determine the occurrence of ochratoxin A in Yemeni green coffee samples.

The determination of Ochratoxin A in Yemeni green coffee beans

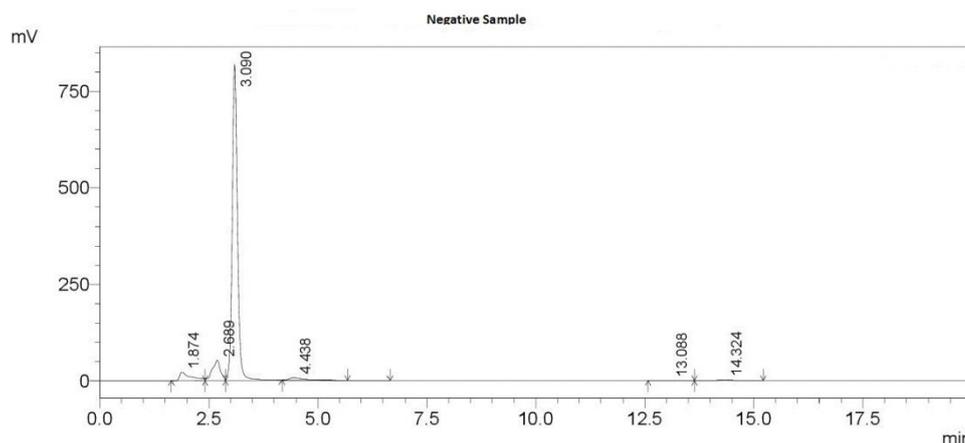
Ochratoxin A (OTA) is an emerging problem in coffee production worldwide. In the past decade, importer coffee companies and researchers have taken a greater interest in the mycological quality of green coffee beans to estimate the risk to humans posed by this mycotoxin.

Table 2: The positive samples which contaminated by Ochratoxin A in Yemeni green coffee beans

Samples Number	Code	Weight of sample	Volume of solution	Concentration of Ochratoxin A
12	H1 / 18	25 gm	5 ml	0.612 ng/g
19	C3 / 7	25 gm	5 ml	0.314 ng/g
20	999	25 gm	5 ml	1.315 ng/g
31	E2 / 11	25 gm	5 ml	0.903 ng/g
35	12	25 gm	5 ml	1.230 ng/g
62	O2	25 gm	5 ml	3.443 ng/g
65	U2	25 gm	5 ml	0.663 ng/g

In this regard, data in table (2) represented that, the positive samples of Yemeni green coffee beans which contaminated by ochratoxin A were 7 out on 70 samples by percentages of 10%, and the concentration

of toxin ranged between 0.314 to 3.443 ng/g by average was 1.211 ng ochratoxin A /per g of Yemeni green coffee beans. The figures below showed ochratoxin A standard positive and negative samples curves.



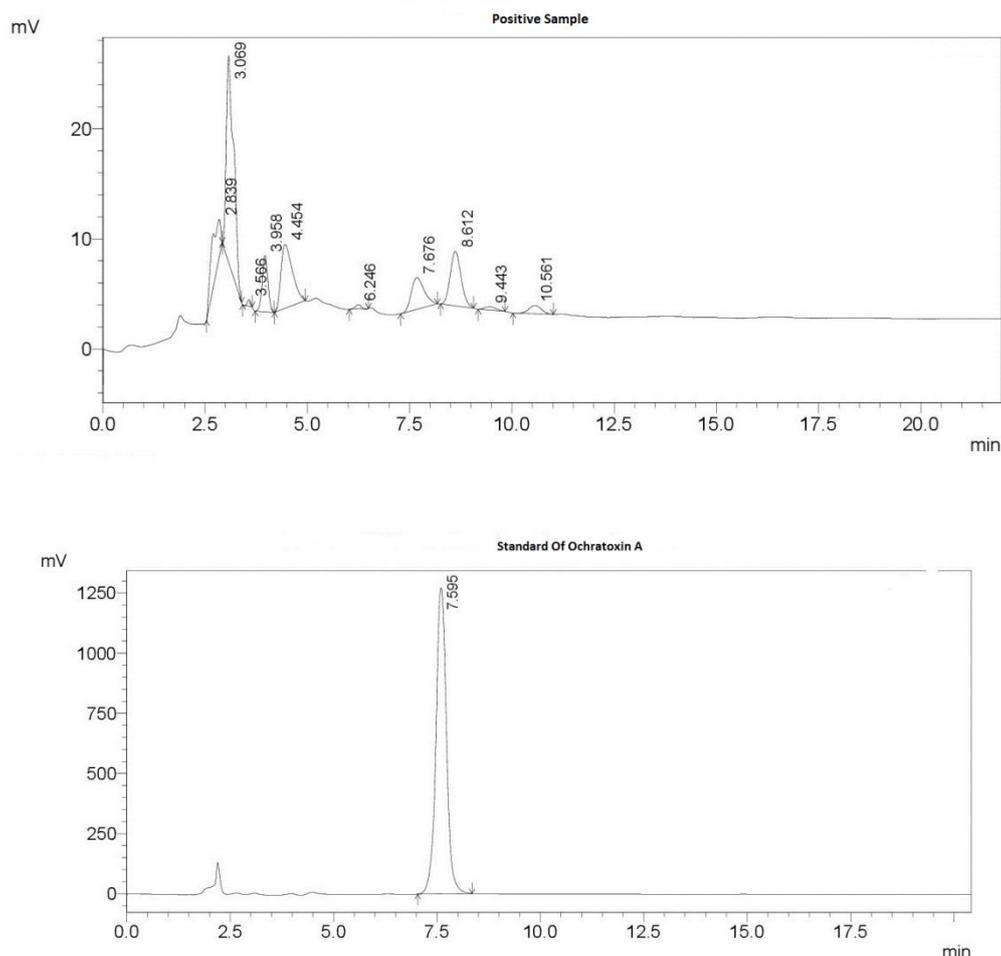


Fig-Chromotogram of Ochratoxin A

Ochratoxin A inhibits protein synthesis both *in vitro* and *in vivo* through competition with phenylalanine and it was also found to increase lipid peroxidation, leading to further cell and mitochondrial damage. Due to health concerns, the FAO/WHO Joint Expert Committee on Food Additives and Contaminants (JECFA) established a provisional tolerable weekly intake of 100 ng/kg body weight (bw). Moreover, the EC Scientific Committee on Food (SCF) recommends that levels of OTA should be reduced as much as possible, i.e., 'below 5 ng/kg bw/day'. Although a minor contributor to the dietary intake of OTA, coffee has received special attention in the last few years. As a consequence, some countries such as Italy, Switzerland, Finland and Greece have set regulatory limits with maximum OTA values ranging from 4 to 20 ppb (ng/g). The EC might establish a limit in the near future. According to estimates of the Institute for Scientific Information on Coffee (ISIC) implemented by FAO, if the EC establishes a regulation for OTA on the proposed level (5 ppb), 7% of coffee batches worldwide would exceed this amount. The rejected shipments would lead to economic losses to producing countries of over one billion dollars and an extra 500 million dollars to the EC alone on laboratory costs. Brazil is the largest coffee producer and exporter

with a 22% average market share in the last seven years. Research on OTA occurrence in green, roasted and soluble coffee produced in Brazil has been carried out. In 132 samples of Brazilian green coffee, 27 were contaminated with OTA at levels of 0.7 to 47.8 ng/g. Samples of soluble coffee and roast and ground coffee contained OTA levels between 0.31 and 1.78 ng/g and 0.99 and 5.87 ng/g, respectively. Most of studies on the incidence of OTA in Brazilian coffee have shown that the contamination in coffee is not significant. However, to date, no investigation has been reported on Brazilian green coffee exclusively destined for export, which would be directly impaired by the regulatory limits of importing countries. Dietary exposures to ochratoxin A were estimated to be 3.88 and 8.97 ng/kg body weight/week for average secondary school student and high consumers respectively. These values were far below the provisional tolerable weekly intake (PTWI) of 100 ng/kg body weight established by JECFA [3]. It can be concluded that secondary school students are unlikely to experience major toxicological effects of ochratoxin A. Goll-Cke *et al.* [44] study measured the OTA content with HPLC in 37 samples of Brazilian green coffee exclusive destined to the export market and also verified a possible relation between coffee

defects and OTA content. The results showed an OTA concentration ranging from < 0.16 ng/g (detection limit) to 6.24 ng/g (average of 3.20 ng/g) for 37 samples. Of the five samples observed for defects, toxin content of *sound* beans ranged from 0.22 to 0.80 ng/g (average 0.46 ng/g) and of *defective* beans from 0.42 to 17.46 (average 4.52 ng/g). Morphological differences among *sound* and *defective* beans showed no susceptibility for mould invasion under optical microscopy observation. One *black* bean depicted the presence of mould and spores on observation under Scanning Electron Microscope (SEM). According to this investigation, Brazilian green coffee for export complies with most limits in place. Since the first analysis of ochratoxin A in unripe (green) coffee beans, studies have shown that only a variable fraction of coffee samples contain the mycotoxin. Reported levels have been from 0.5 to 23.0 ng/g [45], 9.9 to 46 ng/g [48], 0.2 to 5.5 ng/g [47], 0.1 to 17.4 ng/g [19], 0.1 to 4.6 ng/g [48] and 0 to 48 ng/g [49]. An average contamination of 2.4 ng/g was found in 50 coffee bean samples [50]. Simultaneous measurements of ochratoxin A and the presence of ochratoxigenic fungi are rare [51], but essential if a full understanding is to be made of which isolates will produce ochratoxin A and under what conditions. The average level of ochratoxin A contamination found in many studies agrees with results obtained in previous Brazilian and international research, although the percentage of samples contaminated was lower than that found by Micco *et al.* [47]; Nakajima *et al.* [19] and Trucksess *et al.* [48]. No samples contained ochratoxin A levels above 5.0 ng/g, the upper limit proposed in European legislation for coffee and cereal grains suggesting that good agricultural practices and subsequent postharvest handling during the natural (dry) processing of this coffee were performed in most cases [37, 52]. Other studies reveal that coffee consumers are subject to Ochratoxin than those who do not consume coffee regularly. The amount of Ochratoxin that coffee drinkers could consume from contaminated coffee could reach 5 µg/kg, which is mostly unsafe. That is regardless of some studies that claim the effectiveness of thermal canning / packaging and roasting in reducing the concentration of mycotoxin. Nonetheless, until now there has been no proof of the role of roasting on minimizing the level of Ochratoxin which is approximately 10-90%, as this depends of various variables such as temperature and roasting degrees as well as other factors and conditions of contamination. Therefore, coffee consumers are 10-20% subject to Ochratoxin. The European Union has introduced limits for OTA in many of these foods, such as 5 µg OTA per kg roasted coffee and 10 µg OTA per kg soluble coffee [53]. Certain countries also limit OTA in green coffee (8–20 µg/kg); [37]. Production of coffee is the primary source of income for about 25 million families (mostly smallholder farmers) in more than 50 developing nations. Hence, from 2001–2005, the Food and Agriculture Organization of the United Nations (FAO) coordinated an international project to

develop strategies to reduce OTA in coffee (<http://www.coffee-ota.org/>).

CONCLUSION

We can conclude that, Yemeni green coffee samples (*Coffea arabica* L.) under this investigation shows a highly grade of quality. Because the percentages of fungi infection in coffee samples were low and the toxigenic fungi also was low within these fungi. Finally the occurrence of ochratoxin A was very low comparing with the other results by many researchers in different countries.

ACKNOWLEDGMENTS

This work was supported by NAM S&T Centre, New Delhi, India. We are thankful to the director, and all the staff in Indian Institute of Chemical Technology (CSIR - IICT), Hyderabad, India, for providing the infrastructural facilities.

REFERENCES

1. Shundo L, de Almeida AP, Alaburda J, Lamardo LCA, Navas SA, Ruvieri V *et al.*; Aflatoxins and ochratoxin A in Brazilian paprika. Food Control, 2009; 20(12):1099–1102.
2. Pitt JI, Hocking AD; Fungi and food spoilage, 3rd edition, New York : Springer, 2009.
3. JECFA - Joint FAO/WHO Expert Committee on Food Additives; Evaluation of certain mycotoxins in food. WHO Technical Report Series: 906. Geneva: World Health Organization, 2002.
4. Davis AP, Govaerts R, Bridson DM, Stoffelen P; An annotated taxonomic conspectus of the genus *Coffea* (Rubiaceae). Botanical J Linnean Soc, 2006; 152(4): 465-512.
5. Davis AP Rakotonasolo F; Two new species of *Coffea* L. (Rubiaceae) from Northern Madagascar. ADANSONIA, sér.3, 2001; 23(2): 337-345.
6. Fujioka K, Shibamoto T; Chlorogenic acid and caffeine contents in various commercial brewed coffees. Food Chemistry, 2008; 106(1): 217–221.
7. Monteiro MC, Farah A; Chlorogenic acids in Brazilian *Coffea arabica* cultivars from various consecutive crops. Food Chemistry, 2012; 134: 611–614.
8. USAID; Moving Yemen coffee forward. Assessment of the coffee industry in Yemen to sustainably improve incomes and expand trade, 2005.
9. Manandhar JB, Hartman GL, Wang TC; Conidial germination and appressorial formation of *Colletotrichum capsici* and *C. gloeosporioides* isolates from pepper. Pl Dis Repr., 1995; 79(4): 361-366.

10. Gilman CJ; A Manual of Soil Fungi, 2nd edition. The Iowa State University Press, Ames. Iowa, U.S A., 1957: 392.
11. Booth C; The Genus *Fusarium*. Commonwealth Mycological, Institute, Kew, Surrey, England, 1971: 273.
12. Barnett HL, Hunter BB; Illustrated Genera of Imperfect Fungi. 4th edition, ABS Press. The Am. Phyto. Soc. Minnesota, 1986.
13. Nelson PE, Toussoum TA, Marasas WFO; *Fusarium* spp. An Illustrated Manual for Identification. Philadelphia (PA): Pennsylvania Univ., 1993: 189.
14. Samson RA, Hoekstra ES, Frisval JC, Filtenborg O; Introduction to food-borne fungi. 1st edition, Delft, Netherlands: Centra al bureau voor Schimmelcultures Baarn, 1995.
15. AOAC; Association of Official Analytical Chemists, official methods of analysis. 18th edition, Gaithersburg, MD, USA, 2005.
16. Cardenas J; The world coffee crisis. World Coffee Conference, London, UK, 2001. Available from http://www.ico.org/event_pdfs/cardenas.pdf
17. Nogaim QA, Thabet H, Nagi A, Qasim B, Al-Qudami MH, Al-Abadi O *et al.*; Determination of Microbial Contamination in Yemeni Green Coffee. *Advanced Journal of Biological Sciences Research*, 2013; 1 (002): 022-029.
18. Davis ND, Searcy JW, Diener UL; Production of Ochratoxin A by *Aspergillus ochraceus* in a semi-synthetic medium. *Journal of Applied Microbiology*, 1969; 17(5): 742-744.
19. Nakajima M, Tsubouchi H, Miyabe M, Ueno Y; Survey of aflatoxin B1 and ochratoxin A in commercial green coffee beans by high-performance liquid chromatography linked with immunoaffinity chromatography. *Food and Agricultural Immunology*, 1997; 9(2): 77–83.
20. Silva CF, Schwan RF, Dias ES, Wheals AE; Microbial diversity during maturation and natural processing of coffee cherries of *Coffea arabica* in Brazil. *International Journal of Food Microbiology*, 2000; 60(2-3): 251–260.
21. FAO (Food and Agriculture Organization of the United Nations); Guidelines for the Prevention of Mould Formation in Coffee, 2006. Available from ftp://ftp.fao.org/ag/agn/coffee/guidelines_final_en.pdf.
22. Bokhari FM; Mycotoxins and Toxicogenic Fungi in Arabic Coffee Beans in Saudi Arabia. *Advances in Biological Research*, 2007; 1(1-2): 56-66.
23. Perrone G, Susca A, Cozzi G, Ehrlich K, Varga J, Frisvad JC *et al.*; Biodiversity of *Aspergillus* species in some important agricultural products. *Studies in Mycology*, 2007; 59: 53–66.
24. Battilani P, Barbano C, Marin S, Sanchis V, Kozakiewicz Z, Magan N; Mapping of *Aspergillus* section Nigri in Southern Europe and Israel based on geo-statistical analysis. *International Journal of Food Microbiology*, 2006; 111(Suppl 1): S72–S82.
25. Dalcero A, Magnoli C, Hallak C, Chiacchiera SM, Palacio G, Rosa CAR; Detection of ochratoxin A in animal feeds and capacity to produce this mycotoxin by *Aspergillus* section Nigri in Argentina. *Food Additives and Contaminants*, 2002; 19(11): 1065–1072.
26. Battilani P, Pietri A, Bertuzzi T, Languasco L, Giorni P, Kozakiewicz Z; Occurrence of ochratoxin A-producing fungi in grapes grown in Italy. *Journal of Food Protection*, 2003; 66(4): 633–636.
27. Taniwaki MH; An update on ochratoxigenic fungi and ochratoxin A in coffee. *Advances in Experimental Medicine and Biology*, 2006; 571: 189–202.
28. Samson RA, Houbraken JAMP, Kuijpers AFA, Frank JM, Frisvad JC; New ochratoxin A or sclerotium producing species in *Aspergillus* section Nigri. *Studies in Mycology*, 2004; 50: 45–61.
29. Taniwaki MH, Pitt JI, Teixeira AA, Iamanaka BT; The source of ochratoxin A in Brazilian coffee and its formation in relation to processing methods. *International Journal of Food Microbiology*, 2003; 82(2): 173–179.
30. Martins ML, Martins HM, Gimeno A; Incidence of microflora and of ochratoxin A in green coffee beans (*Coffea arabica*). *Food Additives and Contaminants*, 2003; 20(12): 1127–1131.
31. Magnani M, Fernandes T, Prete CSEC, Homechim M, Ono EYS, Vilas-Boas LA *et al.*; Molecular identification of *Aspergillus* spp. isolated from coffee beans. *Scientia Agricola*, 2005; 62(1): 45–49.
32. Ilic Z, Bui T, Tran-Dinh N, Dang MHV, Kennedy I, Carter D; Survey of Vietnamese coffee beans for the presence of ochratoxigenic *Aspergilli*. *Mycopathologia*, 2007; 163(3): 177–182.
33. Leong SL, Hocking AD, Scott ES; *Aspergillus* producing ochratoxin A: isolation from vineyard soils and infection of Semillon bunches in Australia. *Journal of Applied Microbiology*, 2007; 102(1): 124–133.
34. Joosten HMLJ, Goetz J, Pittet A, Schellenberg M, Bucheli P; Production of ochratoxin A by *Aspergillus carbonarius* on coffee cherries. *International Journal of Food Microbiology*, 2001; 65(1-2): 39–44.
35. Pardo E, Marin S, Ramos AJ, Sanchis V; Occurrence of ochratoxigenic fungi and ochratoxin A in green coffee from different

- origins. Food Science and Technology International, 2004; 10(1): 45–49.
36. Leong SL, Hien LT, An TV, Trang NT, Hocking AD, Scott ES; Ochratoxin A-producing aspergilli in Vietnamese green coffee beans. Letters in Applied Microbiology, 2007 b.; 45: 301–306.
 37. Bucheli P, Taniwaki MH; Research on the origin, and on the impact of post-harvest handling and manufacturing on the presence of ochratoxin A in coffee. Food Add Contamin., 2002; 19:655–665.
 38. Sánchez-Hervás M, Gil JV, Bisbal F, Ramón D, Martínez-Culebras PV; Mycobiota and mycotoxin producing fungi from cocoa beans. International Journal of Food Microbiology, 2008; 125: 336–340.
 39. Varga J, Kevei E, Rinyu E, Téren J, Kozakiewicz Z; Ochratoxin production by *Aspergillus* species. Applied and Environmental Microbiology, 1996; 4461–4464.
 40. Bokhari F, Aly MM; Trials towards reduction of fungal growth and Aflatoxin G1 production in Arabic coffee using different additives. African Journal of Food Science, 2009; 3(3): 68-76.
 41. Batistaa LR, Chalfounb SM, Pradoc G, Schwand RF, Whealse, AE; Toxicogenic fungi associated with processed (green) coffee beans (*Coffea arabica* L.). International Journal of Food Microbiology, 2003; 85(3): 293–300.
 42. Sua´rez-Quiroz M, ´lez-Rios OG, Barel M, Guyot B, Schorr-Galindo S, Guiraud JP; Study of ochratoxin A-producing strains in coffee processing. International Journal of Food Science and Technology, 2004; 39(5): 501–507.
 43. Noonim P, Mahakarnchanakul W, Nielsen KF, Frisvad JC, Samson RA; Isolation, identification and toxigenic potential of ochratoxin A-producing *Aspergillus* species from coffee beans grown in two regions of Thailand. International Journal of Food Microbiology, 2008; 128(2): 197–202.
 44. Goll-Cke APB, Taniwaki MH, Tavares DQ; Survey on Ochratoxin A In Brazillan Green Coffee Destined For Exports. Cinc. Tecnol. Aliment., Campinas, 2004; 24(4): 641-645.
 45. Canta´fora A, Grossi M, Miraglia M, Benelli L; Determination of ochratoxin A in coffee beans using reversed-phase high performance liquid chromatography. La Rivista della Societa´ Italiana di Scienza dell’Alimentazione, 1983; 12: 103–108.
 46. Tsubouchi H, Terada H, Yamamoto K, Hisada K, Sakabe Y; Caffeine degradation and increased ochratoxin A production by toxigenic strains of *Aspergillus ochraceus* isolated from green coffee beans. Mycopathologia, 1985; 90:181–186.
 47. Micco M, Grossi M, Miraglia M, Brera C; A study of the contamination by ochratoxin A of green and roasted coffee beans. Food Additives and Contaminants, 1989; 6: 333–339.
 48. Trucksess MW, Giler J, Young K, White KD, Page SW; Determination and survey of ochratoxin A in wheat, barley and coffee. Journal of AOAC International, Baltimore, 1999; 82(1): 85-89.
 49. Romani S, Sacchetti G, Lo´pez CC, Pinnavaia GG, Rosa MD; Screening and occurrence of ochratoxin A in green coffee beans of different origins and types. J Agric Food Chem., 2000; 48: 3616–3619.
 50. Leoni LAB, Furlani RPZ, Valente Soares LM, Oliveira PLC; Ochratoxin A in Brazilian roasted beans and instant coffee. Food Additives and Contaminants. London, 2000; 17(10): 867-870.
 51. Urbano GR, Taniwaki MH, Leitaˆo MFDF, Vicentini MC; Occurrence of ochratoxin A-producing fungi in raw Brazilian coffee. J Food Prot., 2001; 64(8): 1226–1230.
 52. Bucheli P, Kanchanomai C, Meyer I, Pittet A; Development of ochratoxin A during Robusta (*Coffea canephora*) coffee cherry drying. Journal of Agricultural and Food Chemistry, 2000; 48(4): 1358–1362.
 53. European Union; Commission Regulation (EC) No 123/2005 of 26 January 2005 amending Regulation, (EC) No 466/2001 as regards ochratoxin A. Official Journal of the European Union, L 025, 28 Jan 2005.