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Padronização de um método analítico para quantificação de ocratoxina A em grãos de café verde por cromatografia líquida de alta eficiência

Standardization of an analytical method to quantify ochratoxin A in green coffee beans by high performance liquid chromatography

Cuantificación de ocratoxina A en granos verdes de café mediante cromatografía líquida de alta eficiencia

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Resumo

O Brasil é atualmente o maior produtor e exportador de café, sendo o segundo maior consumidor da bebida. A importância de se garantir a segurança alimentar ao consumidor tem influenciado em pesquisas para melhorar e monitorar a qualidade do produto final. Visto que o café é um dos produtos mais contaminados por fungos que podem produzir micotoxinas, representando riscos à saúde humana e animal. O objetivo do trabalho foi padronizar um método cromatográfico para avaliar e quantificar ocratoxina A em 13 amostras de grãos de café cru armazenados em galpão. As amostras de café foram moídas e o analito foi extraído em solução de metanol:bicarbonato de sódio 3% (1:1 v/v). Foi utilizado um cromatógrafo líquido de alta eficiência para a quantificação da ocratoxina A. O método foi validado avaliando-se os parâmetros de seletividade, linearidade, exatidão e limites de detecção e quantificação. O método apresentou robustez aos parâmetros avaliados e entre amostras analisadas, foram encontrados valores superiores ao limite estabelecido pela legislação somente em uma das amostras (75,19 µg.Kg-1). O método de armazenamento de grãos de café cru em galpão se apresentou adequado no presente trabalho, pois 12 amostras apresentaram baixas concentrações de ocratoxiana A, estando dentro dos limites da legislação. Assim, é garantida a segurança alimentar sem nenhuma contaminação severa de ocratoxina A.

Palavras-chaves: HPLC; Fungo; Toxina; Alimento.

Abstract

Nowadays, Brazil is the largest producer and exporter of coffee, also the second largest consumer of the beverage. The importance of ensuring food safety for consumers has influenced research to improve and monitor the final product quality. Coffee is a product that presents a high risk of fungal contamination, which can result in the presence of mycotoxins and poses risks to human and animal health. Therefore, this study aimed to standardize a chromatographic method to test and quantify ochratoxin A in 13 samples of green coffee beans. The green coffee beans were stored in sheds without temperature or humidity control. Samples were ground, and the analyte was extracted by a 3% methanol:sodium bicarbonate (1:2 v/v) solution. Ochratoxin A was quantified in a high performance liquid chromatograph. The method was validated by testing the selectivity, linearity, accuracy, limits of detection and quantification. The method presented robustness to the tested parameters and among the analyzed samples. Ochratoxin A was detected above the limit established by the legislation (75.19 µg kg-1) only in one sample. Overall, the storage of green coffee beans in these sheds was adequate, since 12 samples had a low content of ochratoxin A and they were within the limit established by legislation. Therefore, food safety was guaranteed without any severe mycotoxin contamination.

Keywords: HPLC; Fungus; Toxin; Food safety.

Resumen

Brasil actualmente es el mayor productor y exportador de café, siendo el segundo mayor consumidor. La importancia de garantizar la seguridad alimentaria al consumidor ha influenciado las investigaciones para mejorar y monitorear la calidad del producto final. El café es uno de los productos más susceptible a ser contaminado por hongos que pueden producir micotoxinas, lo cual representa un riesgo para la salud humana y animal. El objetivo de este trabajo fue estandarizar un método cromatográfico para evaluar y cuantificar ocratoxina A en 13 muestras de granos verdes de café, almacenados en bodegas. Las muestras fueron molidas y el analito fue extraído en solución de metanol:bicarbonato de sodio 3% (1:1 v/v). La cuantificación de ocratoxina A fue realizada mediante un cromatógrafo líquido de alta eficiencia. El método fue validado evaluando los parámetros de selectividad, linealidad, exactitud y límites de detección y cuantificación. El método presentó robustez en los parámetros evaluados, y solamente en una de las muestras analizadas fueron encontrados valores superiores al límite establecido por la legislación (75,19 µg.Kg-1). El almacenamiento

de granos de café verde en bodegas mostró ser adecuado en el presente trabajo, pues 12 muestras tuvieron bajos contenidos de ocratoxina A y estuvieron dentro de los límites establecidos por la legislación. De esa forma, se garantiza la seguridad alimentaria sin ninguna contaminación severa por micotoxinas.

Palabras clave: HPLC; Hongo; Toxina; Alimento.

1. Introduction

Coffee is one of the most consumed beverages in the world, and the drink has its guaranteed space on the consumers' table in Brazil. According to the Ministério da Agricultura, Pecuária e Abastecimento (MAPA), coffee arrived in Brazil in 1727 brought from the French Guiana. The climatic conditions and geographical relief were favorable for coffee cultivation, which spread it throughout the country and resulted in a predominant production for years in the Vale do Paraíba region.

Brazil is considered the largest coffee producer and exporter and the second largest coffee consumer in the word, moving US$5.2 billion in 2017. Nowadays, the concern with food quality is not only focused on the required prerequisites such as appearance, taste, aroma, texture, nutritional value and safety, but also chemical and microbiological contaminations. Food contamination caused by fungi results in major economic losses, a waste of raw material and changes in the product quality and safety, directly impacting the agricultural and food industries (Das et al., 2015). Even more, some of these filamentous fungi can produce mycotoxins that pose a serious risk to human and animal health, which can cause diseases or even lead to death (Bennet & Klich, 2003).

Mycotoxin production depends on fungal growth, so it can be produced in the field, before or during harvesting, or during storage. Mycotoxins can remain in the food even after the fungi that produce it has been eliminated. This can be explained for its thermal stability, resistance to heat treatments, or dehydrations processes (Bennet & Klich, 2003; Iamanaka & Taniwaki, 2010).

Ochratoxin A is a mycotoxin classified by the International Agency for Research on Cancer (IARC) as a group 2B human carcinogen. It can cause development of kidney disease, which is considered a contributing factor to a chronic disease in which the kidneys decrease in size and weight with diffuse fibrosis and urinary tract tumors (Iarc, 1993). Ochratoxin A is also related to nephrotoxic properties in animals. In addition, it behaves as a hepatoxin,

immunosuppressive, teratogenic and carcinogenic substance. Studies indicate that this toxin induces cancer in liver, kidneys, mammary glands and testicles of animals (Jeleń & Grabarkiewicz-Szczęsna, 2005; Amezqueta et al., 2012).

Ochratoxin A can be found in oats, barley, rye, wheat, coffee beans, nuts, dried peanuts, beans, spices, dried fruits and wine (Amezqueta et al., 2012). The Agência Nacional de Vigilância Sanitária (ANVISA) assigns maximum limits for mycotoxins in food. This regulation is applied to companies that import, produce, distribute and marked food products. Ochratoxin A concentration for roasted coffee beans and soluble coffee are limited to 10 µg kg-1 (Brasil, 2011; Brasil, 2017).

Verification of ochratoxin A in food can be performed by several chromatographic methods, such as thin-layer chromatography, gas chromatography coupled to mass spectrometry, high performance liquid chromatography coupled to a mass spectrometry and mass spectrometry coupled to inductive plasma; and by enzymatic immunological assays, such as ELISA (EFSA, 2004; Soleimany & Abas, 2012; Prelle et al., 2013; Pereira & Cunha 2014). However, the most common is high performance liquid chromatography combined with immunoaffinity columns and florescence detectors. Studies show that this technique has been used successfully to analyze the mycotoxin presence in food matrices (Prelle et al., 2013; Yashin et al., 2017).

The sample complexity requires a prior treatment, comprising several steps, before the analyte is determined. Most analytical methods used to analyze ochratoxin A has several steps in common, such as sampling, homogenization, extraction, separation / purification and detection. However, these steps can be considered as a source of variation of the results (Prelle et al., 2013; Pereira & Cunha 2014).

In order to be considered appropriate and in compliance, the analytical technique needs to provide safe results and quantify low concentration of compounds in a reliable and reproducible manner. The performance criteria established for the method must include all parameters, such as the limit of detection, coefficient of variation, coefficient of repeatability, reproducibility and the percentage of recovery. Using these parameters, laboratories would be free to adopt the most appropriate analytical method for their facilities (Codex, 2019). Thus, the standardization of a chromatographic method allows the reliability and reproducibility of results. Therefore, this study aimed to standardize a chromatographic method using HPLC and a fluoresce detector to quantify ochratoxin A in 13 samples of green coffee beans stored in sheds for 24 months.

2. Material and Methods

2.1 Plant Samples

Thirteen samples of green coffee beans, Coffee arabica variety, were collected in 2016. The samples were stored in sheds without control of temperature or humidity for 24 months. The samples were obtained from the cities of Bambui, Doresopolis, Piumhi and São Roque in the state of Minas Gerais, Brazil.

2.2 Ochratoxin A extraction

The green coffee beans were ground and sieved to get a 200 mesh size. Ochratoxin A was extracted by stirring 25.00 g of sample for 10 min in 200 mL of 3% methanol:sodium bicarbonate solution (1:1, v/v) at 254 rpm. Subsequently, 10 mL of the filtrate was diluted 10 times in a phosphate-buffered saline solution (PBS) and passed through an immunoaffinity column (OchratestTM – Vicam, Watertown. USA) at a flow rate of 2-3 mL per min. Prior to the full passage of the sample, the column was washed with 10 mL of ultra-pure water at 5 ml per min. Then, the excess liquid was removed by allowing air passage in the column. Elution was carried out with 4 mL of methanol HPLC grade left in contact with the column for 3 min. Finally, the eluate was evaporated in a water bath at 80 °C and resuspended in 400 µL of mobile phase.

2.3 Ochratoxin A quantification

Ochratoxin A was quantified in a Shimadzu HPLC chromatograph equipped with an LC-20AT high-pressure quaternary pump, DGU-20A5 degasser, CBM-20A interface, SIL-20A-HT automatic injector, CTO-20A oven and an RF-20A fluorescence detector. It was used an Agilent-Zorbax Eclipse XDB-C18 column (4.6 x 250 mm, 5 µm) connect to an Agilent-Zorbax Eclipse XDB-C18 (4,6 x 12, 5 mm, 5 µm) pre-column. Wavelengths of excitations and emissions were 332 and 476, respectively. Elution was performed with an isocratic system of 35:35:29:1 v/v (methanol:acetonitrile:water:acetic acid) at 0.8 ml min-1 (Passamani et al., 2014). Injections of 20 µL were done in triplicate. Quantification was

carried out by external calibration from the standard injected at different concentrations to construct an analytical curve by linear regression.

2.4 Chromatographic validation

Selectivity, linearity, limits of detection (LD), limits of quantification (LQ) and accuracy were used to validate the chromatographic method and ensure the good analytical quality of the results (Brasil, 2003; Ribani et al., 2004; Harris, 2008).

Selectivity was assessed by comparing the analytical curve of the sample with and without the addition of the standard. Linearity, obtained by external standardization, was determined by correlating the signal (peak area) and the concentration of the compound, and expressed by the equation of the analytical curve and its respective coefficient of determination(r2). The limits of detection (LD) and quantification (LQ) were determined from the parameters of the analytical curve. Both limits were calculated using the mathematical equations: LD= 3.3 x s/S and LQ= 10 x s/S, where is the estimate standard deviation of the response and S is the angular coefficient of the analytical curve. Last, accuracy was tested by recovery assays using three randomly selected samples that were fortified with ochratoxin A at 3 different concentrations. The recovery was determined considering the relationship between the amount of present addicted analyte in the sample and the response using the following mathematical correlation: Recovery (%) = [(observed concentration) / (expected concentration) x 100 (Brasil, 2003; Ribani et al., 2004; Harris, 2008).

2.5 Statistical analysis

Ochratoxin A values were submitted to an analysis of variance, and means were compared by the Tukey test at a 5% probability level. The experimental design was completely randomized (DRC) and Sisvar software was used (Ferreira, 2011).

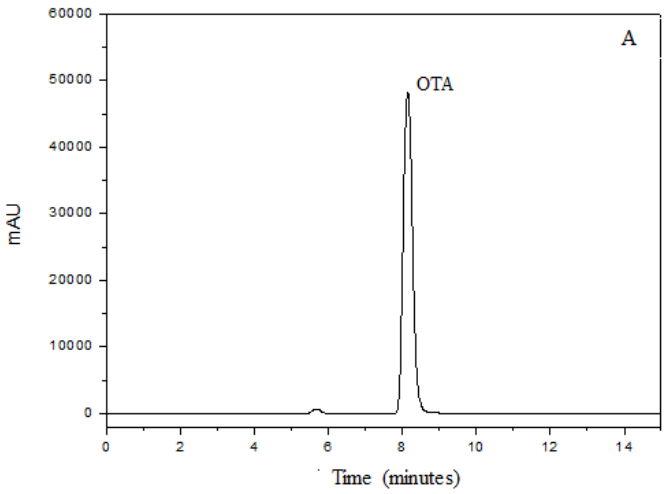
3. Results and Discussion

3.1 Method validation

The reliability of an analytical method is of considerable importance to quantify compounds, which through a robust method maintain the reproducibility and quality of results. The standardization of a method is a way to perform an analysis and publish its results with confidence and responsibility (Ribani et al., 2004).

It was possible to observe the ochratoxin A separation and its chromatographic profile from a standard solution of the analyte under study (Figure 1). The average retention time for ochratoxin A was 8.0 ± 0.1 min. Other authors reported values of 8.5 min (Medina, 2015), 5 min (Galarce-Bastos et al., 2014), 7.2 min (Casal et al., 2014) and 11.2 min (Benites et al., 2017), lower and even higher values than the one found in this study. However, it is noteworthy that the equipment and chromatographic conditions were different.

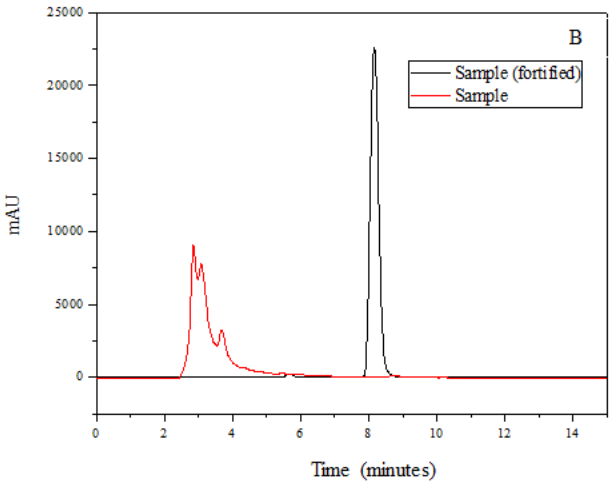
Figure 1. Chromatograms obtained after analysis (A: Ochratoxin A standard solution).



Source: Author (2020).

Under the employed chromatographic conditions, it was found that the coffee samples did not show interfering substances in the retention time of the ochratoxin A. This can be observed by the chromatogram of the sample free of the analyzed compound and of this same sample fortified with the standard analyte at 75 ng mL-1 (Figure 2), attesting the selectivity of the method.

Figure 2. Chromatograms obtained after analysis (B: Ochratoxin A- free sample and the standard added at 75 ng mL-1 in the sample.



Source: Authors (2020).

Through the construction of the analytical curve, it was possible to test the linearity of the method by estimating the coefficient of determination referring to the equation of the line (y = 3796,59x – 3149,52). The coefficient of determination was 0.999, which shows the strong linear correlation between the concentration of the analyzed compound and the peak area, a value above 0.99 is recommended (Brasil, 2003). Also, this coefficient of determination is higher than those reported by Benites et al. (2017) (0.995) and Vecchio et al. (2012), Casal et al. (2014), Galarce-Bastos et al. (2014) (0.998). Therefore, the acquired analytical curve is efficient for quantification of ochratoxin A in green coffee beans.

The LD and LQ were estimated using the parameters from the constructed analytical curve, which were 0.24 and 0.8 µg kg-1, respectively. These values are similar to those reported by Benites et al. (2017), being 0.266 and 0.5 µg kg-1for LD and LQ, respectively. LD and LQ were considerably low, showing a good sensitivity of the method for the analysis of ochratoxin A in coffee samples. Ribani et al. (2004) attribute the small differences in the parameters to the various chromatographic conditions, such as apparatus and methodologies.

The accuracy was evaluated through recovery assays to ensure that any matrix interferences did not influence the ochratoxin A detection. The recovery ranged from 80 to 90% for the three concentrations in the tested samples. Based on the acceptable limits for recovery (70 to 120%), it can be observed that the method presented a good recovery for the analyzed compound and the partial values and average values were within the acceptable limit range. For more complex samples, this acceptable limit range can be from 50 to 120%.

Medina (2015) found recovery values between 78 and 94% in tests done at 5 concentration levels, while Benites et al. (2017) reported values between 95.5 and 109.8%.

Through the validation analysis it is possible to notice that the method presented selectivity, linearity, limits of detection and quantification, recovery and robustness, which generated reproducible and reliable results in the quantification of ochratoxin A.

3.2 Ochratoxin A quantification in green coffee beans

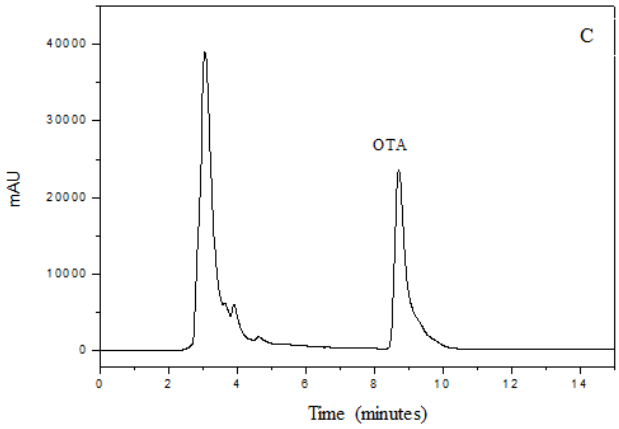
The quantification results of ochratoxin A in 13 samples of stored green coffee beans are shown in Table 1. Ochratoxin A concentration ranged from 0.69 to 75.19 µg kg-1, where sample 4 (Figure 3) presented a value considerable superior to the limit allowed by legislation for roasted and green coffee beans (10 µg kg-1).

Table 1. Concentration of ochratoxin A in green coffee beans.

Samples	Ochratoxin A (µg kg^{-1})
1	1.02 ± 0.08d
2	2.12 ± 0.07c
3	0.69 ± 0.00e
4	**75.19 ± 1.19a**
5	1.10 ± 0.03d
6	0.77 ± 0.01e
7	0.83 ± 0.01e
8	2.79 ± 0.24b
9	0.91 ± 0.01d
10	0.76 ± 0.01e
11	0.83 ± 0.01e
12	0.72 ± 0.01e
13	0.92 ± 0.04d

*Means followed by the same lower case letter do not differ from each other by the Tukey test at a 5% probability level. Source: Authors (2020).

Figure 3. Chromatograms obtained after analysis (C: Ochratoxin A chromatogram of sample 4).



Source: Authors (2020).

Storage conditions of green coffee beans may promote the proliferation of filamentous fungi and consequently favor the optimal conditions for the production of mycotoxins. Fungi are widely distributed in the environment, including water, air and soil. Therefore, food can be contaminated with a wide fungal variety originating from environmental sources, which under favorable conditions can grow in the food and cause its deterioration. Fungi can proliferate on substrates and conditions that other microorganisms do not develop, such as water activity (Aw) from 0.65 to 0.99, considerable low pH, and temperatures ranging from 0 to 40 °C. Hence, these attributes make fungi capable of causing spoilage in food with different levels of humidity and climatic conditions (Medina, 2015).

Water activity and temperature are the factors that most affect fungal growth, sporulation and toxin production (Magan et al., 2011). In general, high humidity (20 – 25%), high relative humidity (70 – 90%) and warm temperatures (22 – 30 °C) increase fungal proliferation and mycotoxin production (Vanesa and Ana, 2013). According to Oliveira et al. (2019) Aspergillus carbonarius showed optimal growth at aw ranging from 0.935 to 0.965 and temperatures between 25 and 32 °C while Aspergillus ochraceu presented better growth at aw of 0.940 to 0.990 and temperatures from 21 to 30 °C. On the other hand, the conditions where these microorganisms showed the highest risk index of ochratoxin A production were: A. carbonarius (aw: 0.95-0.99 and temperature: 22 – 32 °C) and A. ochraceus(aw: 0.97-0.99 and temperature: 25 – 30 °C). Considering the changes in humidity and temperature throughout the year, these favorable conditions for the production of ochratoxin A can be considered a problem in the southern region of Minas Gerais because in the harvest months there is a

considerable increase in the region's temperature and humidity, which may have been a factor for the ochratoxin A detected in the coffee beans that were analyzed.

Passamani et al. (2014) evaluated the production of ochratoxin A by Aspergillus niger and A. carbonarius at different pH, temperatures and water activity. The authors reported an ochratoxin A production of 7 µg g-1 and 1 µg g-1, respectively for each fungi, at a water activity of 0.99, pH 5.35 and 15 °C. These values were higher than those found in our study, but it was expected since the mentioned study established the most favorable conditions for the fungi to produce ochratoxin A.

Batista and Chaulfoun. (2007) quantified ochratoxin A in samples from different coffee fractions that were dried under different conditions (soil, asphalt and cement). Ochratoxin A ranged from 0.1 to 5.0 µg kg-1 of coffee regardless the drying conditions. These values were below those found in this study and within the limit required by legislation.

Barcelo et al. (2017) analyzed the contamination of ochratoxin A in roasted coffee, green coffee beans with and without defects. The concentrations of ochratoxin A ranged from 17.14 to 21.02 µg kg-1 for the defective green coffee beans while the green coffee beans without defects did not present ochratoxin A. After roasting the ochratoxin A ranged from 5.57 to 6.79 µg kg-1, resulting in a reduction of approximately 70% of the contamination. The authors concluded that the roasting process is a practice that decreases contaminants in coffee beans, especially ochratoxin A.

4. Conclusion

All coffee samples presented ochratoxin A contamination, but only one of them showed a concentration above the limit allowed by legislation. Therefore, the adoption of good agricultural practices during all coffee processing can significantly influence the risk of contamination by microorganisms, consequently the production of mycotoxins.

Considering the validation results, the method performed in this study for the identification and quantification of ochratoxin A was adequate for the analysis of green coffee beans.

As coffee is one of the most consumed beverages in the world, the need to assess contaminants is important for the consumer's safety. The method to identify ochratoxin A needs to be efficient, robust, and reproducible in many parts of the world because in each

country a different form of post-harvest is employed, which can contribute to the proliferation of toxin-producing fungi.

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