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Occurrence of Ochratoxigenic Fungi and Ochratoxin A in Green Coffee from Different Origins

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Fungal infection and ochratoxin A (OTA) contamination were determined in green coffee samples from different origins, in which OTA-producing fungi were also identified. About 72% of the beans analysed by direct plating presented fungal infection, including species of *Aspergillus*, *Penicillium* and *Rhizopus*. The genus *Aspergillus* was presented in more than 90% of infected coffee beans. *Aspergillus ochraceus* and *Aspergillus* section *Nigri* isolates represented 2.8 and 65.4%, respectively from the total number of isolates from the coffee beans. The capacity to produce OTA was determined in 260 isolates of *A.* section *Nigri* and 19 of *A. ochraceus* by the agar plug method, giving positive results for 6% of the *A.* section *Nigri* isolates and 16% of the *A. ochraceus*. OTA production was analysed by high performance liquid chromatography. OTA contamination of green coffee beans was analysed by enzyme immunoassay. OTA levels in all samples analysed were above the limit of detection (0.6 μg/kg), with a mean OTA concentration of 6.7 μg/kg.

Key Words: *Aspergillus* section *Nigri*, *Aspergillus ochraceus*, green coffee, ochratoxin A

INTRODUCTION

Ochratoxin A (OTA) is a mycotoxin produced by fungi of the genera *Aspergillus* and *Penicillium*, which could contaminate coffee (Levi et al., 1974; Mislivec et al., 1983), cereals and other substrates. It has been detected in a great variety of agricultural products from different geographical regions of the world and originally isolated as a metabolite of *Aspergillus ochraceus* (Van der Merwe, 1965). Nevertheless, other *Aspergillus* species have been identified since then as OTA producers, such as *Aspergillus carbonarius* (Heenan et al., 1998), *Aspergillus alliaceus* and *Aspergillus niger* (Abarca et al., 1994).

Ochratoxin A is known for its carcinogenic, nephrotoxic, and immunotoxic characteristics in animal cells. The International Agency for Research on Cancer (IARC, 1993) has classified it as a possible human carcinogen (group 2B).

Ochratoxin A has been extensively documented as a contaminant of green coffee beans, in levels ranging from 0.1 to 80 μg/kg (Tsubouchi et al., 1987; Micco et al., 1989; Nakajima et al., 1997; Stegen et al., 1997; Blanc et al., 1998; Jørgensen, 1998; Trucksess et al., 1999; Romani et al., 2000), while levels of OTA in roasted coffee have ranged from 0.05 to 23.5 μg/kg (Tsubouchi et al., 1988; Studer-Rohr et al., 1995; Patel et al., 1997; Trucksess et al., 1999; Leoni et al., 2000; Otteneder and Majerus, 2001; Lombaert et al., 2002). Most remarkable, OTA has been detected in the final coffee brew prepared by common methods (Tsubouchi et al., 1987; Studer-Rohr et al., 1995; Trucksess et al., 1999; Lombaert et al., 2002). Hence, the consumption of coffee could increase human exposure to OTA. It is estimated that 12% of total OTA intake comes from coffee consumption (FAO, 1999).

Maximum levels for OTA have been set by Comission Regulation EC No. 472 (European Community, 2002) for cereals (5 μg/kg) and their by-products (3 μg/kg) and for dried vine fruits (10 μg/kg), while legislation and levels to be set for coffee and coffee products, wine, beer, grape juice, cocoa and cocoa products and spices are currently being considered.

Bucheli et al. (2000) suggested that isolation and identification of the microorganisms producing OTA on coffee cherries could represent an important step towards understanding how to protect coffee cherries from OTA formation. Unfortunately, some attempts undertaken to isolate known potential ochratoxigenic molds, such as *Aspergillus ochraceus*, from fresh and dried coffee cherries were unsuccessful (Mantle, 1998), since a cause and effect relationship between ochratoxigenic *A. ochraceus* and OTA in green coffee was not found, therefore other microorganisms or moulds might be responsible. OTA-producing fungi in several coffee samples analysed from different farms in Brazil revealed there were two or at most three species involved in the production of OTA in coffee: *A. ochraceus*, *A. carbonarius* and rarely, *A. niger* (Taniwaki et al., 1999).

Recent investigations pointed out the growing requirements and potential OTA production of *A. ochraceus* and *A. carbonarius* on green coffee (Mantle and Chow,

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2000; Joosten et al., 2001; Taniwaki et al., 2001). According to these authors, a minimum water activity of 0.80 (about 14% moisture content) is required for OTA production on green coffee.

The objectives of this research were to evaluate the presence of ochratoxin A in green coffee samples and to identify the responsible fungal species.

MATERIALS AND METHODS

Samples

Fifty seven green coffee samples were used in this study: 41 Arabica coffee (*Coffea arabica*) and 16 Robusta coffee (*C. canephora*). Samples were collected in different factory roasters and from different origins: Angola, Bolivia, Brazil, Colombia, Costa Rica, Ecuador, Ethiopia, Guatemala, India, Indonesia, Ivory Coast, Java, Nicaragua, Uganda and Vietnam.

Methods

Direct Plating

Coffee beans were externally disinfected by immersion in a 3% sodium hypochlorite solution for 2 min, then aseptically rinsed into two sterile water washes. Finally, 15 beans of each coffee sample were transferred to Petri plates (5 beans per plate) containing malt extract agar (MEA) and incubated at 25°C. After 7 days, growing fungal colonies were transferred to Czapeck yeast agar and incubated at 25°C in the dark for 7 days, then the fungal isolates were identified to genus level. The genus identification of *Aspergillus* isolates was based on the shape of the conidiophores as observed with a binocular microscope (× 40). Identification to species level was carried out according to morphological and cultural criteria (Raper and Fennell, 1965; Al Musallan, 1980; Klich and Pitt, 1988; Kozakiewicz, 1989).

Test for OTA Production by the Isolated Fungi

The strains of *A. ochraceus* and *A.* section *Nigri*, potential producers of OTA, were grown on Czapek Yeast Extract (CYA) agar at 25°C for 7 days. Three agar plugs (diameter 5 mm) were aseptically removed from the inner, middle and outer area of each colony. Plugs were introduced into 4 mL vials. Methanol (1 mL) was added and the vials were shaken for 5 s. After 60 min the extracts were shaken again, filtered (Millex HV filter 0.45 µm, Millipore, Bedford, MA, USA) and injected into a HPLC instrument.

The HPLC system consisted of a Waters (Mildford, MA, USA) 515 pump, a 717 plus auto sampler injection module, a 474 fluorescence detector (λ_{exc} 330 nm; λ_{em} 460) and a C_{18} analytical column (Waters Spherisorb 5 µm, ODS2, 4.6 × 250 mm) all under control of Waters Millenium[32] software. The mobile phase consisted of an isocratic mixture of 57% acetonitrile, 41% water and 2% acetic acid that was pumped at 1.0 mL/min. OTA was identified on the basis of fluorometric response compared with that of OTA standards.

The isolates of *A.* section *Nigri*, which were positive for OTA production were further classified according to morphological and cultural criteria (Raper and Fennell, 1965; Al Musallan, 1980; Klich and Pitt, 1988; Kozakiewicz, 1989).

Method for the Analysis of OTA in Coffee Beans

Ochratoxin A was analysed by an enzyme immunoassay (RIDASCREEN Ochratoxin A, R-Biopharm, GmbH, Darmstadt, Germany). Two grams of ground coffee from each sample (10–12 beans) were used for OTA extraction with 1 N HCl and dichloromethane (1 : 2), centrifuged and filtered to collect the dichloromethane layer. Then an equivalent volume of 0.13 M $NaHCO_3$ (pH 8.1) was added, shaken and centrifuged. Finally, 100 µL of the upper aqueous phase was diluted with 400 µL of 0.13 M $NaHCO_3$; 50 µL of each sample were used in the assay. The protocol established by the kit manufacturer was followed.

Statistical Analysis

Data of fungal infection and OTA contamination of the coffee samples were analysed by general linear model regression (GLM) using SAS version 8.2 (SAS Institute, Inc, Cary, NC, USA) for determination of the effects of the specie and origin of the samples. Significant mean differences were assessed by Student Newman Keuls test, with a significant level of 0.05.

RESULTS AND DISCUSSION

The coffee beans analysed by direct plating were contaminated with fungi, including species of *Aspergillus*, *Penicillium*, *Fusarium*, *Rhizopus* and yeasts. Other studies showed that these genera were the most important contaminants in this product (Mislivec et al., 1983; Micco et al., 1989; Bucheli et al., 2000). In our study, a high level of fungal infection was observed (70–73%, Table 1). Similarly Urbano et al. (2001) found a high level of contamination (100%) by yeast and moulds in green coffee samples analysed at different stages of maturation and processing. Mislivec et al. (1983) found a 93% level of infection in green coffee.

Internal fungal contamination of coffee beans resulted to be similar in both species. A total of 169 beans (70.4%) out of 240 analysed of Robusta coffee were contaminated, while from 615 Arabica beans analysed, 73.3% were infected.

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Table 1. Total fungi and frequency of isolation (%) from green coffee.

Coffee	No. of Beans		*A. ochraceus* (%)	*A.* section *Nigri* (%)	*A. flavus* (%)	*Rhizopus* spp. (%)	*Penicillium* spp. (%)	Others (%)
	Analysed	Infected						
Arabica	615	451	2.2	67.4	21.5	3.8	3.6	3.6
Robusta	240	169	3.6	86.9	8.3	7.7	0	0
Total	855	620	3.1	72.7	17.9	4.8	2.6	2.6

A high incidence of the genus *Aspergillus* was found in coffee beans, more than 93% of beans being contaminated (Table 1). Isolates of *Aspergillus ochraceus* showed a low incidence in Arabica and Robusta infected beans, while isolates of *A.* section *Nigri* represented 67.4% of infected beans from Arabica coffee and 86.9% from Robusta samples. The high fungal contamination present in Robusta coffee could be related with the sun drying of cherries, which when not correctly carried out can potentially lead to OTA contamination (Bucheli and Taniwaki, 2003). Urbano et al. (2001) detected an incidence of 10.3% *A. ochraceus* isolates in raw coffee from the total analysed beans, most of them detected in Arabica coffee samples coming from the drying stage.

Among the total number of isolates, 2.8% (19) were *A. ochraceus*, while 65.4% (451) belonged to *A.* section *Nigri* (Table 2). From the 304 isolates of *A.* section *Nigri* found in Arabica coffee beans, 260 were tested for their capacity to produce OTA and 19 (7.3%) resulted positive. Out of 147 isolates of *A.* section *Nigri* from Robusta beans, 64 were tested and 2 of them (3.1%) were OTA-producing. *A. ochraceus* isolates were tested for OTA production and found 3 positive isolates (23.1%) from Arabica coffee positive results and none from Robusta samples. OTA-producing isolates of *A.* section *Nigri* were further classified, 6 of them belonged to *A. carbonarius* and 15 to *A. niger* aggregate. Urbano et al. (2001) isolated 155 strains of *A. ochraceus* in coffee samples, 42 were tested for their ochratoxigenic capacity (88.1% positive). Among 344 isolates of *A.* section *Nigri*, 87 were tested for the OTA production and only 11.5% gave positive results. Joosten et al. (2001) only identified one strain of *A. ochraceus,* from Robusta coffee cherries (before and during drying) which did not produce any detectable OTA, while all *A. carbonarius* strains isolated produced significant amounts of it.

In spite of the relatively low incidence of OTA-producing isolates in green coffee samples (22.8%), all samples analysed presented OTA above the limit of detection of the enzyme immunoassay used (0.6 μg/kg). Determination of OTA by high-performance liquid chromatography using immunoaffinity columns allowed higher limits of detection (0.1–0.2 μg/kg; Pittet et al., 1996; Nakajima et al., 1997) than the immunoassay test, therefore it could be appropriate for quality control purposes in green coffee.

Table 2. *A. ochraceus* and *A.* section *Nigri* infection in green coffee beans.

Coffee	Total Fungi	*A. ochraceus*		*A.* section *Nigri*	
		Total	%[a]	Total	%[a]
Arabica	510	13	2.6	304	59.6
Robusta	180	6	3.3	147	81.7
Total	690	19	2.8	451	65.4

[a]The percentage was referred to the total number of isolates.

Bucheli et al. (1998) did not find potential OTA-producers, however OTA was detected in all green coffee samples analysed during storage trials. In turn, Urbano et al. (2001) detected a high percentage of OTA producers, 88.1% of *A. ochraceus* and 11.5% of the isolates of *A. niger* tested, and 75% of the coffee samples presented OTA levels higher than limit of detection (0.2 μg/kg). Similar to our work, most of samples had levels of OTA below the limit suggested by the European Union for the exportation of raw coffee, which is 8 μg/kg. Frank (1999) detected the presence of *A. ochraceus* in the beans, but not all the samples had detectable levels of OTA. These works concluded that the infection by *A. ochraceus* and *A. niger* did not necessarily indicate high OTA contamination.

In our study the average OTA level in green coffee was of 6.7 μg/kg. OTA levels ranged from 1.3 to 31.5 μg/kg (Table 3). OTA contents found in Arabica and Robusta coffee samples were not significantly different; 52.6% of the samples showed OTA values lower than 5 μg/kg, 59.7% had OTA levels below 8 μg/kg (the limit suggested by the European Union for the exportation of raw coffee) and a 7% had OTA levels higher than 20 μg/kg.

Previous studies about the incidence of OTA in green coffee found that only a 4.4% of samples analysed were contaminated with OTA, but their limit of detection was 20 μg/kg (Levi et al., 1974). More recent surveys, with lower limits of detection, showed a higher incidence of OTA in green coffee, with a percentage of contaminated samples ranging from 30 to 65.4% (Micco et al., 1989; Studer-Rohr et al., 1995; Nakajima et al., 1997; Bucheli et al., 1998; Romani et al., 2000). The concentration of OTA in green coffee reported in these studies ranged from 0.1 to 80 μg/kg.

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Table 3. Occurrence and frequency of OTA levels in green coffee samples.

Coffee	Samples Analysed	OTA Content		Frequency of OTA Levels							
				< 5 μg/kg		5–10 μg/kg		10–20 μg/kg		> 20 μg/kg	
		Range	Mean ± SD	No. of Samples	%	No. of Samples	%	No. of Samples	%	No. of Samples	%
Arabica	41	1.3–31.5	6.9 ± 7.1	22	53.7	10	24.4	6	14.6	3	7.3
Robusta	16	1.7–23.3	6.1 ± 6.3	8	50.0	7	43.8	–	–	1	6.3
Total	57	1.3–31.5	6.7 ± 6.6	30	52.6	17	29.8	6	10.5	4	7.0

Table 4. Occurrence of OTA in green coffee beans from different origins.

Origin	Samples	OTA Levels (μg/kg)		
		Min	Max	Mean ± SD
Africa	12	2.4	23.3	10.9 ± 6.7
America	31	1.3	27.7	5.4 ± 5.3
Asia	14	1.6	31.5	6.0 ± 7.9

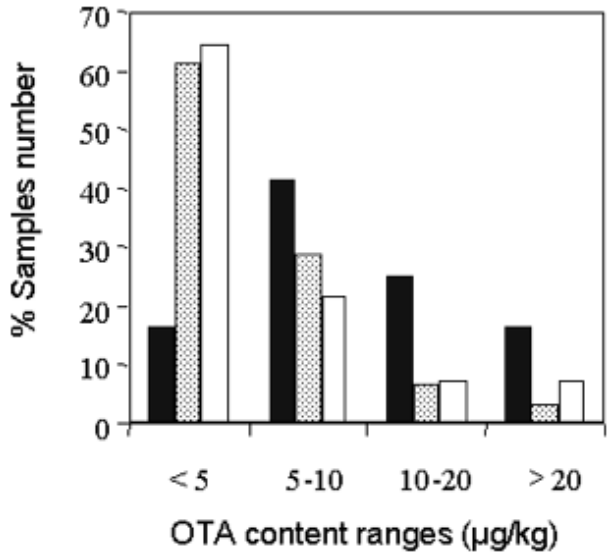

Figure 1. OTA frequency distribution in African (■), American (▨) and Asian (□) green coffee samples.

Analysis of variance demonstrated that the origin of coffee samples had a significant effect in OTA content. The comparison of the means showed that African samples had significantly higher OTA levels than those from America or Asia (Table 4). Similar conclusions were drawn by Romani et al. (2000). The highest frequency for African samples was in the range of 5–10 μg/ kg while for Asian and American was < 5 μg/kg (Figure 1). Fungal infection for African samples was not, however, significantly higher than that for Asian and American samples.

Despite the differences, this and other surveys demonstrate considerable contamination of green coffee with OTA. The poor correlation between OTA content and OTA producers may indicate that OTA contamination is not the result of storage, but it is more likely linked to the conditions undergone during green coffee production (harvesting and drying of coffee fruits) (Bucheli et al., 1998). Moreover this work confirmed that besides *A. ochraceus*, isolates of *A. niger* aggregate and *A. carbonarius* might be responsible for OTA contamination in green coffee.

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