SURVEY ON OCHRATOXIN A IN BRAZILIAN GREEN COFFEE DESTINED FOR EXPORTS[1]

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SUMMARY

The presence of ochratoxin A (OTA) in foods has led some countries to establish regulatory limits. Although coffee is not a major source of OTA in human consumption, the European Community (EC) may establish limits in the near future, with possible economic impact on producing countries. This 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 < 0.16ng/g (detection limit) to 6.24ng/g (average of 3.20ng/g) for 37 samples. Of the five samples observed for defects, toxin content of *sound* beans ranged from 0.22 to 0.80ng/g (average 0.46ng/g) and of *defective* beans from 0.42 to 17.46 (average 4.52ng/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.
Keywords: mycotoxin; Brazil; coffee.

RESUMO

INVESTIGAÇÃO DE OCRATOXINA A EM CAFÉ VERDE BRASILEIRO DESTINADO À EXPORTAÇÃO. A presença de ocratoxina A (OTA) em café, detectada nos últimos anos, tem levado alguns países a estabelecer limites regulatórios. Embora o café não seja uma fonte importante de OTA no consumo humano, a União Européia poderá estabelecer limites, causando impacto econômico em países produtores desses grãos. O presente estudo analisou OTA utilizando CLAE em 37 amostras de café verde brasileiro destinado exclusivamente à exportação e verificou a possível relação entre grãos defeituosos e concentração de OTA. Os resultados mostraram que a concentração de OTA esteve entre < 0,16ng/g (limite de detecção) e 6,24ng/g (média 3,2ng/g). Das cinco amostras avaliadas quanto à relação defeitos X OTA, o nível da toxina esteve entre 0,22 e 0,80ng/g (média 0,46ng/g) entre grãos *sadios* e 0,42 e 17,46ng/g (média 4,52ng/g) entre grãos *defeituosos*. Diferenças morfológicas entre grãos *sadios* e *defeituosos* observados em Microscopia Ótica não demonstraram susceptibilidade à invasão fúngica entre os *defeituosos*. Entre os 15 grãos observados em Microscopia Eletrônica de Varredura (MEV), apenas um (*preto*) revelou presença de fungos e esporos. Os resultados desta investigação apontam que o café verde brasileiro destinado à exportação encontra-se dentro dos limites para OTA já estabelecidos.
Palavras-chave: micotoxina; Brasil; café.

1 – INTRODUCTION

Ochratoxin A (OTA) is a nephrotoxic and possible carcinogenic mycotoxin mostly found in cereals and cereal products in Europe. It is produced by some species of *Aspergillus* and *Penicillium* as a secondary metabolite. 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 [2]. Due to health concerns, the FAO/WHO Joint Expert Committee on Food Additives and Contaminants (JECFA) established a provisional tolerable weekly intake of 100ng/kg body weight (bw) [4]. Moreover, the EC Scientific Committee on Food (SCF) recommends that levels of OTA should be reduced as much as possible, i.e., 'below 5ng/kg bw/day'.

Reports have shown OTA incidence in other foodstuffs such as wine [8], beer [5], cocoa [8], dried wine fruits [8] and green and roasted coffee [9, 15]. Although a minor contributor to the dietary intake of OTA, coffee has received special attention in the last few years [11]. As a consequence, some countries such as Italy, Switzerland, Finland and Greece have set regulatory limits with maximum OTA values ranging from 4 to 20ppb (ng/g). The EC might establish a limit in the near future.

According to estimates of the Institute for Scientifc Information on Coffee (ISIC) implemented by FAO, if the EC establishes a regulation for OTA on the proposed level (5ppb), 7% of coffee batches worldwide would exceed this amount [1]. 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 [13]. 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 47.8ng/g [7]. Samples of soluble coffee and roast and ground coffee contained OTA levels between 0.31 and 1.78ng/g and 0.99 and 5.87ng/g, respectively [12]. Most of studies on

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the incidence of OTA in Brazilian coffee have shown that the contamination in coffee is not significant [6, 7, 14, 16]. 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. The objectives of the present work were to quantify OTA in green coffee samples obtained from export batches and to search for morphological susceptibility of *defective* beans to microbial invasion.

2 – MATERIAL AND METHODS

2.1 – Samples

Thirty seven samples (1kg each) of Brazilian green coffee were obtained from export companies in Santos (Brazil), comprising 6 states, 2 harvest years, arabica and robusta, 'organic' and government stock samples as shown in *Table 1*. Samples were collected according on warehouses at random from 100bags/each at different parts of the warehouse in July 2001. Samples were obtained from the 1999/2000 and 2000/2001 harvests for all origins except for Bahia (dry process) (2000/2001) and government stock (1987/1988).

TABLE 1. Composition of samples according to origin and type.

Origin	Number of samples	Type
São Paulo	9	Arabica
Minas Gerais	9	Arabica
Espírito Santo	6	Arabica & Robusta
Rondonia	3	Robusta
Parana	3	Arabica
Bahia (wet process)	3	Arabica
"Organic"	2	Arabica
Bahia (dry process)	1	Arabica
Government stock	1	Arabica

2.2 – Sub-sampling

Of the total sampling lot, five samples were submitted to classification by a coffee expert at an export company in Santos (Brazil) and the beans were separated into *black*, *sour* and *sound*. *Black* and *sour* beans are considered coffee defects, probably a result of over-ripe cherries, deficient drying in field and/or undesirable fermentation, which could lead to infection of OTA producing fungi and consequently OTA formation [13]. *Black* and *sour* beans of each sample were grouped together composing a *defective* sub-sample while the *sound* ones composed the *sound* sub-sample. It was not possible to classify and separate more than five samples due to lack of enough defected beans to conduct the analysis (minimum 25g).

2.3 – Extraction and cleanup of OTA in coffee

The extraction and cleanup procedures of OTA in the coffee samples were performed according to PITTET et al. [10]. Twenty five grams of the finely blended samples were mixed with 200mL of methanol/3% aqueous sodium hydrogen carbonate (50:50). The suspension was blended for 3 min at medium speed with a Polytron homogenizer and then filtered through a 55mm Whatman GF/B glass microfiber filter under low vacuum. Five milliliters of the filtrate were transferred to a cylinder and the volume then completed to 100mL with phosphate-buffered saline (PBS). The whole extract was applied to the immunoaffinity column at 2-3mL/min with the aid of a vacuum pump. The column was then washed with 10mL distilled water, followed by 4mL methanol. The eluate was collected and evaporated to dryness under a nitrogen stream at 40°C. The residue was resuspended in 1mL of the High Performance Liquid Chromatograph (HPLC) mobile phase. The peak areas were plotted on a standard curve and the concentrations were obtained.

2.4 – HPLC

The HPLC equipment used was a Shimadzu SCL-10AVP (Shimadzu Corporation, Japan) chromatograph with an automatic injector. Toxin detection was obtained with a Shimadzu RF-10AXL fluorescence detector operating at excitation and emission wavelengths of 330nm and 470nm respectively. A SupelcosilÔ LC-18 (5µm particle size, 4.6mm x 250mm) column (Supelco, USA) was used with a Hypersil guard column (5µm particle size, 4.6mm x 25mm). For the mobile phase a solution in the proportion of 42% acetonitrile/58% 4mM sodium acetate/acetic acid (19:1) was applied. The injection volume of the extract was 50µL at a flow rate of 1.0mL/min. The OTA concentration in samples was determined using peak area and then plotting it on a standard curve.

2.5 – Analytical quality control

For recovery tests, uncontaminated samples were spiked with two different concentrations of standard solution corresponding to 8ng/g and 80ng/g and analysed in duplicate using the same procedure as described for samples. The average recovery was 77.9% and 90.9% respectively. The detection limit of the method was obtained analysing coffee samples with minimal concentration of OTA (< 1ng/g). The standard deviation (0.058) of ten repetitions was multiplied by the corresponding number listed on the t (Student) table for 99% of confiability. For ten repetitions and nine degree of freedom the t Student value was 2.821. The detection limit of method (DTM) was 0.16ng/g. This is somewhat lower than to that reported (0.2ng/g) by PITTET et al. [10]. An identity confirmation was carried out adding 0.1mL of concentrated hydrochloric acid (37%) to the OTA residue, and the vial was closed and kept overnight at room temperature. After evaporating the

reaction mixture to dryness and dissolving the residue in 30μL of HPLC solvent, 20μL of it were analysed for OTA methyl ester formation according to a procedure described by ZIMMERLI & DICK [17]. A positive confirmation of identity was provided by the disappearance of the OTA peak at a retention time of 6min and the appearance of a new peak (OTA methyl ester) at 8min.

2.6 – Optical and Scanning Electron Microscopy (SEM)

In total, 15 beans classified as *sound*, *black* and *sour* were observed separately under optical microscopy and SEM. For optical analysis, beans were placed in the microtomy (no previous inclusion), and cut into sections of about 5μm. Observations were made without previous staining; morphology was enhanced by means of polarization lenses. As for SEM preparation, the coffee beans were cut in half, de-fatted with acetone for 3 hours and placed in an incubator overnight. After spattering with gold the beans were observed under SEM at 10kV of acceleration.

3 – RESULTS AND DISCUSSION

3.1 – Quantitative determinations

From the 37 original coffee samples analysed, 36 presented OTA content ranging from < 0.16ng/g to 0.85ng/g and only one sample showed a content of 6.24ng/g (average 3.20ng/g). Twenty samples (54%) did not presented OTA level as they were below the detection limit of 0.16ng/g and 97.5% of samples showed OTA concentration below 5ng/g (*Figure 1*). Although the sampling lot was composed of raw coffee from several producing regions in Brazil, two different coffee treatments, and more than one harvest and two varieties, no correlation was found among any of these variables versus OTA levels.

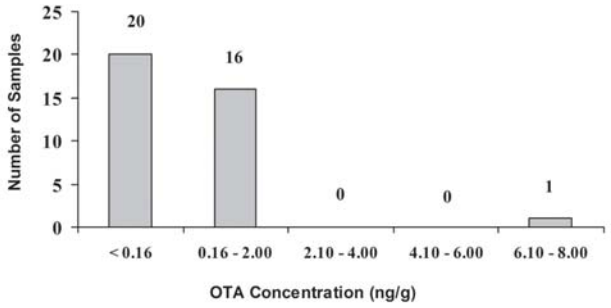

FIGURE 1. Data obtained from a lot of 37 samples of Brazilian export raw coffee from several producing regions (average of duplicates) (detection limit of 0.16ng/g).

The results of the OTA investigation of *sound* and *defective* sub-samples are presented in *Table 2*. Except for one, all sub-samples, *sound* or *defective* showed higher contents of OTA than the original samples, most likely due to the heterogenicity of contamination, which is often a concern in OTA investigations. Average OTA level of *sound* sub-samples was 0.46ng/g against 4.52ng/g of the *defective* sub-sample. HEILMANN, REHFELDT & ROTZOLL [3] observed correlation of OTA levels and coffee defects with the use of a colour sorting machine to separate *defective* beans and measuring OTA levels. LEONI et al. [7] however, found no correlation between OTA contamination and *defective* beans using total defects counting according to the Brazilian classification system.

TABLE 2. Concentration of OTA (ng/g) on original green coffee samples and after separation into two sub-samples: sound and defective (black and sour) (detection limit of 0.16ng/g).

Sample	Original content	*Sound* sub-sample	*Defective* sub-sample
1	0.31	0.61	n.d.
2	< 0.16	0.42	0.42
3	< 0.16	0.22	17.46
4	< 0.16	0.25	3.88
5	< 0.16	0.80	0.84

3.2 – Optical and Scanning Electron Microscopy

Visually, *sound* beans had the typical green colour and good appearance. *Black* ones, known to be a result of over-riping or deficient drying, presented a dark colour. *Sour* beans, a consequence of the same process failures, presented a brown, shiny aspect. The results of optical microscopy (Jenaval Zeiss Optical Microscope) depicted no significant morphological differences in the endosperm of *sound*, *sour* and *black* beans as shown in *Figures 2 to 4*. (Differences in colour represent the best polarized light used in each observation). The cell walls indicated by arrows in *Figure 3* show the characteristic shape and thickness of *Coffea*, while the middle lamellae is defined by the brilliant cresyl blue dye. In *Figure 4* the arrows point to tissues of endosperm whose cells have lost their organization. Diffusion of cell contents occurred due to the high level of moisture. Furthermore, no mould, bacteria or yeast contaminations were found using this technique. With SEM (Jeol JSM-T300 Scanning Electron Microscope), the morphology of *sound* and *sour* coffee beans also showed an organized endosperm with cells containing the contracted cytoplasm (*Figures 5 and 6*). The dotted arrow in figure 5 shows the thick cell walls in *Coffea* while cytoplasm is also depicted (solid arrow) though not preserved. The *black* beans showed a somewhat compressed endosperm and badly defined cellular contents (*Figure 7*). Oil is emerging from cells, as pointed to by arrows, also observed with *sound* and *sour* beans. Of the 15 coffee beans, only one (*black*) depicted the presence of moulds and spores under SEM (not shown).

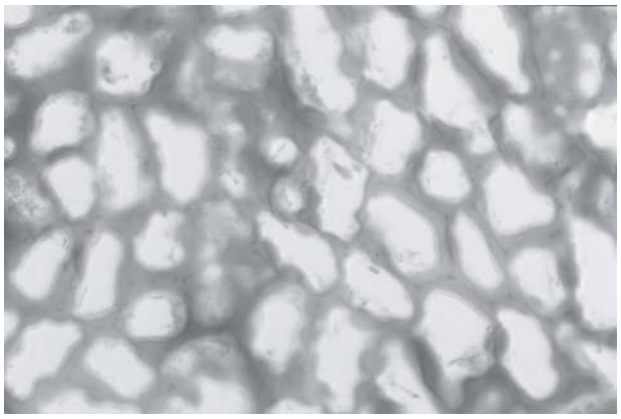

FIGURE 2. Optical microscopy of sound Robusta coffee bean (objective of 25x)

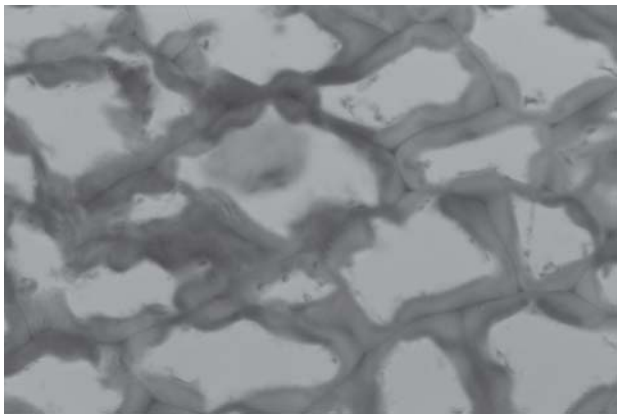

FIGURE 3. Optical microscopy of sour Robusta coffee bean (objective of 40x)

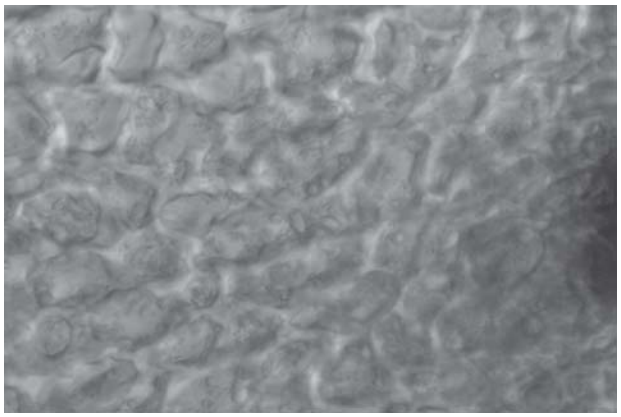

FIGURE 4. Optical microscopy of black Robusta coffee bean (objective of 25x)

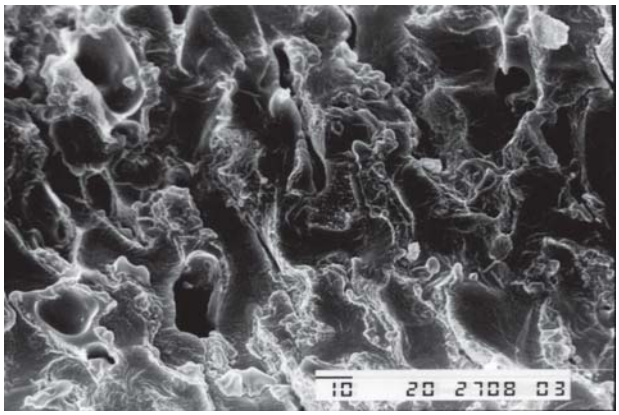

FIGURE 5. Endosperm of sound raw coffee bean on SEM (750x) (Bar= 0µm)

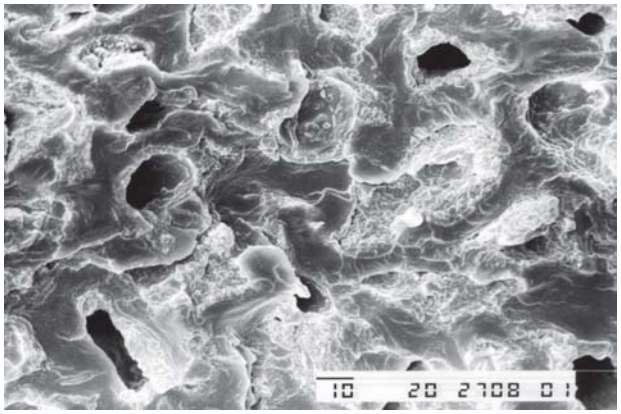

FIGURE 6. Endosperm of sour raw coffee bean on SEM (750x) (Bar = 10µm)

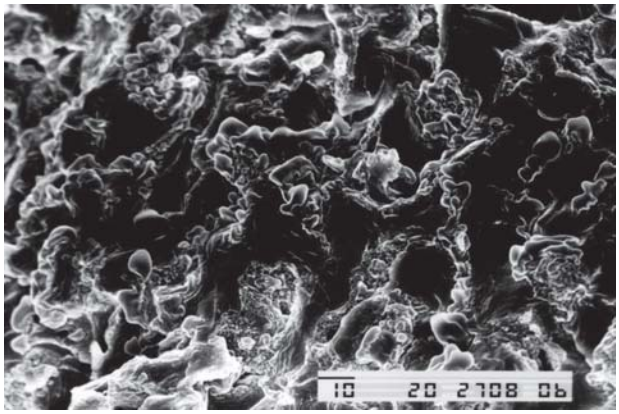

FIGURE 7. Endosperm of black raw coffee bean on SEM (750x) (Bar = 10µm)

4 – CONCLUSIONS

There seems to be a correlation between higher quality of export coffee beans and lower concentration of OTA, since the results of the present study exclusive on export coffee show consistent lower OTA content than previous data on raw coffee produced in the country [6, 7, 14, 16]. However, *defective* beans characterised as *sour* and *black*, which are less present in export coffee, do not necessarily correspond to higher OTA contamination. It was not possible to correlate the visual aspect of *sour* and *black* beans with obvious damage to the endosperm structure, which could indicate susceptibility to mould invasion. In this investigation OTA levels showed no correlation with the green coffee origin nor to the coffee variety and/or after-harvest process. According to the present survey, Brazilian raw coffee for export complies with most regulatory limits in place.

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