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Ozonation of Brazil nuts: Decomposition kinetics, control of *Aspergillus flavus* and the effect on color and on raw oil quality

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ABSTRACT

The objective of this study was to evaluate the decomposition kinetics of ozone gas for preservation of Brazil nuts, the effect on *Aspergillus flavus* and possible effects on color and on raw oil quality. With regards to the saturation process, an exponential reduction of the saturation time was observed, while the saturation concentration increased linearly in the porous medium as the initial gas concentration was increased. Ozone half-life in the Brazil nuts at 25 °C was equivalent to 4.20 min. As for the effect of ozone on *A. flavus*, at the concentration of 8.88 mg L^{-1}, for 240 min, the gas was able to cause a reduction in the count of microorganisms greater than 3.10 log cycles. Changes in the structure of the microorganisms and color of the colonies were also evidenced. Ozonation did not alter the quality of Brazil nut oil or its lipid profile. From the obtained results, it was concluded that: the initial ozone concentration influences the time and saturation concentration; ozone efficiently inactivates *A. flavus* in Brazil nuts; and in the conditions adopted for the present study, ozone does not affect the quality of the raw oil, including the lipid profile.

1. Introduction

The most important product of *Bertholletia excelsa* H.B.K. is its nut, known as the Brazil nuts. Brazil nuts can be consumed, either as is or as an ingredient in products such as granolas, ice cream, chocolates, cakes, candies and cookies (Ferberg, Cabral, Gonçalves, & Deliza, 2002; Pacheco & Scussel, 2007; Santos, Lopes, Azevedo, & Santos, 2010). The main Brazil nuts co-products are cold-pressed oil, flour and milk (Freitas, Freitas-Silva, Miranda, & Coelho, 2007; Felberg, Antoniassi, Deliza, Freitas, & Modesta, 2009; Santos et al., 2010; Sartori, De Alencar, Bastos, Regitano D'Arce, & Skibsted, 2018). Brazil nuts consist of 66.6 g 100 g^{-1} lipid and 14.2 g 100 g^{-1} protein (Thomson, 2011). In addition, according to Thomson (2011), the protein in Brazil nuts is high in the sulfur-containing amino acids cysteine and methionine. These nuts present high values of selenium (Se), iron (Fe), magnesium (Mg) and manganese (Mn), as well as substantial quantities of phenolic compounds (Chunhieng, Hafidi, Pioch, Brochier, & Didier, M., 2008; John & Shahidi, 2010). It is noteworthy that phenolic compounds are widely studied due to their potential beneficial health effects, including prevention of cancer, cardiovascular disease, diabetes, immune disorders and neurodegenerative disease (De Camargo & Lima, 2019; Shahidi, Vamadevan, Oh, & Peng, 2019).

Brazil nuts are mainly produced in extractive-based systems, with only a small number of producing sites located in the northern Brazilian states. The traditional production system is basically focused on family units and has not received many technological investments. Due to the low technological level of the production chain and climatic conditions during the harvest period, where the climate at the production site is characterized by relative humidity of 97% and temperatures between 25 and 30 °C, Brazil nuts are susceptible to contamination, mainly by fungi (Souza, Cartaxo, & Leite, 2004). This raw material is an excellent substrate for fungi and is subject to contamination by mycotoxins, especially aflatoxins, which are extremely toxic and carcinogenic, and are predominantly produced by *Aspergillus flavus* and *A. parasiticus*

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(Massi et al., 2014; Payne, 1998).

In view of the health risks to consumer and the objective of purchasing products with high nutritional quality and free of contaminants, several countries established a maximum allowable limit for aflatoxins, which implied an increase in stringency for Brazil nut imports. Therefore, adequate control during all stages of the production chain is essential to avoid aflatoxin contamination of Brazil nuts and to meet standards for consumption and commercialization, including export. Drying, refrigeration and storage of Brazil nuts in appropriate package, including vacuum packing, has been used to control fungal growth, as well as reducing changes in product quality such as oxidation of lipid fraction (Lin et al., 2012; Lorini, Wobeto, Rosa, Hatem, & Botelho, 2018; Sartori et al., 2018). An alternative that has been presented as a method for the control of aflatoxins in foods is ozone (Alencar, Faroni, Soares, Silva, & Carvalho, 2012; Chen et al., 2014; Zorlugenç, Zorlugenç; Öztekin, & Evliya, 2008).

Ozone is an allotropic form of oxygen, which can be generated synthetically by the corona discharge method (Kim, Yousef, & Dave, 1999). This compound is highlighted because it is highly reactive and in a gas form at 20 °C it has a half-life time of less than 20 min (Novak & Yuan, 2007). The decomposition process can be accelerated when ozonation occurs in medium containing organic materials, such as products of plant origin, and depends on factors such as the type of organic material present in the medium, moisture content and temperature (Alencar, Faroni, Martins, Costa, & Cecon, 2011; Kim et al., 1999; Paes, Faroni, Martins, Cecon, & Heleno, 2017).

The potential of ozone to prevent the synthesis of aflatoxins is related to its proven antimicrobial action. The antimicrobial action of ozone is due to its high oxidative potential, which makes it capable of oxidizing glycolipids, glycoproteins and amino acids of the cell wall and membrane, as well as other constituents of the cellular content (Kim et al., 1999). In plant-based products, ozone has been shown to be effective in inhibiting fungal species of *Aspergillus*, *Fusarium, Geotrichum, Myrothecium*, *Mucor* and others (Alencar et al., 2012; Raila et al., 2006; Sanchez, Alencar, Pinelli, Ferreira, & Roberto, 2016; Wu, Doan, & Cuenca, 2006), as well as viruses and bacteria (Khadre, Yousef, & Kim, 2001; Kim et al., 1999; Whangchai, Saengnil, & Uthaibutra, 2006; Öztekin, Zorlugenc, & Zorlugenc, 2006).

Although there are several studies in literature that evaluate the effect of ozone on foods, especially its effect on different microorganism groups, there are few reports which consider the ozonation of Brazil nuts. Studies are needed that evaluate the decomposition kinetics of the gas in porous medium composed of a mass of Brazil nuts and the control of fungi, including those potentially aflatoxigenic. The determination of parameters such as concentration, saturation time and half-life are fundamental for the implementation of ozonation by industry to evaluate the technical feasibility and design of the system (Alencar et al., 2011). Another fundamental aspect is the effect on product quality, mainly due to the high oxidative potential of ozone and the composition of Brazil nuts, composed of approximately 66.0 g 100 g^{-1} lipids of which unsaturated fatty acids predominate (Freitas & Naves, 2010; Thomson, 2011).

Considering that ozonation represents an alternative for the control of fungi and consequently prevents the synthesis of aflatoxins, and that the presence of this substance in Brazil nuts is considered important from the point of view of public and commercial health, the objective of the present study was to evaluate the decomposition kinetics of ozone in Brazil nuts, as well as the effect on *A. flavus* and its potential effects on color and on raw oil quality.

2. Material and methods

The study was divided into two stages. In the first stage, ozone decomposition kinetics was studied in porous medium composed of a mass of Brazil nuts. In the second stage, the effect of ozone on *Aspergillus flavus* and possible effects on product quality were evaluated.

2.1. Brazil nut samples

One batch of Brazil nuts with moisture content around 4.40 g 100 g^{-1} from the 2016/2017 harvest was used. Brazil nuts were purchased from the Agricultural Producers Cooperative of the northern region of the State of Mato Grosso-Coopervia, Brazil.

2.2. Ozonation process

Ozone gas was obtained by means of an ozone generator with a coupled oxygen concentrator (Model 0&L 5.0 RM – Ozone & Life, São José dos Campos, SP, Brazil) based on the Dielectric Barrier Discharge method. The ozone concentration was determined by the iodometric method, described by Clescerl, Greenberg, and Eaton (2000).

2.3. Study of the ozone decomposition kinetics in porous medium containing Brazil nuts

Brazil nut samples of 1 kg were packaged in glass containers with a capacity of 3.0 L and screw-on lids, with connections for gas inlet and outlet. After passing ozone through the product, the gas was directed to a thermal destroyer manufacturing on demand (Ozone & Life, São José dos Campos, SP, Brazil). The ozonation process of the samples is shown in Fig. 1. Ozone gas concentrations of 2.42, 4.38, 8.88 and 13.24 mg L^{-1} were used with a flow rate of 3.0 L min^{-1}, at a temperature of 25 °C with three replicates. Ozone concentrations were determined from preliminary tests (data not shown). The process of porous medium saturation with ozone was evaluated initially, followed by the gas decomposition process.

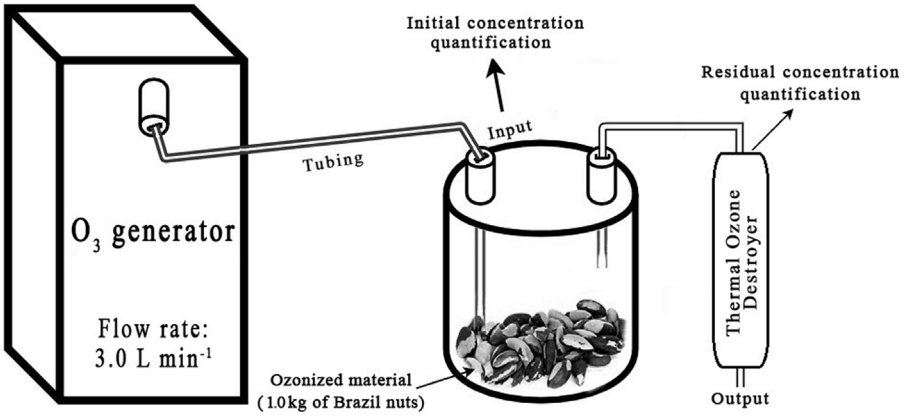

Fig. 1. Schematic of the ozonation of Brazil nuts.

2.3.1. The porous medium saturation process

The saturation process of the medium composed of a mass of Brazil nuts was evaluated. The residual ozone concentration was determined by the iodometric method until it remained constant. To relate the residual ozone gas concentration to time, a sigmoidal equation was fitted to the data obtained (Eq (1)):

$$C = \left[\frac{a}{1 + e^{-(t-b)/c}}\right] \quad \text{Eq. 1}$$

Where C = concentration of ozone gas (mg L^{-1}); t = time (min); a, b and c = constants of the equation.

According to Venegas, Harris, and Simon (1998), parameters a, b and c can be defined as follows: the parameter a corresponds the total change in concentration between the lower and the upper asymptotes; the parameter b is the time at the inflection point of the sigmoidal equation; and the parameter c is proportional to the time range within which most of the concentration change takes place. From the constants b and c it was possible to obtain the saturation time for each gas concentration (Eq. (2)), and subsequently the saturation concentration (Venegas et al., 1998):

$$t_{Sat} = b + 2c \quad \text{Eq. 2}$$

Where, t_{Sat} = saturation time (min).

2.3.2. Evaluation of the ozone decomposition kinetics

Evaluation of the ozone decomposition kinetics was performed after saturating the medium with the gas, according to Santos, Martins, Faroni, Andrade, and Carvalho (2007). The residual gas concentration was quantified after time intervals without gas injection during which ozone decomposition occurred. Zero-order, first order and second order kinetic models were fitted to the ozone concentration data as a function of time (Wright, 2004, p. 441). The decomposition kinetic models and their integrated and linearized equations, and half-lives are presented in Table 1.

2.4. Analysis of the effect of ozone on Aspergillus flavus

The strain of *A. flavus*, named CCUB1405, was isolated from symptomatic commercial peanuts in the selective culture media *Aspergillus flavus* and *parasiticus* Agar (AFPA) (Pitt & Hocking, 2009, p. 519), and was characterized by sequence analysis of the ITS and beta-tubulin (BenA) partial gene. A sequence of the used isolate was deposited in the GenBank database under accession number CCUB1405. Preliminary tests confirmed that the strain CCUB1405 is aflatoxin producing.

2.4.1. Inoculation of Aspergillus flavus in Brazil nuts

For inoculation of *A. flavus* in Brazil nuts, 10 mL of sterile distilled water was added to each plate, and the conidia suspension of the isolate was then adjusted to the concentration of 10^3 conidia mL^{-1}. Brazil nuts were immersed for 10 min in the conidial suspension (Michel & Radcliffe, 1995). After inoculation, the samples remained at room temperature for seven days in such a way that the microorganisms proliferated.

Table 1
Kinetic models of decomposition and respective integrated and linearized equations with corresponding half-lives.

Order	Differential equation	Integrated and Linearized Equation	Half-life
0	$\frac{dC}{dt} = -k$	$C = C_0 - kt$	$t_{1/2} = \frac{C_0}{2k}$
1	$\frac{dC}{dt} = -kC$	$lnC = lnC_0 - kt$	$t_{1/2} = \frac{ln(2)}{k}$
2	$\frac{dC}{dt} = -kC^2$	$\frac{1}{C} = \frac{1}{C_0} + kt$	$t_{1/2} = \frac{1}{kC_0}$

2.4.2. Ozonation of Brazil nuts contaminated with Aspergillus flavus

Brazil nut samples of 100 g were inoculated and individually distributed in truss bags to be submitted to the ozonation process. Ozone concentrations equivalent to 2.42, 4.38, 8.88 and 13.24 mg L^{-1} were used for periods of 0, 60, 120, 180 and 240 min, with flow rate of 3.0 L min^{-1} and temperature of 25 °C.

2.4.3. Determination of the Aspergillus flavus count before and after ozonation

The *Aspergillus flavus* count in Brazil nuts after ozonation was determined by quantifying the fungi via plate dilution (Pitt & Hocking, 2009, p. 519). Brazil nut samples of 25 g were subjected to several serial dilutions in 0.1 g 100 g^{-1} peptone water. Then, the selected dilutions were plated in *Aspergillus flavus* and *parasiticus* Agar (AFPA) culture medium, and immediately incubated at 30 °C for 42 h. The results were expressed in log CFU g^{-1}.

2.4.4. Analysis of the structure of Aspergillus flavus before and after ozonation

The effect of ozone on the fungi structure was analyzed using a Leica DM2500 optical microscope (Leica Microsystems, Wetzlar, Germany), with a clear camera and coupled image capture system, and a 40x objective. To evaluate the effect of ozone on coloration of fungi colonies the Leica MDG33 stereomicroscope with incident light was used (Leica Microsystems, Wetzlar, Germany).

2.5. Qualitative analyzes of the ozonated Brazil nuts

Brazil nuts not inoculated with *Aspergillus flavus* but submitted to the same procedures used in the evaluation stage of the fungicidal effect of ozone were used. The samples were evaluated for moisture, coloration and qualitative variables of the raw oil.

2.5.1. Moisture content

To determine the moisture content of Brazil nuts, the oven method was used with forced air circulation, at a temperature of 103 ± 2 °C until reaching constant weight, according to ISO 665–2000 (UNECE, 2000).

2.5.2. Coloration

Color evaluation of the Brazil nuts was carried out in a Colorquest XE spectrophotometer (HunterLab, Reston, United States), obtaining values of the coordinates L (measurable in terms of white to black intensity), a (measurable in terms of intensity from red to green) and b (measurable in terms of yellow and blue intensity) of the Hunter system. From the coordinates L, a and b it was possible to obtain the hue angle h (Eq. (3)), color saturation or chroma C (Eq. (4)) and color difference △E (Eq. (5)) (Francis, 1975; Little, 1975; Maskan, 2001; Mclellan, Lind, & Kime, 1995).

$$h = arctang(b/a) \quad \text{(Eq. 3)}$$

$$C = \sqrt{(a^2 + b^2)} \quad \text{(Eq. 4)}$$

$$\Delta E = \sqrt{(L - L_0)^2 + (a - a_0)^2 + (b - b_0)^2} \quad \text{(Eq. 5)}$$

In which L_0, a_0 and b_0 are the coordinates obtained before ozonation of the nuts.

2.5.3. Qualitative analysis of the raw oil

Initially, total lipids were extracted using the method of Bligh and Dyer (1959). The extracted oil was evaluated with respect to the free fatty acids content, the peroxide index and the iodine index. A lipid profile of the oil extracted from the product submitted to ozonation was also determined.

The determination of free fatty acids (FFA, mg KOH g^{-1}) was performed according to AOCS (1993, p. 2v), based on the Ca 5a-40

method. Evaluation of the peroxide value was carried out according to AOCS (1993, p. 2v), method Cd 8–53, with results expressed in mEq kg^{-1}. The iodine index (II) was determined according to the AOCS (1993, p. 2v), Method Cd 1b-87 and expressed in g I_2 100 g^{-1}.

2.5.3.1. Determination of the lipid profile. To determine the lipid profile, raw oil extracted from non-ozonated (0 mg L^{-1}) and ozonated nuts at the concentrations of 2.42, 4.38, 8.88 and 13.24 mg L^{-1} for 240 min was analyzed. Initially the fatty acids were esterified from the oil extracted by the method of Bligh and Dyer (1959), according to Mendonça, Araújo, Borgo, and Araújo (2015).

The GC-2014ATF/SPL Gas Chromatograph (Shimadzu Corp., Japan) with AOC 20i autoinjector, flame ionization detector (FID-2014) and Rt-2560 chromatographic column (Restek, Bellefonte, PA, USA), measuring 100 m long, with 0.25 mm internal diameter and 0.20 μm film thickness, was used in the chromatographic analysis. The chromatographic conditions established were: Split type injection mode with injection temperature of 200 °C; the column heating ramp was programmed to start at 140 °C until reaching 200 °C. The detector temperature was 220 °C and helium was used as the carrier gas, with flow in the column of 1.2 mL min^{-1}. The chromatographic run was equivalent to 45 min. Oil samples were diluted in 2 mL hexane before injection. The volume injected was 1 μL. Identification of each fatty acid was performed by comparison with the retention time of the fatty acid standard Supelco 37 component FAME mix (Supelco®, USA). The results were expressed in g of each fatty acid, in relation to 100 g of the total fatty acids.

2.5.3.2. Statistical analysis. The experiment was performed in a Completely Randomized Design. In the first stage, in which the ozone decomposition kinetics was studied, four initial gas concentrations (2.42, 4.38, 8.88 and 13.24 mg L^{-1}) were evaluated, with three replicates. In the second stage, which evaluated the fungicidal effect of ozone and the effect on product quality, a 4 × 5 Factorial Scheme was used, with four gas concentrations (2.42, 4.38, 8.88 and 13.24 mg L^{-1}) and five ozonation periods (0, 60, 120, 180 and 240 min), with three replicates. An analysis of variance (ANOVA) was performed at 5% probability, followed by a regression analysis or Tukey's test. The software StatPlus v.5 (AnalystSoft Inc., Canada) was used to perform the analysis of variance; and the software SigmaPlot v.10 (Systat Software Inc, Germany) was used to obtain the regression equations and plot the graphs.

3. Results and discussion

3.1. Saturation of the porous medium

The regression curves referring to the ozone saturation process for Brazil nuts were determined (Table 2). The ozone concentration increased as the ozonation period increased for all gas concentrations. These results can be explained by the fact that ozone initially reacts with active sites on the product surface, resulting in rapid gas degradation and elimination of active sites (Strait, 1998; Kells, Mason, Maier, & Wolososhuk, 2001). Also according to these authors, after elimination of the active sites the rate of gas degradation in the porous medium decreases, and consequently the concentration in the porous medium tends to increase. The trend observed for Brazil nuts regarding the saturation concentration was similar to that observed for other products, such as popcorn, peanuts, rice and wheat flour (Alencar et al., 2011; Paes et al., 2017; Ravi, Venkatachalam, & Rajamani, 2015; Silva, Faroni, Sousa, Prates, & Abreu, 2019).

With regards to the saturation time values obtained from the equations contained in Table 2, an exponential reduction was observed as the initial gas concentration was increased (Fig. 2). For the initial ozone concentration of 2.42 mg L^{-1}, the saturation time was 40.3 min, whereas for 13.24 mg L^{-1} it was equivalent to 15.2 min. With regards to the saturation concentration, there was a linear increase with the increase of the initial gas concentration. An increase of 1.0 mg L^{-1} implies an increase in the saturation concentration of the porous medium containing Brazil nuts of approximately 0.53 mg L^{-1}.

It is important to know the ozone concentration in the porous medium, since this variable is fundamental for establishing conditions for the control of microorganisms and degradation of aflatoxins in Brazil nuts. The saturation concentrations obtained in the present study, in the range between 0.87 and 6.74 mg L^{-1}, are higher than the ozone concentration used by Kells et al. (2001) in corn grains, which was equivalent to 0.11 mg L^{-1}, sufficient to cause a 63% reduction in surface infection by the potentially aflatoxigenic species of *A. parasiticus* after three days of ozonation.

Table 2
Adjusted regression equations and respective coefficients of determination (R^2) for the residual ozone concentrations (mg L^{-1}) during the process of saturating the medium containing Brazil nuts with 4.40 g 100 g^{-1} (w.b.) moisture content in function of the exposure period, for ozone concentrations of 2.42, 4.38, 8.88 and 13.24 mg L^{-1} and flow rate of 3.0 L min^{-1}.

Initial gas concentrations (mg L^{-1})	Adjusted regression	R^2	SEE
2.42	$\hat{y} = \frac{1.183}{1+e^{-\left(\frac{x-20.127}{10.074}\right)}}$	0.96	0.0828
4.38	$\hat{y} = \frac{2.569}{1+e^{-\left(\frac{x-11.313}{8.284}\right)}}$	0.91	0.2560
8.88	$\hat{y} = \frac{5.497}{1+e^{-\left(\frac{x-10.120}{5.596}\right)}}$	0.95	0.4542
13.24	$\hat{y} = \frac{7.650}{1+e^{-\left(\frac{x-7.629}{3.801}\right)}}$	0.96	0.5201

SEE = Standard error of estimate.
n = 3 sample replicates.

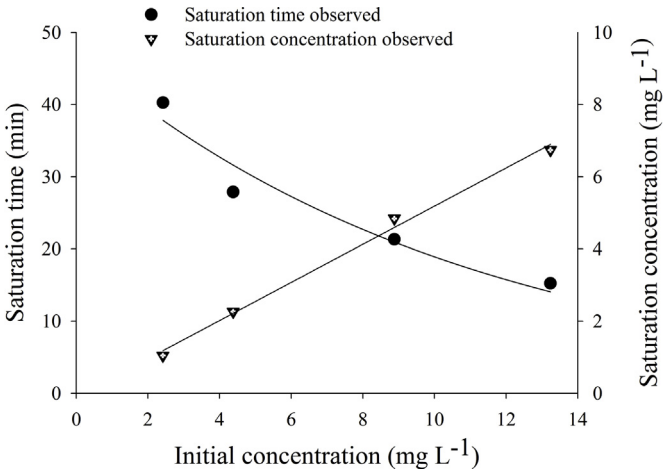

Fig. 2. Time (T_{Sat}) and concentration (C_{Sat}) of ozone saturation in the porous medium containing Brazil nuts with 4.40 g 100 g^{-1} (w.b.) moisture content, in function of the initial gas concentration.
$\hat{y}_{T_{Sat}} = 47.1839e^{-0.0915^{*}x}$ $R^2 = 0.93$ SEE = 3.2960
$\hat{y}_{C_{Sat}} = -0.097 + 0.528^{**}x$ $R^2 = 0.99$ SEE = 0.2326 * Significant ($p < 0.05$) ** Significant ($p < 0.01$) SEE – Standard error of estimate.

3.2. Ozone decomposition kinetics

The regression equations of the different kinetic models adjusted to the residual ozone concentrations in the porous medium as a function of the decomposition period, for the different gas concentrations, are shown in Table 3. It was verified that the first order kinetic model was that which presented the best fit to the residual ozone concentration data during the decomposition process, for all initial concentrations, according to the coefficient of determination (R^2). Other authors also

Table 3
Regression equations of the decomposition kinetic models adjusted to the data on residual ozone concentration in function of the decomposition period (x) in medium containing Brazil nuts with 4.40 g 100 g^{-1} (w.b.) moisture content.

Initial gas concentrations (mg L^{-1})	Order	Adjusted regression	R^2	SEE	Half-life (min)
2.42	0	$\hat{y} = 0.360 - 0.028^{**}x$	0.58	0.1505	6.43
	1	$\hat{y} = -0.298 - 0.175^{**}x$	0.92	0.2777	3.96
	2	$\hat{y} = -19.786 + 10.333^{*}x$	0.82	27.5098	1.91
4.38	0	$\hat{y} = 1.683 - 0.136^{**}x$	0.50	0.8481	6.20
	1	$\hat{y} = 0.333 - 0.171^{*}x$	0.89	0.3223	4.05
	2	$\hat{y} = -5.376 + 2.609^{*}x$	0.78	7.9065	2.06
8.88	0	$\hat{y} = 3.455 - 0.262^{**}x$	0.56	1.4699	6.59
	1	$\hat{y} = 0.906 - 0.165^{**}x$	0.86	0.4328	4.20
	2	$\hat{y} = -0.986 + 0.500^{*}x$	0.79	1.6715	1.97
13.24	0	$\hat{y} = 6.688 - 0.477^{**}x$	0.81	1.4565	7.01
	1	$\hat{y} = 1.340 - 0.151^{*}x$	0.89	0.3321	4.59
	2	$\hat{y} = -0.722 + 0.248^{*}x$	0.71	1.0227	2.91

SEE = Standard error of estimate.
*Significant ($p < 0.05$).
** Significant ($p < 0.01$).
n = 3 sample replicates.

observed a similar result in porous medium for rice, corn and wheat flour (Paes et al., 2017; Ravi et al., 2015; Santos et al., 2007).

It was possible to obtain the half-life in the porous medium (Table 3) using the coefficients of the kinetic models, according to Table 1. The decomposition rate constant remained in the range of −0.151 to −0.175 min^{-1} for the first-order kinetic model. It is emphasized that for the first-order kinetic model, half-life is independent of the initial concentration of the compound evaluated (Wright, 2004, p. 441). Thus, ozone half-life in the Brazil nuts with 4.40 g/100 g^{-1} moisture content at 25 °C, calculated from the values obtained for the different gas concentrations, was equivalent to 4.20 ± 0.28 min. The half-life of ozone in the porous medium composed of a mass of Brazil nuts was lower than the values obtained for peanuts, equivalent to 7.1 min (moisture content of 7.1 g 100g^{-1}/25 °C), corn equal to 5.57 min (moisture content of 12.8 g 100g^{-1}/25 °C) and rice equivalent to 13.8 min (moisture content of 11.4 g 100g^{-1}/31 °C) (Alencar et al., 2011; Ravi et al., 2015; Santos et al., 2007). One of the factors that probably favors the rapid decomposition of ozone when in contact with Brazilian nuts is the composition, which may present 15.0–17.0 g 100 g^{-1} proteins and 63–70 g 100 g^{-1} lipids (Chunhieng, Hafidi, Pioch, Brochier, & Didier, 2008). Meirelles (2015) observed a negative correlation between lipid content and half-life of ozone. Higher lipid content resulted shorter half-life of ozone in porous medium. In this context, according to Tiwari et al. (2010), the gas reacts with the chemical components present in the outer layer of the product. Thus, ozone moves through porous medium slowly.

3.3. Count of *Aspergillus flavus* after ozonation

The counts of *A. flavus* (log CFU g^{-1}) in ozonated Brazil nuts at concentrations of 2.42, 4.38, 8.88 and 13.24 mg L^{-1} for up to 240 min were evaluated (Fig. 3 and Table 4). There was a significant reduction ($p < 0.05$) in the microorganism counts and the most significant reduction was observed in the concentrations of 8.88 and 13.24 mg L^{-1}. When the concentrations of 2.42 and 4.38 mg L^{-1} were used, the estimated reductions in microorganism counts were 1.39 and 1.89 log cycles, respectively, after 240 min of ozonation (Table 4). However, when utilizing the concentrations of 8.88 and 13.24 mg L^{-1}, reductions were estimated to be greater than 2.80 log cycles after 60 min. For the period of 240 min the reduction in microorganism count was greater than 3.10 log cycles.

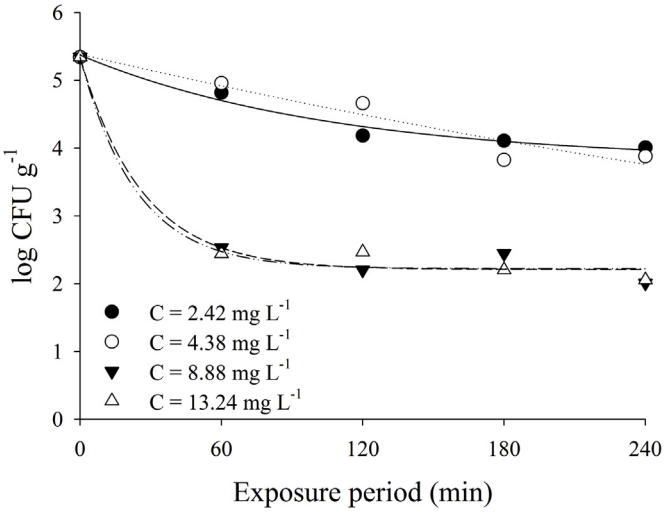

Fig. 3. Count of *Aspergillus flavus* (log CFU g^{-1}) in function of the exposure period in Brazil nuts ozonized at the concentrations of 2.42, 4.38, 8.88 and 13.24 mg L^{-1} and flow rate of 3.0 L min^{-1}.

Table 4
Regression equations and their respective coefficients of determination for determination of the counts of *Aspergillus flavus* (log CFU g^{-1}) in function of the exposure period (x) in Brazil nuts ozonized at concentrations of 2.42, 4.38, 8.88 and 13.24 mg L^{-1} and flow rate of 3.0 L min^{-1} and estimated counts.

Gas concentrations (mg L^{-1})	Adjusted regression	R^2	SEE
2.42	$\hat{y} = 3.805 + 1.567e^{(-0.009x)}$	0.97	0.1304
4.38	$\hat{y} = 0.431 + 4.956e^{(-0.002x)}$	0.93	0.2512
8.88	$\hat{y} = 2.209 + 3.135e^{(-0.038x)}$	0.99	0.2183
13.24	$\hat{y} = 2.223 + 3.119e^{(-0.042x)}$	0.98	0.1998

Estimated counts of *Aspergillus flavus* (log CFU g^{-1})

Gas concentrations (mg L^{-1})	Ozonation period (min)				
	0	60	120	180	240
2.42	5.37	4.72	4.34	4.12	3.99
4.38	5.39	4.83	4.33	3.89	3.50
8.88	5.34	2.53	2.24	2.21	2.21
13.24	5.34	2.47	2.24	2.22	2.22

SEE = Standard error of estimate.
n = 3 sample replicates.

In the counting of fungi present in the nuts submitted or not to the ozonation process, only colonies with orange coloration on the back of the Petri dish were considered, characteristic of the species *A. flavus* and *A. parasiticus* (Pitt & Hocking, 2009, p. 519). Thus, ozone was efficient in the inactivation of potentially aflatoxigenic microorganisms in Brazil nuts. Alencar et al. (2012) also observed a significant effect of ozone in the inactivation of *Aspergillus* spp., with a reduction of approximately 3.0 cycles in ozonized peanut grains when applied at the concentration of 21 mg L^{-1}, for 96 h.

Inactivation of microorganisms during ozonation occurs based on the oxidation of constituents of the cell wall and membrane, and also from elements of cellular content, while cell lysis may occur during ozone exposure. Among the compounds that can be oxidized by ozone are polyunsaturated fatty acids, polysaccharides, enzymes and nucleic materials such as thymine and cytosine (Ishizaki, K., Shinriki, N., Ikehata, A., & Ueda, 1981; Khadre et al., 2001). It is important to highlight that the molecular ozone and/or radicals generated during decomposition can act in the oxidation process of these cellular

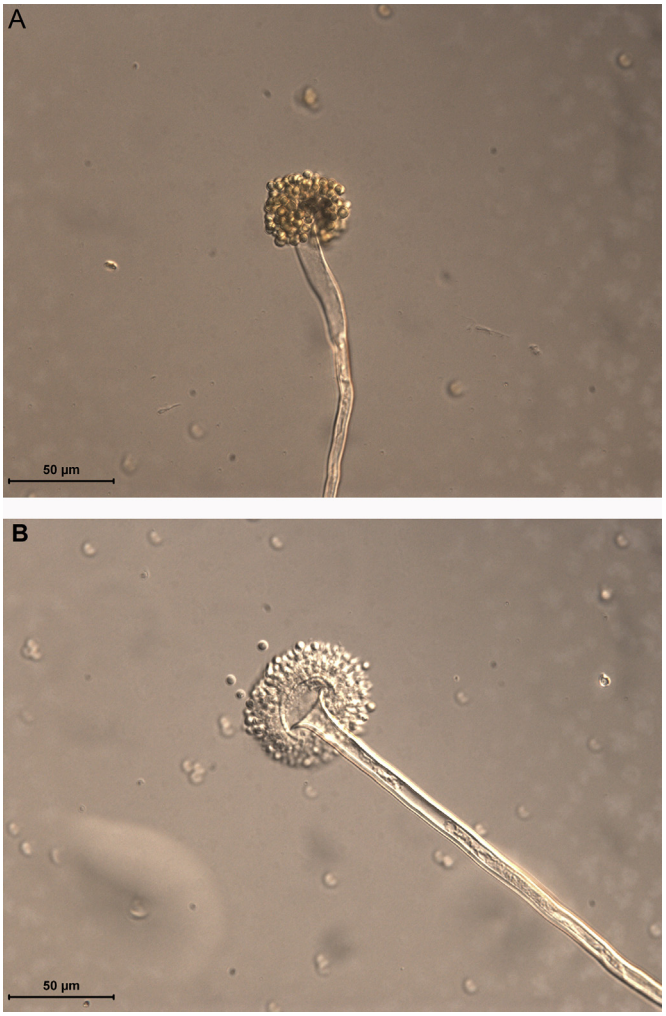

Fig. 4. Morphological aspects, obtained by optical microscopy with 40x objective, of *Aspergillus flavus* collected in Brazil nuts before (A) and after (B) exposure to ozone gas, at the concentration of 13.24 mg L^{-1}, for 240 min.

constituents (Pascual, Llorca, & Canut, 2007).

3.4. Morphological structure of Aspergillus flavus before and after ozonation

The morphological aspects obtained by optical microscopy of *A. flavus* collected in Brazil nut exposed to an ozone gas concentration of 13.24 mg L^{-1} for 240 min were evaluated (Fig. 4). Changes in the color of the colony were verified, certainly associated with oxidation of vital cell structures. The alteration in color is confirmed in Fig. 1A (Supplementary Material), in which it is possible to observe a change in coloration of the fungi colony after ozonization. In the non-ozonated nuts there was a greenish color characteristic of *A. flavus* colonies. However, in ozonized nuts the color of the fungi colonies suffered a depigmentation with expressive bleaching. Other authors also observed alterations in the coloration of fungi colony species during ozonation. Zotti, Porro, Vizzini, and Mariotti (2008) attributed the alteration in color of *A. niger* colonies to oxidation of the pigment melanin and of *A. flavus* to the oxidation of anthraquinone, which according to Shier, Lao, Steele, and Abbas (2005) is an intermediate pigment in the synthesis of aflatoxins. Alencar et al. (2012) also observed changes in color of *Aspergillus* spp. when peanuts were ozonized at the concentration of 21 mg L^{-1}, for 96 h.

In addition to the changes in the color of the colony, there were changes in the components of the filamentous structure of the microorganism, such as conidiophore, vesicle and conidia, as a result of ozone oxidation. It is noteworthy that the oxidation of the morphological structure after ozonation justifies the reduction in *A. flavus* count, as shown in Fig. 3 and Table 4.

Table 5
Regression equations for moisture content (g 100 g^{-1}) in function of the exposure period (x) of Brazil nuts ozonized at concentrations of 2.42, 4.38, 8.88 and 13.24 mg L^{-1} and flow rate of 3.0 L min^{-1} and estimated values before ozonation (0 min) and after 240 min.

Gas concentrations (mg L^{-1})	Adjusted regression	R^2	SEE	Estimated values (g $100g^{-1}$)	
				Ozonation period (min)	
				0	240
2.42	$\hat{y} = 4.016$	–	–	4.02	4.02
4.38	$\hat{y} = 4.515$	–	–	4.52	4.52
8.88	$\hat{y} = 4.314 - 0.005^{*}x$	0.82	0.1958	4.31	3.54
13.24	$\hat{y} = 4.197$	–	–	4.20	4.20

SEE = Standard error of estimate.
*Significant ($p < 0.05$).
n = 3 sample replicates.

3.5. Quality of the ozonated Brazil nuts

Regarding the moisture content of the nuts, a significant reduction ($p < 0.05$) was observed only for the concentration of 8.88 mg L^{-1}. Before ozonation, the estimated value of moisture content of the nuts was 4.31 g 100 g^{-1} and after 240 min was equivalent to 3.54 g 100 g^{-1} (Table 5). This fact can be attributed to the relative humidity of the ozone-containing gas mixture that passed through the product, which was reduced during the oxygen concentration process before gas generation. Thus, it was observed that the reduction in moisture content of the nuts is not simply due to the action of the gas, but to the predominant effect of the low relative humidity during ozonation. It should be noted that the composition of Brazil nuts, with a lipid content of around 66.0 g 100 g^{-1} (Freitas & Naves, 2010), favors water loss during ozonation. A similar result was obtained by Alencar et al. (2011) in peanut grains ozonized at concentrations of 13 and 21 mg L^{-1} for up to 96 h.

Regarding the effect of the process on product color, the variables hue angle (h) and color difference (ΔE) varied significantly ($p < 0.05$) due to the ozonation period, regardless of gas concentration (Fig. 5). The hue angle (Fig. 5A) and color difference (Fig. 5B) showed a tendency to increase as the ozonation period increased. However, the color saturation or chroma did not show significant variation during 240 min of ozonation, with a mean value of 14.52 ± 0.71. There was also significant variation ($p < 0.05$) in the color difference when the effect of the initial gas concentration alone was analyzed (Fig. 6). The mean value of color difference in the nuts exposed to a concentration of 8.88 mg L^{-1} differed from those obtained when utilizing concentrations of 2.42 and 4.38 mg L^{-1}. These changes over the ozonation period can be attributed to the oxidation of pigments of the dark brown skin that surrounds the nuts, in a manner similar to that observed in peanut grains by Alencar et al. (2011) and by Sanchez et al. (2016).

There were no significant variations due to the interaction between ozone concentration and exposure period to the gas, as well as these factors alone, when the free fatty acids, the peroxide index and the iodine index of the raw oil were analyzed, according to ANOVA ($p > 0.05$) (Table 1A of Supplementary Material). The free fatty acids remained in the range of 0.66 ± 0.02 to 0.91 ± 0.13 mg KOH g^{-1}, below the limit established by the *Codex Alimentarius* for raw oil which is 4.00 mg KOH g^{-1} (FAO, 1999). Regarding the peroxide index, the mean value was 16.30 ± 2.57 mEq kg^{-1}, which is higher than the

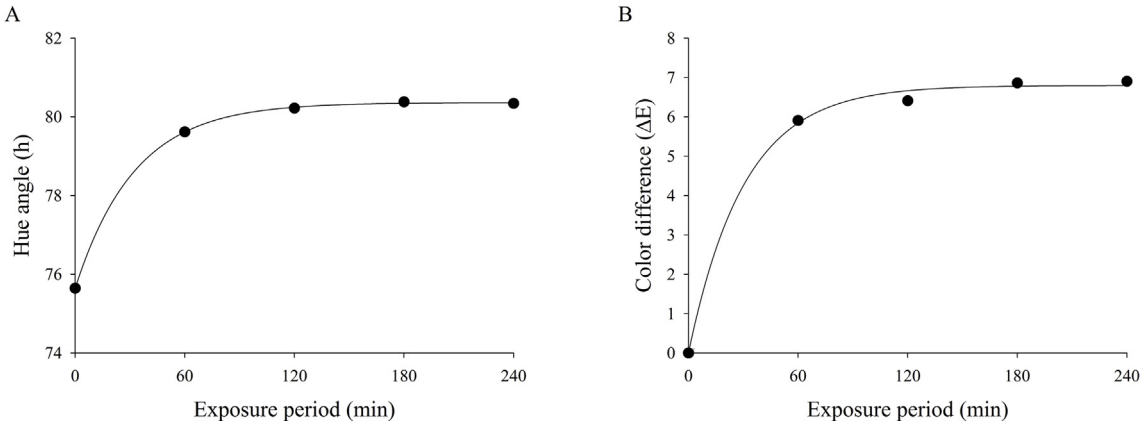

Fig. 5. Hue angle (h) and color different (ΔE) in Brazil nuts ozonized for up to 240 min.
$\hat{y}_h = 75.644 + 4.717(1 - e^{(-0.031x)})$ $R^2 = 0.99$ SEE = 0.0339 $\hat{y}_{\Delta E} = 6.796(1 - e^{(-0.036x)})$ $R^2 = 0.99$ SEE = 0.1704 SEE = Standard error of estimate.

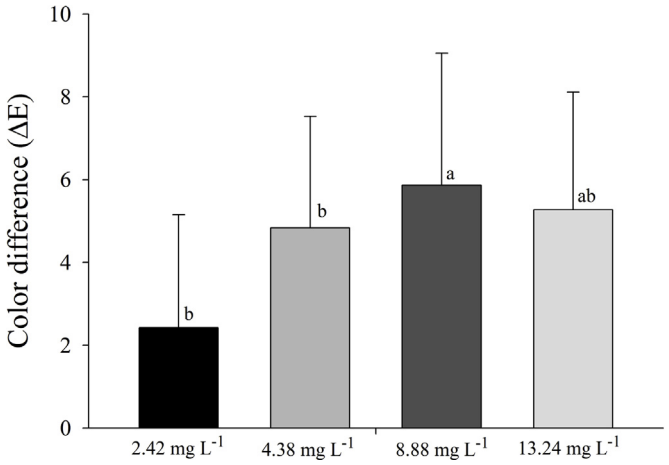

Fig. 6. Color difference (ΔE) of Brazil nuts ozonized at the concentrations of 2.42, 4.38, 8.88 and 13.24 mg L^{-1} and flow rate of 3.0 L min^{-1}.
Mean values followed by the same letter do not statistically differ by the Tukey's test at 5% probability.

limit established by the *Codex Alimentarius* of 15 mEq kg^{-1}. This result can be attributed to the initial quality of the raw material, and therefore should not be attributed to the ozonation process under the conditions adopted in the present work. With regards to the iodine index, values between 96.78 ± 0.65 and 105.75 ± 2.71 gI_2 100 g^{-1} were obtained. These values are similar to that obtained by Muniz et al. (2015) for Brazil nut oil of 95.00 gI_2 100 g^{-1}. Results related to the raw oil quality are in agreement with those observed by other authors for ozonated maize and peanuts (Alencar et al., 2011; Chen et al., 2014; Faroni, Pereira, Sousa, Silva, & Urrichi, 2007). On the other hand, Sanchez et al. (2016) observed an increase in the peroxide index of raw oil extracted from ozonized peanut grains at 6.41 mg L^{-1} for 12 h. However, these authors obtained values lower than 10 mEq kg^{-1}.

Regarding the lipid profile of the raw oil, there was no significant difference according to ANOVA ($p > 0.05$) when the effect of different ozone concentrations per 240 min period was analyzed (Table 6). It was possible to quantify, independent of ozonation, the palmitic (C16:0), stearic (C18:0), oleic (C18:1) and linoleic (C18:2) fatty acids. For raw oil extracted from non-ozonated Brazil nuts, mean values equivalent to 14.40 ± 0.13, 11.18 ± 0.06, 23.85 ± 0.12 and 50.55 ± 0.24 g 100 g^{-1} were obtained for the C16:0, C18:0, C18:1 and C18:2 fatty acids, respectively. When the ozone concentration of 13.24 mg L^{-1} was used for 240 min, mean values of 14.49 ± 0.49, 11.71 ± 0.09, 24.01 ± 0.30 and 49.79 ± 0.88 g 100 g^{-1} were obtained for the C16:0, C18:0, C18:1 and C18:2 fatty acids, respectively. Fig. 7 shows the chromatograms related to the lipid profile of the raw oil obtained from non-ozonated (A) and ozonized (B) Brazil nuts at the concentration of 13.24 mg L^{-1}, for 240 min.

Thus, although ozone presented high oxidative potential, ozonation was not able to cause oxidation in such a way as to alter the lipid profile of the oil. This result is extremely important for maintaining the quality of Brazil nuts and the fact that Brazil nut oil is predominantly composed of unsaturated fatty acids (Freitas & Naves, 2010). It is noteworthy that unsaturated fatty acids are susceptible to oxidation, and in this process there is an alteration in odor and flavor (Matz, 1992, p. 864). Mendez, Maier, Mason, and Woloshuk (2003) also observed no significant alteration in the lipid profile of raw oil extracted from corn, soybeans and wheat ozonized at a concentration of 50 ppm for 30 days.

Future studies with the purpose of to evaluating the effect of ozonation on other compounds present in Brazil nuts, such as tocopherols and phenolic compounds, in the formation of volatile aroma compounds and in the sensory analysis of the product, are fundamental to complement the information obtained in the present work.

4. Conclusions

From the obtained results it is possible to conclude that the initial ozone concentration influences the saturation time and the saturation concentration in porous medium composed of a mass of Brazil nuts. Ozone was shown to be efficient in the inactivation of *A. flavus* in Brazil nuts, able to cause a reduction of more than 3.1 log cycles when adopting the concentration of 8.88 mg L^{-1} and the exposure period of 240 min. Thus, ozonation is an alternative technique for the inactivation of *A. flavus*, and can therefore act to prevent the synthesis of aflatoxins. In general, under the conditions adopted in the present study ozone does not affect the quality of the raw oil, including the lipid profile.

CRediT authorship contribution statement

Juliana Martins de Oliveira: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Writing - review & editing. **Ernandes Rodrigues de Alencar:** Supervision, Conceptualization, Methodology, Formal analysis, Validation, Writing - original draft, Writing - review & editing. **Luiz Eduardo Bassay Blum:** Conceptualization, Methodology, Investigation, Formal analysis. **Wallas Felippe de Souza Ferreira:** Investigation, Formal analysis, Writing - review & editing. **Silvia de Carvalho Campos Botelho:** Investigation, Formal analysis. **Aline Mondini Calil Racanicci:** Investigation. **Eliana dos Santos Leandro:** Formal analysis, Writing - original draft. **Marcio Antônio Mendonça:** Investigation. **Eder Stolben Moscon:** Investigation. **Lincoln Vicente Araújo dos Santos**

Table 6
Lipid profile of raw oil obtained from non-ozonated Brazil nut (0.00 mg L^{-1}) and exposed to ozone at concentrations of 2.42, 4.38, 8.88 and 13.24 mg L^{-1} and flow rate of 3.0 L min^{-1} for 240 min.

Gas concentrations (mg L^{-1})	Fatty acids (g $100g^{-1}$)			
	C16:0^{ns}	C18:0^{ns}	C18:1^{ns}	C18:2^{ns}
	Mean ± SD			
Non-ozonated (0.00)	14.40 ± 0.13	11.18 ± 0.06	23.85 ± 0.12	50.55 ± 0.24
2.42	14.43 ± 0.03	11.02 ± 0.27	23.83 ± 0.08	50.72 ± 0.21
4.38	14.40 ± 0.47	11.30 ± 0.59	23.75 ± 0.29	50.56 ± 0.26
8.88	14.14 ± 0.10	11.20 ± 0.02	24.28 ± 0.24	49.98 ± 0.24
13.24	14.49 ± 0.49	11.71 ± 0.09	24.01 ± 0.30	49.79 ± 0.88

C16:0 –Palmitic acid; C18:0 –Stearic acid; C18:1 - Oleic acid; C18:2 - Linoleic acid.
^{ns} No significant difference according to ANOVA ($p > 0.05$).
SD - Standard Deviation.
n = 3 sample replicates.

Bizerra: Investigation, Formal analysis. **Caroline Rosa da Silva:** Investigation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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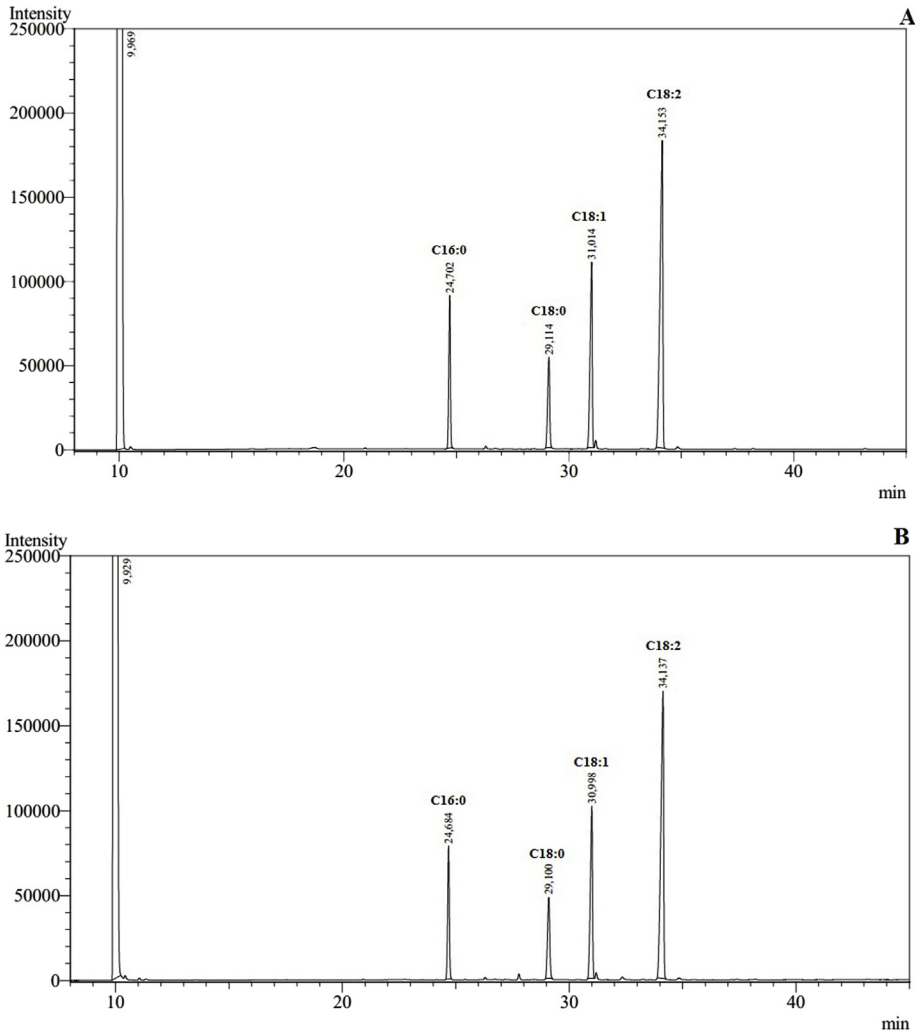

Fig. 7. Chromatograms of the lipid profile of raw oil obtained from non-ozonized (A) and ozonized (B) Brazil nuts at the concentration of 13.24 mg L^{-1} and flow rate of 3.0 L min^{-1}, for 240 min.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2020.109106.

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