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Short communication

Effect of different roasting levels and particle sizes on ochratoxin A concentration in coffee beans

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ABSTRACT

Contamination of roasted coffee with ochratoxin A (OTA) is directly related to the processing quality throughout the coffee production chain, from the farming to the roasting processes. The aim of this study was to evaluate the effects of roasting and particle size on the residual concentration of ochratoxin A in roasted and ground coffee. Coffee beans were artificially contaminated with *Aspergillus ochraceus*. The beans were roasted to three levels (light, medium and dark) and ground into three types (fine, medium and coarse) after an incubation period. OTA quantification was performed using high-performance liquid chromatography. The combination of dark roast and coarse particle size had the lowest concentration of OTA, 3.06 μg/kg with a 97.17% reduction. The results of this study show that roasting and particle size, rather than roasting alone, are critical for the residual concentration of OTA in roasted and ground coffee beans.

1. Introduction

The development of microbial infections in coffee beans may compromise both their visual appearance and their taste and aroma. Among the microorganisms associated with coffee beans, filamentous fungi are primarily responsible for causing damage and producing mycotoxins (Batista, Chalfoun, Prado, Schwan, & Wheals, 2003).

Coffee bean contamination with ochratoxin A may occur throughout the entire production chain and is directly related to the care and quality of the crop management, harvest, post-harvest storage and type of roasting.

The three main species of ochratoxin A (OTA)-producing fungi associated with coffee are *Aspergillus carbonarius*, *Aspergillusochraceus* and, rarely, *Aspergillus niger* (Chalfoun & Batista, 2003; Joosten, Goetz, Pittet, Schellenberg, & Bucheli, 2001; Prado, Marín, Ramos, & Sanchis, 2004; Suárez-Quiroz et al., 2004; Urbano, Taniwaki, Leitão, & Vicentini, 2001).

The toxic effects of OTA appear to be related to its ability to inhibit protein synthesis by competing with phenylalanine in the reaction catalyzed by phenylalanyl-tRNA synthetase and other systems that require this amino acid. OTA also increases lipid peroxidation, leading to greater mitochondrial and cell damage (Dirheimer, 1996; Turner, & Subrahmanyam, 2009). Furthermore, this mycotoxin is also considered nephrotoxic, cytotoxic, carcinogenic, teratogenic and immunosuppressive (Abrunhosa, Santos, & Venâncio, 2006; Dachoupakan et al., 2009; Ghali, Hmaissia-Khlifa, Ghorbel, Maaroufi, & Hedili, 2008; Lino, Baeta, Henri, Dinis, Pena, & Silveira, 2008; Suárez-Quiroz et al., 2004; Zhang et al., 2009). OTA was classified by the International Agency for Research on Cancer (IARC) as a member of group 2B, i.e., a possible human carcinogen (International Agency for Research on Cancer – IARC, 1993).

The results from studies of ochratoxin A in food have aroused concern in Brazil. The National Health and Food Safety Surveillance

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Agency (Agência Nacional de Vigilância Sanitária, ANVISA, 2011) has set a maximum limit of ochratoxin A at 10 μg/kg in roasted and soluble coffee. The European Union has set a limit of 5 μg/kg for roasted and ground coffee beans and 10 μg/kg for soluble coffee (Commission of the European Communities – CEC, 2006, 364 p.).

Several studies have shown that the process of roasting is effective in reducing the OTA concentration (Blanc, Pittet, MunozBox, & Viani, 1998; Castellanos-Onorio et al., 2011; Ferraz et al., 2010; Heilmann, Rehfeldt, & Rotzoll, 1999; Pérez de Obanos, González-Peñas, & López de Cerain, 2005; Tsubouchi, Yamamoto, Hisada, Sakabe, & Udagawa, 1987; Viani, 2002), but there is still a lack of more conclusive studies about the effects of the various stages of roasting, grinding and methods of preparing the beverage on toxin stability.

Thus, the current study aimed to evaluate the effect of different roasting levels and particle sizes on the residual concentration of ochratoxin A in roasted and ground coffee beans to prepare filtered coffee beverages.

2. Materials and methods

2.1. Coffee samples

Twenty kilograms of coffee beans (*Coffea arabica* L.) from the 2009/2010 harvest, classified as a harsh beverage, initial humidity (11%), was prepared by the Agricultural Cooperative (Cooperativa Agrícola) located in the State of Minas Gerais-Brazil and used in this study.

2.2. Isolation and identification of potentially ochratoxigenic fungi in coffee beans

The direct plating technique in dichloran-rose bengal-chloramphenicol (DRBC) culture medium (Merck) was used for isolating contaminant fungi in the coffee beans. One hundred coffee beans were assessed without superficial disinfection and 100 coffee beans with superficial disinfection, as described by Samson, Hoekstra, Frisvad, and Filtenborg (2000). The plates were incubated at 25 °C for 7 days. The results were expressed as the percentage of beans contaminated by filamentous fungi, according to Pitt and Hocking (1997).

2.3. Identification of species from the Aspergillus genus

Species from the *Aspergillus* genus were identified using pure cultures and incubated in Czapek yeast agar (CYA) media at temperatures of 25 °C and 37 °C for 7 days and malt extract mycological peptone agar (MEA) at 25 °C for 7 days. The macroscopic and microscopic characteristics were observed after growth and were described according to Klich (2002) and Samson et al. (2007).

2.4. Evaluation of isolate ochratoxigenic potential using the plug agar method

Isolates from the *Aspergillus* genus *Circumdati* section were inoculated in yeast extract sucrose agar (YES) medium, and those from the *Nigri* section were inoculated in CYA medium; both types of isolates were incubated at 25 °C for 7 days and evaluated as described by Filtenborg and Frisvad (1980). Ten microliters of OTA standard solution (Sigma–Aldrich) was added to a predetermined point on the thin layer chromatography plate (Silica Gel 60, Merck). TEF (90% toluene, ethyl acetate and formic acid, 60:30:10) was the mobile phase employed. The confirmation of OTA production was performed under λ 366-nm ultraviolet light in a CAMAG chromatovisor (UF-Betrachter). Those isolates considered to produce OTA exhibited retention factors (RF) and fluorescence spots similar to those of the OTA standards.

2.5. Preparation and inoculation of fungal spores with increased ochratoxigenic potential in coffee beans

A. ochraceus CSM15 isolated from coffee beans were plated on 3 15-cm-diameter MEA media plates and incubated at 25 °C for 7 days. Forty milliliters of distilled water with 0.1% Tween 80 was added to each plate after growth. This suspension was gas-filtered, and 200 mL of water with 0.1% Tween 80 was added to the plate, thereby preparing the 240 mL inoculum. The spore concentration of 10^7 CFU [colony-forming units]/ml was assessed using a hemocytometer.

Six kilograms of coffee beans (*Coffea arabica* L) was sterilized in an autoclave (121 °C for 15 min) and divided into 3 trays for use in the inoculation step. Two hundred 40 mL of inoculum was added to each tray containing 2 kg of coffee beans. Sterilized water was added to reach the appropriate humidity (20%) for the development and production of OTA by *A. ochraceus* CSM15. After homogenization in the trays, the samples were transferred to burlap bags and stored in boxes to retain the humidity for 30 days and rehumidified daily with sterilized water. All beans were homogenized again after 30 days and dried in patios until reaching 11% humidity for the subsequent OTA and roasting process analysis.

2.6. OTA analysis in contaminated raw coffee beans

The OTA analysis using high-performance liquid chromatography (HPLC) was performed in triplicate at the Laboratory of Mycotoxins and Mycology, Ezequiel Dias Foundation (Laboratório de Micotoxinas e Micologia, da Fundação Ezequiel Dias) in Belo Horizonte, MG [Minas Gerais]. The methodology described by Vargas and Santos (2005) was used for the OTA extraction and quantification.

2.6.1. Ochratoxin A (OTA) calibration curve design

An intermediate solution (IS) at a concentration of 1.2014 μg/mL was prepared using an OTA standard stock solution (SS) at a concentration of 120.144 μg/mL, as determined by spectrophotometry according to the Association of Official Analytical Chemists – AOAC (1997, 806 p.), by diluting the SS a hundred times (pipetting 10 μL SS and adding 990 μL of toluene:acetic acid 99:1 v/v).

2.6.2. Extraction

Twelve and a half-gram coffee samples were weighed in a 500-mL Erlenmeyer flask in triplicate and supplemented with 200 mL of a 3% methanol:sodium bicarbonate (1:1, v/v) solution. The Erlenmeyer flask was sealed with a silicone stopper, and the system was submitted to vigorous mechanical stirring for 30 min. The samples were filtered through Whatman grade no. 4 paper after standing for 2 min and then re-filtered using a 47-mm-diameter Whatman grade GF/B microfiber filter under vacuum. Ten milliliters was removed with a volumetric pipette and supplemented with 90 mL phosphate-buffered saline [PBS].

2.6.3. Purification in immunoaffinity columns

The 100 mL of diluted extract was applied onto the immunoaffinity column (IAC) (Ochraprep® – Rhône) at a flow of 2–3 mL/min using vacuum. Subsequently, the column was washed with 10 mL deionized water and dried under vacuum for approximately 30 s.

2.6.4. Ochratoxin A elution

A 20-mL glass syringe was adapted to the IAC by adding 1 mL of HPLC-grade methanol to the column. Back-flushing was performed,

and the methanol remained in contact with the column resin for 3 min. The eluate was collected in a 4-mL vial. The procedure was repeated two more times, and the eluate was evaporated in a water bath at a temperature of 40°C–50 °C under an atmosphere of N_2.

2.6.5. High-performance liquid chromatography

OTA separation and quantification were performed in a Shimadzu high-performance liquid chromatography system coupled to a fluorescence detector (excitation at 333 nm and emission at 476 nm), using the CLASS-VP software. The column used was a C18 Shim-pack VP-ODS column, sized 4.6 mm × 250 mm and 4.6 μm in particle diameter. The pre-column used was a Shim-pack G-ODS, sized 4 mm × 10 mm, with particles of 5 μm in diameter. The column was stabilized with the mobile phase (acetonitrile: methanol: aqueous solution of glacial acetic acid (35:35:30, v/v)) at a flow rate of 0.8 mL/min. Fifty-microliter portions of the standard and sample solutions were injected. Under these conditions, the retention time was approximately 9 min. Quantification was performed by designing a calibration curve and using the peak area and the concentrations of the OTA standard solutions.

An OTA-free coffee sample was contaminated with 3 different concentrations in triplicate to evaluate the efficiency of the methodology used and the laboratory performance. In addition, samples of roasted and ground coffee containing known concentrations of ochratoxin A, according to the Food Analysis Performance Assessment Scheme (FAPAS), England, were also analyzed. This sample is included in the Ochratoxin Report No. 1733, Series 17, Round 33, Proficiency Testing.

2.6.6. Ochratoxin A confirmation

The positive confirmation of OTA was confirmed by the disappearance of the OTA peak at a retention time of approximately 10 min and the appearance of a new peak (ochratoxin A methyl ester) at a retention time of approximately 37 min (Pittet, Tornare, Huggett, & Viani, 1996).

2.6.7. Analytical methodology efficiency

Recovery tests were performed by adding OTA to uncontaminated coffee samples to evaluate the performance of the analytical methodology used. Coffee samples were contaminated in triplicate with three OTA concentrations: 1.78 μg/L, 2.66 μg/L and 3.55 μg/L. The linearity was calculated from the linear regression equation, assessed using the least squares method. The linear correlation coefficient (R^2) was used as an indicator of linearity for the mathematical model.

2.7. Roasting and grinding of the contaminated coffee beans

The coffee beans were roasted in a Probatino model Probat roaster at 3 different roasting levels (light, medium and dark). Agtron System/Specialty Coffee Association of America (SCAA) roast classification color discs were used to establish the sample tonality.

The following relationships between the roasting level and Agtron disc number were established for the samples: light roast – Agtron disc No. 95; medium roast – Agtron disc No. 65 and dark roast – Agtron disc No. 35. The time and temperature at which the coffee beans were exposed in that stage were controlled. The light-roast sample was exposed for 13.5 min at a temperature of 205 °C, the medium-roast sample was exposed for 14 min at a temperature of 217 °C, and the dark-roast sample was exposed for 14 min at a temperature of 224 °C.

The coffees were ground in a Mahlkoning electric mill at 3 different particle sizes (fine, medium and coarse), according to the Coffee Quality Program (Programa de Qualidade do Café, Associação Brasileira da Indústria de Café [Brazilian Association of the Coffee Industry] – ABIC, 2011). Brazilian Association of Technical Standards (Associação Brasileira de Normas Técnicas, ABNT) sieve numbers 12, 16, 20 and 30 and sieve bottom pans were used for the classification of particle size. The finely milled coffees were those with 0% retention in sieves No. 12 and 16, 70% retention in sieves No. 20 and 30 and 30% at the bottom pan. The average particle size coffees had 7% retention in sieve No. 12, 16.73% retention in sieves No. 20 and 30 and 20% at the bottom pan. The coarse particle size coffee had 33% retention in sieve No. 12, 16.55% retention in sieves No. 20 and 30 and 12% at the bottom pan.

2.8. OTA analysis of contaminated coffee beans following roasting and grinding

The same methodology described for the OTA analysis in contaminated raw coffee beans presented in item 2.6 was adopted in this procedure. The analysis were made in 3 repeats.

2.9. Statistical analyses

The data were transformed into ln *y*, and analysis of variance (ANOVA) was performed to ensure that the experimental analysis was performed following the basic prerequisites. A 3 × 3 factorial scheme was used, installed in a completely randomized design (CRD) with 3 repeats.

The data obtained were submitted to ANOVA and compared using Tukey's test at 5% probability. The entire analysis was performed using the R software (version 2.11.1, 20).

3. Results

The level of OTA in the Proficiency Test sample was 7.2 ng/g, for a theoretical value of 9.6 ng/g and an acceptable range from 5.4 to 13.8 ng/g. The recovery and coefficient of variation results (Table 1) from the samples fortified with ochratoxin A at three different concentrations were above 70% and below 20%, revealing that the methodology used is in accordance with Law No. 401/2006 of 02/23/2006 of the Commission Regulation (CEC, 2006; Horwitz, Kamps, & Boyer, 1980).

Thirty-five fungi from the *Aspergillus* genus, including *Aspergillus ochraceus* (25), *Aspergillus melleus* (01), *Aspergillus niger* (05), *Aspergillus tubingensis* (03) and *A. niger* aggregate (01) were isolated and identified in the sample analyzed. All isolates were tested for OTA production, and only *A. ochraceus* was potentially ochratoxigenic (80%). The isolate *A. ochraceus* CSM15 was selected because it showed the most intense spot in the TLC plate and was considered to be the isolate that produced the most OTA. This isolate is deposited in the Culture Collection of the Department of Food

Table 1
Ochratoxin A recovery test in coffee beans.

Contamination (μg/kg)	Result (μg/kg)	Recovery (%)	Mean result (μg/kg)	Mean recovery (%)
1.78	1.36	76	1.44	81 CV 6.2%
1.78	1.42	80		
1.78	1.54	87		
2.66	1.97	74	2.61	98 CV 22.2%
2.66	2.78	104		
2.66	3.09	116		
3.55	4.04	114	3.6	102 CV 11.4%
3.55	3.23	91		
3.55	2.54	100		

Coefficient of variation (CV).

Table 2
Mean OTA values detected in the samples, percent reduction, residual value, temperature and roasting time.

Temperature and roasting time	Sample types	Mean OTA concentration (μg/kg)	Standard deviation	OTA reduction	Residual value
	Green coffee without inoculation	5.41	–	–	
	Inoculated green coffee	108.33	–	–	
205 °C in 13.5 (min)	Light roast/fine particle size	47.41	1.97	56.25%	43.77%
	Light roast/medium particle size	40.78	1.07	62.36%	37.64%
	Light roast/coarse particle size	28.24	3.60	73.93%	26.07%
217 °C in 14 (min)	Medium roast/fine particle size	16.81	1.04	84.48%	15.52%
	Medium roast/medium particle size	10.75	0.69	90.01%	9.93%
	Medium roast/coarse particle size	8.43	0.89	92.22%	7.78%
224 °C in 14 (min)	Dark roast/fine particle size	4.97	0.18	95.41%	4.59%
	Dark roast/medium particle size	3.73	0.36	96.55%	3.44%
	Dark roast/coarse particle size	3.06	0.16	97.17%	2.83%

The results of the levels of ochratoxin A are the average of 3 replicates.

Science Federal University of Lavras/MG (Coleção de Cultura do Departamento de Ciência dos Alimentos da Universidade Federal de Lavras/MG).

The OTA concentration in the raw coffee beans before roasting was found to be 5.41 g/kg, while the content found in the naturally contaminated sample was 108.33 μg/kg after a period of inoculation with *A. ochraceus* CSM15. Therefore, the evaluation of the roasting and particle size effect was based on a sample contaminated with 108.33 μg/kg.

OTA was detected in 100% of the roasted samples analyzed at concentrations ranging from 108.33 μg/kg to 3.06 μg/kg; the detection was performed in triplicate. The sample with the highest concentration was the green coffee inoculated with OTA-producing *A. ochraceus* CSM15, which was considered to be the indicator sample because the experimental treatments were normalized against it. A reduction in the residual OTA value was also found following roasting and grinding (Table 2).

According to Table 3, the effect of the interaction between the roasting level and particle size on the mean OTA concentration in the coffee was statistically significant. The three roasting levels also noticeably resulted in different mean OTA concentrations for each particle size used (Table 4).

A significant difference between the means of the three roasting levels and fine particle size was found using the Tukey's test, and dark roasting was the level with the lowest mean value for the OTA concentration (Table 5).

A significant difference could also be found among the means of the three roasting levels when analyzing the roasting levels with medium particle size. The dark roast also showed the lowest mean value for the OTA concentration, mimicking the results found in Table 6.

Finally, Tukey's test was applied to analyze the dark roast with coarse particle size. The means also showed a significant difference, and the dark roast manifested the lowest mean value for the OTA concentration (Table 7).

Coffees with coarse particle size yielded the lowest mean values for the OTA concentration, regardless of the roasting level. The lowest concentration of toxin resulted from the combination of dark roast and coarse particle size, with a mean value of 3.06 μg/kg. This sample contained an average of 2.83% residual OTA, with a 97.17% reduction in the OTA level.

Table 3
Analysis of variance for the OTA extraction data in coffee, transformed into ln *y*.

Source of variation	DF	SS	RMS	*F*	*Pr* > *F*
Roasting	2	23.5911	11.7956	*Fa* = 2084.75	0.000000
Particle size	2	1.4487	0.7243	*Fb* = 128.02	0.000000
Roasting × particle size	4	0.0848	0.0212	*Fc* = 3.74	0.021866
Residue	18	0.1018	0.0057		
Total	26	25.2264			

CV = 3.04%.

4. Discussion

A. ochraceus CSM15 was selected as a potential ochratoxigenic fungus. Studies have confirmed that this species is able to synthesize OTA in coffee beans (Batista et al., 2003; Batista et al., 2009; Santini et al., 2011; Taniwaki, Pitt, Teixeira, & Iamanaka, 2003; Velmourougane, Bhat, Gopinandhan, & Panneerselvam, 2011).

The OTA contents in raw coffee beans prior to roasting increased from 5.41 g/kg to 108.33 μg/kg following inoculation with *A. ochraceus* CSM15, confirming the efficacy of the incubation conditions for fungal development and OTA production. The increase in the OTA concentration following inoculation also confirms the toxigenic potential of the *A. ochraceus* CSM15 used in this study.

The results show that the roasting process and particle size are important determinants of the residual concentration of OTA, which ranged from 2.83% to 43.77% depending on the roasting level and particle size.

Other reports have previously demonstrated that a reduction in the OTA content occurs following the roasting process, although additional factors should be considered, including the heterogeneity of coffee bean contamination, natural contamination versus artificial contamination, the performance of analytical methods and the roasting conditions (Scott, 1996).

The 97.17% reduction in OTA levels is similar to that found by Levi, Trenk and Mohr (1974) when comparing only the roasting processes. These authors showed that green coffee beans supplemented with OTA and submitted to conditions simulating typical roasting operations (20 min at 200 °C) experienced a 77%–87% OTA reduction. Blanc et al. (1998) only found 16% of the OTA originally contained in green coffee roasted for 14 min at 223 °C. Ferraz et al. (2010) reported a reduction in the OTA content of 53%–99% after 12 min of roasting in samples roasted at 180 °C and 240 °C, respectively.

Table 4
Analysis of variance regarding roasting performance for each particle size.

Source of variation	DF	SS	RMS	*F*	*Pr* > *F*
(Roasting/G_{fine})	2	7.64055	3.82027	*Fd* = 675.1942	0
(Roasting/G_{medium})	2	7.39947	3.69974	*Fe* = 653.8903	0
(Roasting/G_{coarse})	2	8.63587	4.31793	*Ff* = 763.1505	0
Residue	18				
Total	26	25.2264	0.97025		

Table 5
Tukey's test regarding roasting levels with fine particle size.

Roast	Mean	Mean values without transformation into ln *y*
Light	3.858325 a	47.41 μg/kg
Medium	2.820806 b	16.81 μg/kg
Dark	1.603783 c	4.97 μg/kg

Means followed by the same letter do not differ statistically, according to the Tukey's test at 5% probability.

Table 7
Tukey's test regarding roasting levels with coarse particle size.

Roast	Mean	Mean values without transformation into ln *y*
Light	3.335529 a	28.24 μg/kg
Medium	2.128356 b	8.43 μg/kg
Dark	1.117388 c	3.06 μg/kg

Means followed by the same letter do not differ statistically, according to the Tukey's test at 5% probability.

Significant differences in the residual OTA values could be found in all of the treatments studied. The combination of light roasting and coarse particle size yielded the lowest residual OTA value among the treatments involving light roasting, with a 73.93% reduction.

The lowest residual OTA value was also observed in the combination of medium roasting and coarse particle size, with a 92.22% OTA reduction. The combination of dark roasting and coarse particle size was the treatment producing the lowest toxin concentration, with 3.06 μg/kg. This sample contained, on average, 2.83% residual OTA, with a 97.17% OTA reduction.

The interaction between dark roasting and any particle size analyzed led to the lowest OTA concentrations. Stegen van der, Essens, & Lijn van der (2001) found that a larger OTA reduction occurs upon the increased roasting duration in darker-colored samples in their analysis of the effect of roasting on OTA reduction in coffee. This finding may be related to the increased roasting process temperature because the higher the temperature is, the higher the OTA reduction upon thermal treatment will be (Heilmann et al., 1999; Studer-Rohr, Dietrich, Schlatter, & Schlatter, 1995).

According to Romani, Pinnavaia, and Dalla Rosa (2003), the OTA reduction during roasting is primarily attributed to the aggressiveness of the thermal treatment and is usually related to the initial OTA content.

The lower residual OTA values in the treatments with coarse particle size may be explained by the particle size effect on the extraction power. Carvalho and Chalfoun (1985) report that the speed of extraction of coffee solubles is closely related to the grinding level because the higher the grinding intensity is, the more ground the coffee bean and the lower its particle size will be.

The medium roast was the most affected by the different types of particle size in that it showed the largest difference between the mean estimates of fine and coarse particle size. The opposite can be noted of the dark roast. The thermal treatment was the most responsible for the OTA reduction at the high roasting temperature, decreasing the particle size effect.

Some authors have proposed that physical removal through the residue film of coffee beans after roasting is another mechanism of OTA elimination. However, Blanc et al. (1998) showed that this type of removal only partially explains the elimination. Romani et al. (2003) noted the minimal removal of OTA from the residue film of coffee beans (2.08%). These results led to the conclusion that the reduction in the OTA levels during roasting appears to be mainly attributable to thermal destruction.

Another possible explanation for the low residual OTA values has been provided by Studer-Rohr et al. (1995). These authors state that the partial isomerization of OTA occurs at position C-3 during the roasting process, making OTA untraceable using routine methodologies.

Previous studies have shown that roasting (Blanc et al., 1998; Ferraz et al., 2010; Levi et al., 1974), physical removal (Blanc et al., 1998; Romani et al., 2003) and isomerization (Studer-Rohr et al., 1995) reduce the OTA levels.

Only roasting (removal of chaff and thermal destruction) and/or treatment with organic solvents seem to be appropriate technologies with which to clean up OTA-contaminated coffee beans on arrival (Heilmann et al., 1999).

In conclusion the great benefit of the current study was the evaluation of the effects of three particle sizes, which clearly indicated that this parameter affects the residual values of OTA in roasted and ground coffee. The type of roasting and particle size interfere with the residual content of ochratoxin A in the beverage. Therefore, the study helps to establish quality requisites for roasted and ground coffee production within national and international standards of quality, enabling value to be added to a traditional product of Brazilian agriculture.

Table 6
Tukey's test regarding roasting levels with medium particle size.

Roast	Mean	Mean values without transformation into ln *y*
Light	3.707921 a	40.78 μg/kg
Medium	2.373736 b	10.75 μg/kg
Dark	1.313722 c	3.73 μg/kg

Means followed by the same letter do not differ statistically, according to the Tukey's test at 5% probability.

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