University of São Paulo
"Luiz de Queiroz" College of Agriculture

Chemical changes in Brazil nuts and co-products: characterisation and strategies of control and monitoring

Alan Giovanini de Oliveira Sartori

Thesis presented to obtain the degree of Doctor in Science. Area: Food Science and Technology

Piracicaba
2017

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Bachelor of Food Science

Chemical changes in Brazil nuts and co-products: characterisation and strategies of control and monitoring

versão revisada de acordo com a resolução CoPGr 6018 de 2011

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RESUMO

Alterações químicas em castanha do Brasil e coprodutos: caracterização e estratégias de controle e monitoramento

A castanha do Brasil (*Bertholletia excelsa*, H.B.K.) é uma semente de boa qualidade nutricional coletada em florestas tropicais da América do Sul, cuja cadeia produtiva é uma das mais importantes atividades econômicas não madeireiras da Amazônia brasileira. Os principais objetivos desta pesquisa foram: 1) caracterizar a ocorrência de alterações químicas em castanhas do Brasil (CB), óleo de castanha do Brasil obtido por prensagem a frio (OCB) e farinha de castanha do Brasil obtida por extração aquosa (FCB); e 2) investigar estratégias para controlar e monitorar essas alterações ao longo do armazenamento. Para isso, técnicas consolidadas como a espectrofotometria e a cromatografia, e uma técnica relativamente recente, a espectroscopia de ressonância de spin eletrônico (RSE), foram empregadas. Dentre os principais resultados obtidos, foi possível constatar o efeito de diferentes combinações de temperaturas e atmosferas de embalagem sobre a tendência de formação de radicais e sobre a geração de compostos voláteis de aroma relacionados a odor indesejável em CB, e que a temperatura de refrigeração combinada com a embalagem a vácuo foi a mais eficiente na preservação da qualidade da CB. Demonstrou-se que o uso de um método de aprisionamento de spins de RSE pode ser eficiente para monitorar alterações químicas em OCB com histórico conhecido embalado em frascos de vidro transparente ou marrons sob condições de armazenamento comercial. Para FCB, foi demonstrado que pequenas variações na atividade de água (aw) podem afetar significativamente as taxas de oxidação lipídica e de reações de escurecimento não enzimático durante armazenamento. Obteve-se indicação de que para FCB com aw inicial de 0,196, mas não para FCB com aw inicial de 0,101, produtos secundários da oxidação lipídica podem ser substratos para a formação de produtos do escurecimento não enzimático. Como conclusão geral, os resultados obtidos podem ajudar a explicar melhor os processos de deterioração química em CB e seus coprodutos, conforme as condições de armazenamento, e que o uso de um método que requer menor quantidade de amostras, é rápido e não usa solventes é viável para o monitoramento da qualidade de OCB.

Palavras-chave: *Bertholletia excelsa*; Óleo de castanha do Brasil; Farinha de castanha do Brasil; Oxidação lipídica; Espectroscopia de ressonância de spin eletrônico; Vida útil

ABSTRACT

Chemical changes in Brazil nuts and co-products: characterization and strategies of control and monitoring

Brazil nuts (*Bertholletia excelsa*, H.B.K.) are seeds of high nutritional value collected from South American rainforests and its productive chain is one of the most important non-timber economic activities in Brazilian amazon. The main objectives of this research were: 1) characterize the occurrence of chemical changes in Brazil nut kernels (BNK), cold-pressed Brazil nut oil (BNO) and Brazil nut flour obtained by water extraction (BNF); and 2) investigate strategies of control and monitoring these changes during storage. For this, consolidated techniques, such as spectrophotometry and chromatography, and a relatively new analytical technique, the electron spin resonance (ESR) spectroscopy, were employed. As major results, it was found that different combinations of storage temperatures and atmosphere packages have differently affected the tendency of radical formation and off-flavor volatile aroma compounds generation in BNK, and that the combination of refrigeration with vacuum packing was able to keep BNK at their best. It was demonstrated that a spin-trapping ESR spectroscopy method would be suitable to monitor oxidative changes in BNO with known history stored either in clear or in brown glass bottles under retail conditions. For BNF, it was demonstrated that minor variations on water activity (a_w) might significantly affect the rates of both lipid oxidation and nonenzymatic browning reactions during storage. There was indication that for BNF with initial a_w of 0.196, but not for BNF with initial a_w of 0.101, under the studied conditions, secondary products from lipid oxidation might be substrates for nonenzymatic browning products formation. As a conclusion, these results may help to better understand chemical deteriorative processes in BNK and its co-products, according to the storage conditions, and that the use of less sample-demanding, fast and solvent-free analytical method to monitor these changes in BNO is feasible.

Keywords: *Bertholletia excels*; Brazil nut oil; Brazil nut flour; Lipid oxidation; Electron spin resonance spectroscopy; Shelf life

1. INTRODUCTION

Brazil nut is the seed of *Bertholletia excelsa*, H.B.K. (family of *Lecythidaceae*) tree, which grows naturally in rainforests from South America, and its productive chain is environmentally sustainable, since the seeds are collected as they fall out of the trees and no deforestation is needed. Harvesting and processing is an income source for local and traditional communities and the commercialization of in shell and shelled Brazil nut kernels (BNK) is one of the most important non-timber economic activities in the Brazilian Amazon. Although the commercialized amount has increased over the past years, it has potential to grow, when compared with other nuts, such as walnuts, cashew nuts and hazelnuts.

Worldwide consumer interest in Brazil nut has increased since BNK is the greatest food source of selenium. These seeds also contain high amounts of n-6 and n-9 fatty acids, vitamin E, magnesium, phosphorus, zinc, manganese, sulphur-containing amino acids and phytosterols. One way to add value and to increase Brazil nut market is by trading co-products. These co-products may be prepared from whole or even broken BNK, which are sold at a lower price. Some Brazil nut co-products available in the market are the cold-pressed Brazil nut oil (BNO), the water-soluble extract known as Brazil nut milk, and different kinds of Brazil nut flours, which are used for culinary purposes. One kind of Brazil nut flour is obtained from the solid residue of water extraction preparation of Brazil nut milk, which can be finely ground and dried (BNF).

One shortcoming for BNK commercialization is the high fat content of 60-70%, which is mainly composed of unsaturated fatty acids that can oxidize easily and then reduce its shelf life. Despite the growing market importance and nutritional relevance, there are few studies focused on the effect of postharvest/post-processing practices on oxidative changes. Hence, this PhD thesis is the continuation of a research line of the laboratory of Oils and Fats from "Luiz de Queiroz" College of Agriculture, University of São Paulo, focused on the characterization of chemical changes and on the study of ways of control and monitoring these changes in BNK and its co-products during storage. In addition, this research was enriched by the joint work with the Faculty of Science, University of Copenhagen.

The present document is divided into three chapters. For the first chapter our hypothesis was that different storage conditions might affect the tendency of lipid radical formation and the formation of off-flavor volatile aroma compounds (VACs) in shelled BNK. In the second chapter, the focus was to investigate the formation of off-flavor VACs and tocopherol analogs in BNO stored under retail condition, as well as the feasibility of a less sample-demanding, fast and solvent-free spin-trapping electron spin resonance spectroscopy method to monitor these chemical changes. For the third chapter our hypothesis was that minor variations on a_w would not significantly affect the rates of both lipid oxidation and nonenzymatic browning in BNF.

2. MILD STORAGE CONDITIONS AFFECT TENDENCY OF LIPID RADICAL FORMATION AND VOLATILES IN BRAZIL NUTS (*BERTHOLLETIA EXCELSA*)

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Abstract

Brazil nut is an important product from the Amazonian region and its productive chain is an income source for local communities. The effect of combinations of packaging atmospheres (loose or vacuum-packed) and storage temperatures (4±1 ºC or 24±2 ºC) on the tendency of lipid radical formation and on off-flavor volatiles was investigated in shelled Brazil nuts kernels for up to four months. It was observed that refrigeration, but not vacuum packing, was effective to control the tendency of lipid radical formation, as detected by spin-trapping electron spin resonance (ESR) spectroscopy, as well as of peroxides and conjugated dienes. The formation of off-flavor volatiles, as detected by HS-SPME-GC-MS, was also affected by storage conditions, and refrigeration was efficient to reduce 3-octen-2-one. However, the combination of refrigeration with vacuum packing, even using LDPE pouches with high OTR, reduced the formation of hexanal, which is a major off-flavor volatile, and thus should be recommended for storage of Brazil nut kernels for the studied period.

Keywords: ESR; Free radicals; Lipid oxidation; Spin trapping

2.1. Introduction

Brazil nuts are the seeds of *Bertholletia excelsa* Humb. & Bonpl. tree (family of *Lecythidaceae*), which is from the Amazonian region. In Brazil, the majority of commercialized seeds is collected as they fall out of the trees in natural rainforests and no deforestation is needed (FAO, 2013). Global production has doubled in 25 years, from 49,740 tons in 1989 to 109,300 tons in 2014, and Brazil was the second largest producer, with an output of 39,000 ton in 2014 (FAO, 2017).

Regarding biochemical composition, Brazil nut kernel is the greatest food source of selenium, which plays a key role as cofactor for antioxidant glutathione peroxidase (Rotruck et al., 1973). Selenium also has antiviral effects, may be essential for human reproduction, and may reduce risk of autoimmune thyroid disease (Rayman, 2012). Brazil nuts contain high contents of n-6 and n-9 fatty acids, vitamin E, magnesium, phosphorus, zinc, manganese, and

sulphur-containing amino acids (USDA, 2015), besides bioactive compounds, such as phytoesterols (da Costa et al., 2010).

As Brazil nut kernels have high lipid content (60-70%) of which around 40% is linoleic acid (USDA, 2015), they tend to oxidize easily (Vieira and Regitano-d'Arce, 1999). Therefore, postharvest practices regarding storage, such as air removal and refrigeration determinately influence the shelf life of the kernels by retarding the formation of hydroperoxides, which are decomposed to off-flavor volatiles. Despite the growing market importance and nutritional relevance, few studies have investigated the effect of storage on oxidative changes in Brazil nuts (Ribeiro et al., 1993a, Ribeiro et al., 1993b, Ribeiro et al., 1995), and there were not found studies reporting the tendency of lipid radical formation and onvolatiles in these nuts. In addition, the tendency of formation of lipid free radicals, which are precursors for hydroperoxides, can be monitored by electron spin resonance (ESR) spectroscopy, which is a sensitive and solvent-free method (Andersen and Skibsted, 2002). For that, a spin trap compound capable of complexing with short-lived free radicals to form long-lived spin adducts that are detected by ESR is used (Velasco et al., 2004). ESR spectroscopy has been used to detect radical species in several dried foods, but not in nuts (Andersen and Skibsted, 2018).

Therefore, the objective of the present study was to investigate for the first time the effect of retail storage conditions on the tendency of lipid radical formation and on volatiles in shelled Brazil nut kernels.

2.2. Materials and Methods

2.2.1. Materials

One metalized vacuum-packed bag of 20 kg of fresh and shelled Brazil nut kernels of small size (at least 68 kernels in 453 g) was purchased from a local market. After thorough mixing, portions of kernels (300 g) were placed into low-density polyethylene (LDPE) pouches, which are commonly used as packaging material, vacuum or loose, for nuts in Brazil, with an estimated oxygen transmission rate (OTR) of 9843 $cm^3/m^2/24h$ (at 23 ºC and 0% relative humidity). Kernels were vacuum or loose-packed on a sealing machine (model 300 B, Selovac, São Paulo, Brazil) and stored in the dark at ambient conditions (24±2 ºC) or under refrigeration (4±1 ºC). Therefore, four treatments were evaluated:

Ambient temperature, loose packing (treatment AL)

Refrigerated temperature, loose packing (treatment RL)

Ambient temperature, vacuum packing (treatment AV)

Refrigerated temperature, vacuum packing (treatment RV)

Temperature was monitored using a thermo hygrometer (Incoterm, Porto Alegre, Brazil), and effectiveness of sealing was checked by visually evaluating the formation of air bubbles when submerging an extra pouch (not used in the study) in water after sealing each three pouches. Every 30 days and during four months, samples were collected and 200 g were chopped in a domestic mixer and stored under vacuum in LDPE pouches, while 100 g were cold pressed with a hydraulic press (Carver, Wabash, USA) under up to 172 MPa and the obtained oil was filtered and stored in eppendorf flasks. The chopped kernels and the cold pressed oils were kept at –18 ºC in the dark until analysis. All chemicals were of analytical grade or higher as required.

2.2.2. Fatty acid profile

Aiming at characterization, composition of major fatty acids in the cold pressed oil was assessed on time zero using a GC-2010 Plus gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector (FID) and separation was achieved on a Rtx-Wax capillary column (30 m x 0.32 mm, 0.25 μm) (Restek, Bellefonte, USA). Nitrogen was the carrier gas at a flow rate of 1.2 mL/min, and injected sample volume was 1 μL (split 1:20). Initial column oven temperature (60 ºC) was raised to 210 ºC at 20 ºC/min rate and hold for 7 min, and then raised to 240 ºC at 30 ºC/min rate and hold for 12 min. Both injector and FID temperatures were set at 250 ºC. Samples were prepared as described by Hartman and Lago (1973). Methyl esters were identified using peak retention times of the standard FAME mix GLC-87 (Nu-Chek, Elysian, USA) as reference, and quantified by area normalization using methyl tridecanoate (T0627, Sigma-Aldrich, St. Louis, USA) as internal standard.

2.2.3. Tendency of lipid radical formation

The spin-trapping ESR method was based on procedures described by Thomsen et al. (2000), with modifications. Cold-pressed oil (1 g) was gently swirled with 1 mg of N-tert-butyl-α-phenylnitrone (PBN) (80126, Sigma-Aldrich, St. Louis, USA) in brown eppendorf flasks and kept submerged into thermostatted water bath at 70 ºC. After 5h, 50 μL were transferred to capillary micropipettes (Blaubrand, Wertheim, Germany) and measured on MiniScope MS200 ESR spectrometer (Magnettech, Berlin, Germany). Acquisition parameters were center field 3336.90 G; sweep width of 66.42 G; sweep time of 30 s; and modulation amplitude of 1 G. Each measurement was performed as average of six sweeps. Results were expressed as the height of the first peak in the spectra after 5h of incubation, since peak height is related to spin adducts concentration and can be used to compare similar samples (Thomsen, Kristensen and Skibsted, 2000).

2.2.4. Primary lipid oxidation products

Peroxide value (PV) was measured according to the method described by Shanta and Decker (1994), and specific absorption at 232 nm (K_{232}) was determined according to standard method ISO 3656:2011. For both analyses, a Shimadzu UV 1203 spectrophotometer (Kyoto, Japan) was used.

2.2.5. Volatile aroma compounds (VACs)

Procedures to recover and measure VACs formation were based on a method described by de Camargo et al. (2016). To VACs recovery, 2 g of cold pressed oil were added in a 20 mL vial that was flushed with nitrogen for 10 s and immediately capped with a gas-tight aluminum lid with silicone septum. The vial was kept submerged into a thermostatted water bath at 80 ºC under magnetic stirring for 20 min. Then, a solid-phase microextraction (SPME) assembly composed of a divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS, 50/30 μm) fiber (Supelco, Bellefonte, USA) was inserted through the septum and exposed to the headspace for 10 min. This fiber

was inserted into the injector of a GC-2010 gas chromatograph (Shimadzu, Kyoto, Japan) coupled to a mass spectrometer QP 2010 Plus (Shimadzu, Kyoto, Japan) and separation was achieved on a Rtx-5MS capillary column (30 m x 0.25 mm, 0.25 μm) (Restek, Bellefonte, USA). Helium was used as carrier gas at a flow rate of 1 mL/min, and injected sample volume was 1 μL (splitless mode). Initial column oven temperature 35 ºC was held for 3 min, raised to 60 ºC (5 ºC/min) and hold for 3 min, then raised to 200 ºC (8 ºC/min) and hold for 10 min, and then raised to 280 ºC (20 ºC/min) and hold for 5 min. Injector and ion source temperatures were set to 270 ºC and 200 ºC, respectively. Spectral data were obtained over a mass range from 20 to 300 m/z. Every day a blank (a vial without sample submitted under exactly the same conditions) was run before starting analyses. Peaks were tentatively identified by both mass spectra and linear retention index (LRI). Mass spectra were matched with those from Wiley Library (Version 8), considering 85% similarity as cut off. LRI was calculated relatively to standard *n*-alkane series and compared with literature data (Elmore et al., 2000; Gocmen et al., 2004; Ventanas et al., 2007; Xie et al., 2008; Wang et al., 2009; Babushok et al., 2011; Georgiadou et al., 2015), considering differences of 2% as cut off. Hexanal and trans-2-heptenal identities were confirmed by spiking Brazil nut oil samples with reference standards (codes 115606 and 90244, respectively, both from Sigma-Aldrich, St. Louis, USA). Relative amounts were represented in terms of peak area.

2.2.6. Sensory analysis

A panel composed of seven assessors evaluated the samples of chopped kernels. Criteria to choose assessors were to be familiar with sensory analysis, Brazil nuts and rancid odor in foods. A type I incomplete Latin square (t = 21; k = 5; r = 5; b = 21; λ = 1, E = 0.84) design was used (Cochran and Cox, 1964), considering 16 samples (4 treatments x 4 months) + 5 time zero samples (control). Each assessor analyzed three random blocks with five samples each. Rancid odor was the attribute evaluated using a 10-point scale (from 1 to 10), with assessors having access to reference samples of fresh (point 1 of the scale) and highly rancid (point 10 of the scale) kernels. Assessments were conducted in individual booths with monochromatic red light in order to minimize sample color effect. The protocol for this study was previously approved by the Committee of Ethics in Research of the Luiz de Queiroz College of Agriculture (Process n. 47619515.2.0000.5395).

2.2.7. Statistical analysis

Normality (Ryan-Joyner's test) and homoscedasticity (Bartlett's test) were checked. Mean values were evaluated by analysis of variance (one-way ANOVA), and, in case of differences, Tukey's test was used. Pearson correlation was tested between PV, K_{232} and ESR analysis. The level of confidence of 0.05 was considered and all statistical analyses were determined using Minitab® 17 software (Minitab Inc., State College, USA), while figure were generated using Statistica® 64 (StatSoft, Tulsa, USA).

2.3. Results and Discussion

2.3.1. Fatty acid profile

The composition of major fatty acids was determined on time zero (Table 1). The cold pressed oil was majorly composed of linoleic acid (~39%), oleic acid (~32%), palmitic acid (~16%) and stearic acid (~11%), which is in agreement with literature data (USDA, 2015). The high content of linoleic acid indicates that the lipid fraction of the kernels are susceptible to be oxidized, since this fatty acid contains one bisallylic hydrogen atom prone to be lost in order to form an alkyl radical and then start fatty acid deterioration (Choe and Min, 2006).

Tabela 1. Composition of major fatty acids in shelled Brazil nuts

Fatty acid	Time zero
Palmitic (C16:0)	15.91 ± 0.03
Palmitoleic (C16:1)	0.37 ± 0.00
Stearic (C18:0)	11.30 ± 0.01
Oleic (C18:1)	32.83 ± 0.02
Linoleic (C18:2)	39.35 ± 0.00
Arachidic (C20:0)	0.25 ± 0.00
$\sum_{SFA}$	27.45 ± 0.02
$\sum_{MUFA}$	33.20 ± 0.02
$\sum_{PUFA}$	39.35 ± 0.00

Results expressed as mean ± standard deviation (n = 2) of percent mass of total fatty acid mass. *SFA* Total saturated fatty acids, *MUFA* Total monounsaturated fatty acids, *PUFA* Total polyunsaturated fatty acids.

2.3.2. Tendency of lipid radical formation

To the best of our knowledge, this was the first time ESR spectroscopy was used to measure radical species in Brazil nut kernels and in nuts in general (Andersen and Skibsted, 2018). Firstly, non-destructive direct measurement in ESR spectrometer was tested using chopped kernels placed in ESR tubes with 4 mm inner diameter (Wilmad Glass Company, Buena, USA), with notes taken for height and weight to measure density. Nevertheless, low and similar peaks were observed in the ESR spectra (data not shown), which may be due to the exposure of the short-lived radical species to oxygen during chopping, therefore hindering detection by ESR (Andersen and Skibsted, 2002). Thus, the ESR technique using PBN as the spin trap was employed.

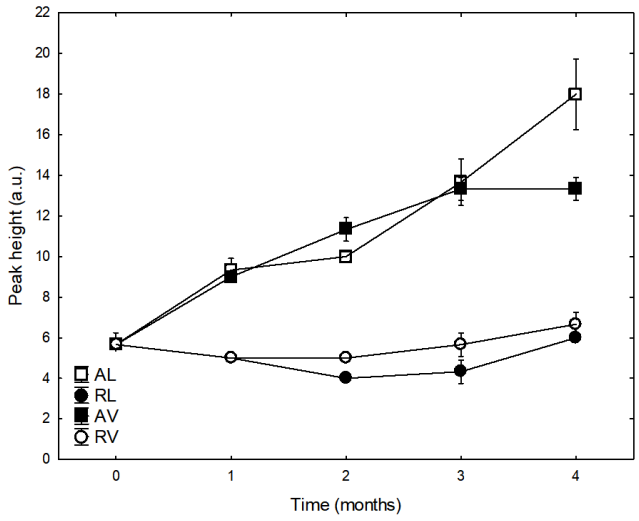

Figura 1. Tendency of lipid radical formation in Brazil nuts stored under different conditions

Results expressed as mean (n = 3) and bars represent standard deviation. *AL* Ambient conditions and loose packing, *RL* Refrigerated temperature and loose packing, *AV* Ambient conditions and vacuum packing, *RV* Refrigerated temperature and vacuum packing

In this study, the effect of tendency of radical formation can be clearly seen on Figure 1 and kernels stored under refrigeration (treatments RL and RV) presented low tendency to lipid radical formation during the entire storage. On the other hand, kernels stored under ambient conditions (treatments AL and AV) showed a sharp increase on tendency of lipid radical formation during the first month and kept increasing up to the third month. Thereafter, it kept increasing for AL, while remained stable for AV, with significant difference between these treatments during the fourth month. Therefore, vacuum packing did not affect the tendency to lipid radical formation during refrigerated storage up to four months and during room temperature storage at least up to three months.

Although the identification of the trapped free radicals is hindered due to their addition to the PBN molecule (Andersen and Skibsted, 2002), it is suggested PBN traps mainly peroxyl radicals, which are formed from the very fast reaction of alkyl radicals with oxygen (Velasco et al., 2005). During lipid oxidation, peroxyl radicals abstract one hydrogen atom from polyunsaturated fatty acids, forming hydroperoxides and another alkyl radical, propagating lipid deterioration (Choe and Min, 2006).

2.3.3. Primary lipid oxidation products

Figure 2 comprises results for PV (2A) and K_{232} (2B), which represent the total content of peroxides and the total content of conjugated dienes, respectively. PV on time zero (2.68±0.18 meq. O_2/kg) was similar to PV reported for cold-pressed oil extracted from fresh Brazil nut kernels (Gutierrez, Regitano-d'Arce and Rauen-Miguel, 1997).

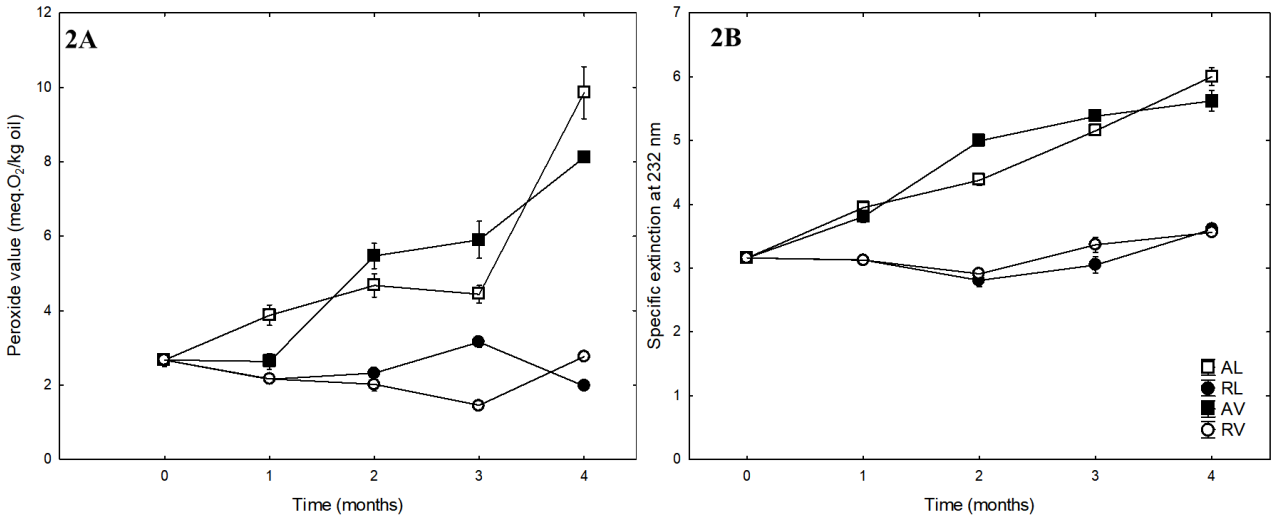

Figura 2. Peroxide value (2A) and specific extinction at 232 nm (2B) in Brazil nuts stored under different conditions

Results expressed as mean (n = 3) and bars represent standard deviation. *AL* Ambient conditions and loose packing, *RL* Refrigerated temperature and loose packing, *AV* Ambient conditions and vacuum packing, *RV* Refrigerated temperature and vacuum packing

During the first month, PV slightly increased for AL (3.88±0.27 meq. O_2/kg) and remained low for the other treatments, with values varying from 2.16±0.11 meq. O_2/kg for RL to 2.64±0.21 meq. O_2/kg for AV. Thereafter, for kernels stored under refrigeration (RL and RV), it tended to remain low during the entire storage, not exceeding 3.16 meq. O_2/kg. For AL, PV maintained from the first to the third month (from 3.88 to 4.68 meq. O_2/kg) and then sharply increased during the fourth month, reaching 9.86±0.70 meq. O_2/kg. Meanwhile, it kept increasing, reaching 8.12±0.07 meq. O_2/kg after four months for AV. Thus, as expected, refrigeration reduced significantly peroxide formation in stored kernels, which is in agreement with Ribeiro et al. (1993a) that observed the effectiveness of refrigeration when compared to room temperature in reducing peroxides formation for Brazil nuts packed in paperboard for four months.

In contrast, treatments under vacuum (AV and RV) did not show a protective effect, likely due to the relatively high OTR of the LDPE pouches used, which might not have kept properly vacuum atmosphere within the package over time, although OTR was not estimated during storage to confirm this hypothesis. However, Chun et al. (2005) reported that even low contents of residual oxygen were enough to form peroxides in fresh peanuts containing low initial PV and stored under vacuum. Accordingly, reducing headspace oxygen from 2 to 1% showed to be more efficient than reduction from 21 to 2% on lipid oxidation rate of linoleic acid (Marcuse and Fredriksson, 1968).

Another method to measure primary lipid oxidation products is K_{232}, which is based on the property of conjugated diene hydroperoxides formed in oils containing linoleic acid to give rise to an absorption peak at 232 nm in the ultraviolet region. Results for K_{232} are comprised at Figure (2B) and show the clear effect of temperature on this parameter, as K_{232} remained low up to four months for kernels stored under refrigeration (treatments RL and RV) and increased significantly for kernels stored under room temperature (treatments AL and AV). Although it is known hydroperoxides formed during lipid autoxidation are conjugated dienes (Choe and Min, 2006), K_{232} significantly correlated with PV only for treatments under ambient conditions (AL: r = 0.875 and AV: r = 0.925) at a level of confidence of 0.05.

However, K_{232} results were similar to those found for the tendency of lipid radical formation (Figure 1), and this similarity was confirmed by high correlation coefficients between these methods for all treatments at a level of confidence of 0.01 (AL: 0.980; AV: 0.975; RL: 0.876, RV: 0.851). One hypothesis for the strong correlation between K_{232} and the ESR analysis for Brazil nut kernels is that peroxyl radicals, although very unstable, might been detected as well as by K_{232}, since these radical species are conjugated dienes (Evans et al., 1985).

2.3.4. Volatile aroma compounds

VACs detected by HS-SPME-GC-MS in the kernels were aldehydes, ketones, alcohols and pyrroles, as shown at Table 2. Almost all samples presented formation of the same compounds, except time zero for 2-decanone, and it varied according to treatment and time of storage. No hydrocarbons were included, since their thresholds are generally high and they are unlikely flavor contributors to Brazil nut kernels (Clark and Nursten, 1976).

Tabela 2. Volatile aroma compounds in shelled Brazil nuts stored under different conditions

Volatile aroma compounds	LRI	Treatment	Time zero	1 month	2 months	3 months	4 months
Aldehydes							
Hexanal*	800.8	AL	2878 ± 129^{d}	4029 ± 170^{Ac}	4568 ± 142^{Ab}	5391 ± 231^{Aa}	5268 ± 320^{Ba}
		RL	2878 ± 129^{d}	3760 ± 289^{Ac}	4227 ± 324^{Abc}	4513 ± 233^{Bb}	5760 ± 293^{Aa}
		AV	2878 ± 129^{c}	4279 ± 141^{Ab}	4443 ± 304^{Aab}	4489 ± 232^{Bab}	4959 ± 233^{Ba}
		RV	2878 ± 129^{c}	2829 ± 165^{Bc}	3198 ± 126^{Bb}	3525 ± 6^{Ca}	2836 ± 320^{Cc}
Trans-2-heptenal*	960.3	AL	390 ± 15^{b}	373 ± 15^{BCb}	401 ± 13^{Bb}	451 ± 13^{Aa}	472 ± 18^{Ba}
		RL	390 ± 15^{b}	377 ± 20^{Bb}	385 ± 10^{BCb}	465 ± 33^{Aa}	422 ± 23^{Cab}
		AV	390 ± 15^{b}	476 ± 19^{Aa}	467 ± 17^{Aa}	462 ± 23^{Aa}	507 ± 3^{Aa}
		RV	390 ± 15^{a}	325 ± 21^{Cb}	349 ± 23^{Cab}	310 ± 6^{Bb}	368 ± 13^{Da}
Trans-2-octenal	1060.9	AL	51 ± 4^{e}	90 ± 6^{Bd}	110 ± 5^{Ac}	158 ± 12^{Aa}	129 ± 5^{Bb}
		RL	51 ± 4^{d}	91 ± 7^{Bc}	118 ± 15^{Abc}	146 ± 14^{Ab}	201 ± 16^{Aa}
		AV	51 ± 4^{c}	123 ± 8^{Aab}	106 ± 2^{Ab}	135 ± 5^{Aa}	124 ± 13^{Bab}
		RV	51 ± 4^{c}	104 ± 15^{Aba}	101 ± 1^{Aa}	81 ± 0^{Bb}	92 ± 2^{Cab}
Nonanal	1105.0	AL	88 ± 5^{bc}	100 ± 8^{Bbc}	102 ± 6^{Bb}	133 ± 7^{Aa}	86 ± 4^{Cc}
		RL	88 ± 5^{b}	99 ± 11^{Bb}	107 ± 1^{ABb}	135 ± 13^{Aa}	136 ± 2^{Aa}
		AV	88 ± 5^{c}	127 ± 3^{Aab}	115 ± 4^{Ab}	128 ± 7^{Aa}	131 ± 4^{Aa}
		RV	88 ± 5^{c}	82 ± 2^{Bcd}	77 ± 2^{Cd}	127 ± 7^{Aa}	99 ± 8^{Bb}
Ketones							
3-Octen-2-one	1041.7	AL	255 ± 25^{d}	314 ± 17^{ABc}	390 ± 23^{Ab}	461 ± 27^{Aa}	472 ± 23^{Ba}
		RL	255 ± 25^{ab}	255 ± 22^{BCab}	229 ± 23^{Cb}	313 ± 31^{Ba}	291 ± 14^{Cab}
		AV	255 ± 25^{d}	339 ± 21^{Ac}	427 ± 9^{Ab}	463 ± 8^{Ab}	508 ± 10^{Aa}
		RV	255 ± 25^{abc}	238 ± 33^{Cbc}	282 ± 4^{Bab}	226 ± 14^{Cc}	291 ± 20^{Ca}
2-Nonanone	1093.4	AL	49 ± 9^{d}	170 ± 7^{Bc}	167 ± 12^{Bc}	335 ± 21^{Ca}	207 ± 1^{Ab}
		RL	49 ± 9^{d}	121 ± 4^{Cc}	148 ± 19^{Bc}	310 ± 29^{Ca}	219 ± 19^{Ab}
		AV	49 ± 9^{e}	275 ± 6^{Ac}	413 ± 35^{Ab}	583 ± 70^{Ba}	163 ± 4^{Bd}
		RV	49 ± 9^{c}	124 ± 3^{Cb}	109 ± 6^{Cb}	2170 ± 194^{Aa}	121 ± 13^{Cb}

2-Decanone	1192.0	AL	Nd	33 ± 2^{Ac}	42 ± 3^{Ab}	52 ± 3^{Ba}	39 ± 0^{Ab}
		RL	Nd	29 ± 4^{Ab}	33 ± 5^{Bb}	51 ± 5^{Ba}	34 ± 4^{Bb}
		AV	Nd	28 ± 1^{Abc}	29 ± 1^{Bb}	52 ± 4^{Ba}	24 ± 0^{Cc}
		RV	Nd	11 ± 4^{Bc}	20 ± 1^{Cb}	126 ± 23^{Aa}	22 ± 0^{Cb}
Alcohols							
1-Octen-3-ol	982.4	AL	266 ± 43^{b}	429 ± 29^{Bb}	500 ± 25^{Aba}	565 ± 39^{Aa}	541 ± 27^{Ba}
		RL	266 ± 43^{d}	406 ± 19^{Bc}	427 ± 54^{Bbc}	514 ± 46^{ABab}	559 ± 26^{Ba}
		AV	266 ± 43^{c}	545 ± 29^{Ab}	521 ± 11^{Ab}	545 ± 32^{Ab}	698 ± 23^{Aa}
		RV	266 ± 43^{b}	409 ± 63^{Ba}	443 ± 27^{Aba}	425 ± 15^{Ba}	442 ± 24^{Ca}
1-Pentanol	770.4	AL	174 ± 7^{d}	265 ± 13^{Bc}	333 ± 10^{Ab}	420 ± 35^{Aa}	368 ± 14^{Ab}
		RL	174 ± 7^{d}	225 ± 19^{Cc}	252 ± 16^{Bbc}	279 ± 26^{Bb}	403 ± 19^{Aa}
		AV	174 ± 7^{c}	322 ± 11^{Ab}	307 ± 34^{ABb}	381 ± 23^{Aab}	441 ± 78^{Aa}
		RV	174 ± 7^{b}	257 ± 14^{BCa}	256 ± 28^{Ba}	197 ± 12^{Cb}	240 ± 10^{Ba}
2-Nonanol	1100.3	AL	4 ± 1^{c}	18 ± 1^{Bb}	20 ± 0^{Ab}	24 ± 3^{Ca}	20 ± 0^{Bab}
		RL	4 ± 1^{c}	12 ± 2^{Cd}	19 ± 1^{Ac}	31 ± 2^{Ca}	26 ± 1^{Ab}
		AV	4 ± 1^{c}	22 ± 1^{Ab}	20 ± 1^{Ab}	85 ± 17^{Ba}	19 ± 0^{Cb}
		RV	4 ± 1^{d}	7 ± 1^{Dc}	10 ± 0^{Bb}	813 ± 63^{Aa}	10 ± 2^{Db}
Pyrrole-derivative							
1-Methyl-1*H*-pyrrole	746.2	AL	179 ± 9^{c}	265 ± 18^{Ab}	240 ± 3^{Bb}	273 ± 28^{Bb}	495 ± 12^{Aa}
		RL	179 ± 9^{d}	261 ± 19^{Ac}	290 ± 21^{Abc}	435 ± 33^{Aa}	327 ± 25^{Bb}
		AV	179 ± 9^{b}	187 ± 16^{Bb}	183 ± 12^{Bc}	268 ± 2^{Ba}	327 ± 49^{Ba}
		RV	179 ± 9^{c}	225 ± 13^{ABab}	199 ± 17^{Cbc}	188 ± 4^{Cc}	245 ± 10^{Ca}

Results expressed as mean ± standard deviation (n = 3) of the peak area (adimensional unit). Means followed by different superscript upper-case letters within the same row are significantly different (one-way ANOVA, $p < 0.05$). Means followed by different superscript lower-case letters within the same column are significantly different (one-way ANOVA, $p < 0.05$). LRI: Linear retention index. *Identification confirmed by comparing with a standard reference. *AL* Ambient conditions and loose packing, *RL* Refrigerated temperature and loose packing, *AV* Ambient conditions and vacuum packing, *RV* Refrigerated temperature and vacuum packing

Aldehydes are important VACs related to (off)-flavor in foods, and some of them, such as hexanal, which is a major product from linoleic acid oxidation (Choe and Min, 2006), are used as markers for secondary lipid oxidation products formation. In this study, hexanal formation remained low for RV during the entire storage, while, after four months, it increased around 100% for RL, despite the low PV and tendency of radical formation at that stage, and 72 and 83% for AV and AL, respectively. Therefore, vacuum packing using LDPE pouches combined with refrigeration was efficient to reduce formation of this saturated VAC, which is relevant in fresh Brazil nut kernels (Clark and Nursten, 1976), but whose increased formation is related with rancidity by sensory analysis (Zajdenwerg et al., 2011).

Although the relevant peak areas, formation of trans-2-heptenal, which is also a product from linoleic acid oxidation (Ullrich and Grosch, 1987), remained low for RV, increased 21 and 30% for AL and AV, respectively, at the end of storage, while no clear tendency could be observed for RL. These low formation rate observed for trans-2-heptenal may indicate it is majorly formed during photoxidation (Lee and Min, 2010). In contrast, the formation of trans-2-octenal, which is also formed during linoleic acid oxidation and may present lower threshold than hexanal (Ullrich and Grosch, 1987), increased 80, 143, 153 and 294% for RV, AV, AL and RL, respectively, after four

months. Finally, nonanal formation tended to increase for RL and AV, while no clear tendency could be observed for AL and RV.

Ketones generally have low thresholds and may contribute to flavor profile of foods. The formation of 3-octen-2-one continuously increased in kernels stored under ambient conditions (AL and AV), while it remained low in kernels stored under refrigeration (RL and RV), which indicate a clear effect of temperature. 3-Octen-2-one is as unsaturated ketone formed during linoleic acid autoxidation (Ullrich and Grosch, 1987), and therefore, may contribute to flavor deterioration of the kernels. 2-Nonanone and 2-decanone were previously identified in Brazil nut extracts and their flavors were assessed as peanutty/fruity and green/fruity, respectively (Clark and Nursten, 1976), thus probably not related to oxidative deterioration.

The formation of 1-octen-3-ol and 1-pentanol increased for all treatments, but less for RV (Table 2). These short-chain alcohols are products of the decomposition of linoleic acid hydroperoxides (Ullrich and Grosch, 1987) and thereby may impact off-flavor formation in the kernels. 2-Nonanol has been identified in Brazil nut extracts, although its aroma has not been assessed yet (Clark and Nursten, 1976). It is noteworthy that some samples showed outstanding peak areas for 2-nonanol (AV and RV 3 months), as well as for 2-nonanone (AV 2 and 3 months, and RV 3 months) and 2-decanone (RV 3 months), which suggests that their formation may be affected by factors others than packaging atmosphere, temperature and time of storage.

Pyrrole-derivatives are heterocyclic aromatic compounds that may be products of the interaction between amino acids and aliphatic aldehydes (Adams et al., 2005). The 1-methyl-1*H*-pyrrole, which was also identified in fresh and dried pistachios (Georgiadou et al., 2015), was likely formed during the drying process and its formation kept increasing during storage, but less for RV.

2.3.5. Sensory analysis

Sensory analysis was conducted to verifiy the effect of undergoing chemical changes on rancidity and results for rancid odor are shown at Table 3. No statistically significant changes were noticed among the samples during the whole storage period, likely due to the early stage of oxidation of the samples, which could be represented by PV. Zajdenwerg et al. (2011) reported that while trained assessors identified oxidized odor in Brazil nut kernels with a PV of 9.9 meq. O_2/kg oil, which is comparable with the highest PV found in this study (9.86 meq. O_2/kg oil for AL 4 months). Furthermore, untrained assessors (consumers) identified the same attribute only when PV was > 17 meq. O_2/kg oil, which indicates consumers are used to eat oxidized Brazil nut kernels (Zajdenwerg et al., 2011).

Tabela 3. Rancid odor attributed by the trained assessors to the Brazil nut samples with respect to storage conditions and period

Treatment	Time zero	1 month	2 months	3 months	4 months
AL	1.7 ± 0.3	1.4 ± 0.9	1.3 ± 0.4	2.0 ± 0.7	1.6 ± 0.5
RL		1.4 ± 0.5	1.2 ± 0.4	2.0 ± 0.7	1.3 ± 0.4
AV		1.8 ± 0.8	1.5 ± 0.5	1.8 ± 0.8	1.8 ± 0.8
RV		1.4 ± 0.5	2.0 ± 1.0	1.3 ± 0.4	1.6 ± 0.9

No significant changes were detected (one-way ANOVA, $p < 0.05$). Data from time zero is an average of the five samples used. AL Ambient conditions and loose packing, RL Refrigerated temperature and loose packing, AV Ambient conditions and vacuum packing, RV Refrigerated temperature and vacuum packing

2.4. Conclusions

The use of refrigeration was effective, while vacuum packing had no effect, to control the tendency of radical formation, as well as of peroxides and conjugated dienes in Brazil nut kernels during storage. The formation of off-flavor VACs was affected by storage conditions and the use of refrigeration reduced the formation of 3-octen-2-one. However, the combination of refrigeration and vacuum packing, even using LDPE pouches with high OTR, reduced the formation of hexanal, which is a major contributor to flavor deterioration, as well as of other off-flavor VACs, and thus should be recommended for Brazil nut kernels storage.

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3. VOLATILES AND TENDENCY OF RADICAL FORMATION IN COLD-PRESSED BRAZIL NUT OIL DURING AMBIENT STORAGE

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Abstract

Cold-pressed Brazil nut oil (BNO) is a value-added non-timber forest co-product of market interest, despite the lack of knowledge about its ambient storage stability. The objective of the present study was to investigate oxidative changes in BNO stored in clear and brown glass bottles under retail conditions with focus on monitoring for the first time the formation of a large set of volatile aroma compounds (VACs), the depletion of tocopherol analogs and the tendency of radical formation. VACs whose formation continuously increased and correlated with storage time were identified by HS-SPME-GC-MS. Depletion of tocopherols was more pronounced for the oil stored in clear glass and the content loss was higher for alpha-tocopherol than for gamma-tocopherol. Furthermore, the tendency of radical formation detected by a spin-trapping electron spin resonance (ESR) spectroscopy method was demonstrated to be a simpler, faster and less sample-demanding tool to monitor the oxidative status of BNO stored under retail conditions and its results significantly correlated with traditional methods for determination of primary oxidation products. However, the simultaneous measurement of key off-flavor volatiles is recommended for samples with unknown history.

Keywords: Lipid oxidation; ESR; Spin trapping; Tocopherol; Rancimat

3.1. Introduction

Brazil nuts (*Bertholletia excelsa* Bonpl.) are important non-timber forest products of the humid tropics and their collection do not require deforestation [1]. These seeds are mainly composed of oil (67%) which upon separation by cold pressing of good quality Brazil nut kernels, is clear and has a pleasant mild aroma [2]. The dominant oil components are linoleic acid (18:2n-6) and oleic acid (18:1n-9). Due to the high content of linoleic acid (~35%) [3], cold-pressed Brazil nut oil (BNO) is highly susceptible to oxidative reactions, which lead to off-flavor formation and consequently consumer rejection [4]. These reactions have their rates affected, among other factors, by the type of packaging. Edible cold-pressed oils are most often stored in glass bottles, which may partially protect the oil against oxidation due to the great oxygen barrier. However, it is relevant to consider glass bottle color and its

effect as light screen since the combination of light and oxygen, even under domestic and retail conditions, is damaging to unsaturated lipids [5].

Oxidative changes in vegetable oils stored under mild conditions are generally investigated by determining both hydroperoxides, as primary oxidation products, and volatiles formed during hydroperoxide degradation, as secondary oxidation products. The hydroperoxides are commonly determined by volumetric or spectrophotometric methods, which present low-to-moderate selectivity and sensitivity and require the use of organic solvents [6]. On the other hand, the volatiles related to off-flavor can be measured by gas chromatography (GC) along with techniques to recover them from samples, such as headspace (HS) techniques, which generally present high sensitivity [6]. Some volatiles are used as markers to monitor off-flavor formation in oils if they are formed in great amounts during oxidative degradation of a particular unsaturated fatty acid found in the sample. However, volatiles include different functional groups such as aldehydes, ketones, alcohols and hydrocarbons and then monitoring a large set of compounds is a better approach to investigate oxidative changes [6].

Meanwhile, reactive intermediate free radicals, formed prior to both hydroperoxides and off-flavor compounds, can be rapidly detected by electron paramagnetic resonance (EPR) spectroscopy; also known as electron spin resonance (ESR) spectroscopy [7]. The use of a spin-trapping compound (or spin trap) capable of complexing with very unstable free radicals to form spin adducts, which are long-lived radicals to be detected by ESR spectroscopy, is a sensitive green method that may not require the use of organic solvents [8]. The classic Rancimat is another rapid and solvent-free method to estimate oxidative status of oils and is based on the use of high temperatures and intensive airflow to accelerate lipid degradation [7]. Furthermore, BNO also contains naturally occurring antioxidants, the tocopherols [3,9], which depletion during storage may also be used for monitoring oxidative changes in vegetable oils [5].

Although the oxidative stability of Brazil nut oil has already been evaluated [2,4,10,11,12], there were not found studies about changes on formation of different volatile aroma compounds and depletion of tocopherol analogs during ambient storage. In addition, to the best of our knowledge, there are no papers reporting the use of an ESR spectroscopy method based on the addition of the spin trap immediately before analysis to determine the tendency of radical formation in stored edible cold-pressed vegetable oils. Therefore, the objective of this study was to investigate the occurrence of oxidative changes in BNO stored in clear and brown glass bottles under retail conditions, with focus on formation of volatile aroma compounds, tocopherol depletion and tendency of radical formation.

3.2. Experimental Procedures

3.2.1. Chemicals and reagents

All reagents were of analytical or HPLC grade, as required. Methyl tridecanoate (code T0627), α-tocopherol (code T3251), γ-tocopherol (code T1782), δ-tocopherol (code T2028), hexanal (code 115606), *trans*-2-heptenal (code 90244), ammonium thiocyanate, iron(II) sulfate heptahydrate, cumene hydroperoxide, *N*-*tert*-butyl-α-phenylnitrone (PBN) (code 80126), and standard *n*-alkanes (C7-C30) (code 49451-U) were Sigma-Aldrich (St. Louis, USA). A solid-phase microextraction (SPME) assembly composed of divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS, 50/30 μm) fiber was supplied by Supelco (Bellefonte, USA). Barium chloride dihydrate, hydrochloric acid, sodium chloride, sodium hydroxide, sulfuric acid,

isooctane and butanol were supplied by Dinamica (Diadema, Brazil); fatty acid methyl esters (FAME) standard mix (GLC-87), by Nu-Chek (Elysian, USA); sodium sulphate and ammonium chloride, by Cromoline (Diadema, Brazil); methanol and hexane, by Panreac (Barcelona, Spain), and propan-2-ol and acetonitrile, by Carlo Erba (Rodano, Italy).

3.2.2. Oil samples and experimental design

Fresh, shelled and dried Brazil nuts (20 kg) were purchased from a local market. According to the supplier, they came from Óbidos, PA, Brazil. They were vacuum-packed in a metalized pouch protected by a cardboard box. Brazil nuts had their skins removed, were cold pressed in a laboratory hydraulic press (Carver, Wabash, USA) under up to 34 MPa at 25 ºC for 10 min, and the oil was filtered through filter paper. In order to reach the same headspace volume, 15 mL of oil were accurately measured and stored in 20 mL round clear or brown glass bottles with 58.3 mm height, 32.0 mm diameter, 1.3 mm thickness (Wheaton Brasil, São Bernardo do Campo, Brazil) capped with a gas-tight cork lid. Light transmission was 91% at 550 nm through clear glass, and 22% at 550 nm and 0.7% at 290-450 nm through brown glass (data from manufacturer). Samples were maintained at room temperature (24 ± 2 ºC) during the study. A 32W fluorescent light bulb was used as light source and samples were exposed to 8 hour-light (luminance was 180 LUX) and 16 hour-dark (luminance < 40 LUX) a day. Luminance was monitored using a lux meter. The storage period was 5 months. On time zero and after one, two, three, four, and five months, three samples of each type of glass bottle were randomly collected and frozen at –18 ºC until analysis.

3.2.3. Fatty acid profile

Fatty acid methyl esters (FAME) were prepared according to the procedures described by [13]. A GC-2010 Plus equipped with a flame ionization detector (FID) was used. Separation was achieved on a Rtx-Wax capillary column (30 m x 0.32 mm, 0.25 μm) (Restek, Bellefonte, USA). Initial oven temperature (60 ºC) raised to 210 ºC at 20 ºC/min rate (hold for 7 min), and then raised to 240 ºC at 30 ºC/min rate (hold for 12 min). Both injector (split 1:20) and detector temperatures were set at 250 ºC. Injection volume was 1 μL. Carrier gas was nitrogen with column flow rate of 1.2 mL/min. Peak retention times were compared with those of a standard FAME mix. Methyl tridecanoate was used as internal standard and quantitation was performed by normalization.

3.2.4. Peroxide Value (PV)

Concentration of hydroperoxides was determined as described elsewhere (1994) [14]. Absorbance was measured at 510 nm. PV was calculated using external standard curve built with cumene hydroperoxide solutions.

3.2.5. Specific extinction at 232 nm (K_{232})

Conjugated dienes were detected according to the standard method ISO 3656:2011. For that, 20 mg of oil were diluted with 25 mL of isooctane, swirled and absorbance at 232 nm was measured. Results were expressed as extinction at 232 nm of 1% solution of oil in isooctane, in a thickness of 1 cm (K_{232}).

3.2.6. Volatile aroma compounds (VACs)

VACs were recovered by headspace (HS) solid-phase microextraction (SPME) and determined by gas chromatography coupled to mass spectrometry (GC-MS) based on the procedures described elsewhere [15]. Two grams of BNO were placed in 20 mL headspace vial, flushed with nitrogen for 10 s, and then the vial was capped with a gas-tight aluminum lid with polytetrafluoroethylene/silicone septum. The vial was kept submerged into thermostatted water bath at 80 ºC under magnetic stirring for 30 min. Then, SPME fiber was inserted through the septum and exposed to the headspace for 10 min. The fiber with adsorbed VACs was inserted into the injector of a GC-2010 coupled to a mass spectrometer QP 2010 Plus. Separation was achieved on a Rtx-5MS capillary column (30 m x 0.25 mm, 0.25 μm) (Restek, Bellefonte, USA). Oven temperature program was: initial temperature 32 ºC (hold for 3 min); raised to 60 ºC at 5 ºC/min rate (hold for 3 min); raised to 200 ºC at 8 ºC/min rate (hold for 10 min); and then raised to 280 ºC at 20 ºC/min rate (hold for 5 min). Injector (splitless mode) and ion source temperatures were set at 270 ºC and 200 ºC, respectively. Mass spectrometer scan was from 20 to 300 m/z. Carrier gas was helium at flow rate of 1.0 mL/min. Peaks were tentatively identified by both mass spectra and linear retention index (LRI). Mass spectra were matched with those from Wiley Library (Version 8), considering ≥ 90% similarity as cut off. LRI were calculated relatively to standard *n*-alkane series [16], and were compared with literature data, considering 1% difference as cut off. Hexanal and *trans*-2-heptenal identities were also confirmed by spiking BNO samples with commercial standards and comparing retention times and peak areas with those of non-spiked samples. Amounts were represented in terms of peak area.

3.2.7. Tocopherols

Tocopherol analysis was carried out according to a method described elsewhere [17]. This method does not distinguish γ- from β-tocopherol. However, the content of β-tocopherol in Brazil nuts is negligible [3]. The HPLC system consisted of LC-20AT, auto sampler SIL-20AC HT, and fluorescence detector RF-20AXL. Separation was achieved on a VP-ODS-2 C18 Shim-Pack column (250x5 mm) (Shimadzu, Kyoto, Japan). Mobile phase was methanol:acetonitrile (1:1) at a flow rate of 1 mL/min. Samples (0.06 g) were diluted with 1.5 mL propan-2-ol, vortexed for 30 s, filtered at 0.45 μm, and then 20 μL were injected. Tocopherols were detected using excitation and emission wavelength of 295 and 325 nm, respectively. Identification was performed by comparing peak retention time and peak area of samples spiked with standard solutions with those of non-spiked samples. Quantitation was carried out by external calibration.

3.2.8. ESR spectroscopy analysis

The tendency of radical formation was determined by ESR spectroscopy. The method was based on procedures described elsewhere [7], with modifications. The spin trap PBN (1 mg/g oil) was dissolved in the samples by gently swirling. As spin adducts formation depends on heating and time [7], tests were conducted aiming at defining the lower incubation temperature and still a reasonable time range for analysis and it was found that 70 ºC during 5h was enough to detect peaks in the ESR spectra for all samples. From preparation time (0 h) and the following hours, 50 μL were transferred every 30 min to capillary micropipettes (Blaubrand, Wertheim, Germany) and screened with MiniScope MS200 ESR spectrometer at 22 ºC. Acquisition parameters were center field, 3336.9 G; sweep width, 66.42 G; sweep time, 30 s; and modulation amplitude, 1 G. Each measurement was performed as average of six sweeps. A software (Magnettech, Berlin, Germany) was used to obtain the height of the first peak of the ESR spectra, which is suitable to measure the concentration of spin adducts when comparing similar samples [7]. Peak height after 5h of incubation was used to follow the formation of spin adducts instead of the lag phase, which is the period before a sharp increase on spin adducts formation, since it showed to follow better storage time for BNO.

3.2.9. Rancimat method

Oxidative stability index was determined using a Metrohm 743 Rancimat instrument [8]. The oil (2 g) was placed into the Rancimat apparatus and heated at 100 ºC under an airflow rate of 20 L/h. Results were expressed as the number of hours until the content of formed volatile organic acids sharply increased, which is known as the Induction Period (IP).

3.2.10. Statistical analysis

Normality of distributions (Ryan-Joyner's test) and homoscedasticity (Bartlett's test) were verified for all data. Differences between treatments were checked by paired *t*-test. Differences within treatments were evaluated by analysis of variance (one-way ANOVA), and Tukey's post hoc test. Pearson's linear correlation was determined in order to detect associations between each two variables. All statistical analyses were performed at the level of confidence of 0.05, using Minitab® 17 software (Minitab, State College, USA). Figures were generated using Statistica® 64 (StatSoft, Tulsa, USA).

3.3. Results & Discussion

3.3.1. Fatty acid profile

Composition of major fatty acids was determined on time zero and after five months of storage (Table 4). The major fatty acids were 18:2 n-6, 18:1 n-9, 16:0 and 18:0 and the relative amounts of each one on time zero were in accordance with literature data [18]. For both treatments (clear and brown glass bottles), total saturated fatty acids

remained stable while total monounsaturated fatty acids and total polyunsaturated fatty acids slightly increased and decreased, respectively, during storage. It may indicate loss of linoleic acid, which is more easily oxidized, when compared with the others fatty acids found in BNO, due to the presence of a bisallylic hydrogen atom. Similar results were obtained for cold-pressed pumpkin seed oil, which has comparable fatty acid profile, stored in glass bottles at room temperature [19]. These results confirm findings of a previous study that showed a decrease on iodine value, but no significant variation between BNO samples packed in clear or brown glass bottles and stored at room temperature up to six months [2]. It is noteworthy to mention that iodine value strongly correlated (r = 0.98) with linoleic acid content in BNOs with different PV and acid values [10].

Tabela 4. Fatty acid composition of cold-pressed Brazil nut oil before and after storage in clear or brown glass bottles

Fatty acid	Time zero	5 months	
		Clear	Brown
16:0	15.77 ± 0.09[a]	16.06 ± 0.03[b]	16.07 ± 0.04[b]
16:1 n-7	0.38 ± 0.00[a]	0.37 ± 0.00[a]	0.37 ± 0.00[a]
18:0	11.28 ± 0.07[a]	11.08 ± 0.03[a]	11.11 ± 0.03[a]
18:1 n-9	32.79 ± 0.06[a]	34.31 ± 0.02[b]	34.30 ± 0.03[b]
18:2 n-6	39.52 ± 0.11[a]	37.93 ± 0.05[b]	37.90 ± 0.04[b]
20:0	0.26 ± 0.00[a]	0.25 ± 0.00[a]	0.25 ± 0.00[a]
SFA	27.31 ± 0.17[a]	27.39 ± 0.07[a]	27.42 ± 0.07[a]
MUFA	33.17 ± 0.06[b]	34.68 ± 0.02[a]	34.68 ± 0.03[a]
PUFA	39.52 ± 0.11[a]	37.93 ± 0.05[b]	37.90 ± 0.04[b]

Results expressed as mean ± standard deviation (n = 2) of percent mass of total fatty acid mass. Means followed by different superscript letters within the same row are statistically different (one-way ANOVA, $p < 0.05$). SFA = total saturated fatty acids; MUFA = total monounsaturated fatty acids; PUFA = total polyunsaturated fatty acids.

3.3.2. Lipid oxidation primary products

On time zero, PV (2.69 ± 0.04 meq. O_2 /kg) was consistent with results reported in the literature for oils extracted from fresh Brazil nuts by cold pressing [10,20]. PV remained stable for the first month of storage (~3 meq. O_2 /kg) and significantly increased from the first to the second month with no significant differences between treatments. From the second to the fourth month, PV increased more for the oil stored in clear glass. After four months, PV for the oil stored in clear glass was almost two times higher (15.08 ± 0.56 meq. O_2 /kg) than for brown glass (7.97 ± 0.47 meq. O_2 /kg), reaching the maximum value defined by the Codex Alimentarius for cold-pressed edible vegetable oils of 15 meq. O_2/kg [21] (Figure 3a).

However, one month later (after five months), while PV for the oil stored in brown glass kept increasing (10.97 ± 1.00 meq. O_2 /kg), PV for the oil stored in clear glass dropped (8.85 ± 0.66 meq. O_2 /kg). These results suggest that hydroperoxide decomposition might be higher than hydroperoxide formation in BNO stored in clear bottles after five months under the considered conditions. Oro et al [22] similarly observed that PV increased from time zero up to 75 days and then decreased, which was associated with a concomitant increase on oxidized taste in cold-pressed pecan oil stored at room temperature.

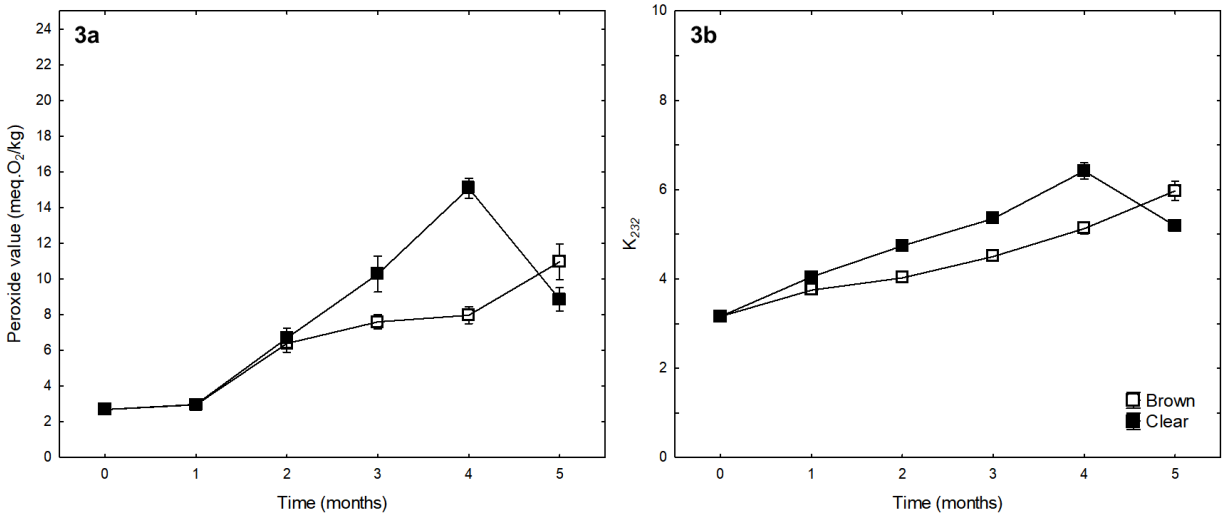

Figura 3. Peroxide value (3a) and specific extinction at 232 nm (3b) in cold-pressed Brazil nut oil stored in clear and brown glass bottles

Results expressed as mean (n = 3) and bars represent standard deviation.

As observed for PV results, the formation of conjugated dienes continuously increased and was significantly lower for the oil stored in brown glass during the first four months (Figure 3b). After five months, K_{232} dropped for the oil stored in clear glass and continued to increase for the oil stored in brown glass. The similarity between PV and K_{232} results is expected since the majority of the hydroperoxides formed during lipid oxidation are known to be conjugated dienes. The contribution of light exposure on PV and K_{232} was also observed for cold-pressed poppy seed oil during nine-month storage at 20 °C [23]. Therefore, depending on storage conditions and duration, monitoring only primary lipid oxidation products may not be suitable to assess the resistance towards rancidity of vegetable oils.

3.3.3. Volatile aroma compounds (VACs)

The formation of VACs was monitored during storage to investigate the extension of oxidative reactions in BNO. Four aldehydes, two ketones, and two alcohols were detected and tentatively identified (Table 5). Nonisothermal index was calculated for each compound and the LRIs were 801.3 for hexanal, 960.9 for *trans*-2-heptenal, 1061.4 for *trans*-2-octenal, 1105.4 for nonanal, 1042.3 for 3-octen-2-one, 968.6 for 1-heptanol, 983.0 for 1-octen-3-ol, and 1093.5 for 2-nonanone. Calculated LRIs were compared with literature data for apolar GC columns with stationary phases containing 5% phenyl groups [16,24,25]. Hydrocarbons present in the samples were not included in this study since they are unlikely to affect Brazil nut aroma [26]. To the best of our knowledge, only one previous study has characterized volatile aroma profile of Brazil nuts [26]. Clark & Nursten [26] evaluated fresh Brazil nut kernels and found hexanal and 2-nonanone, but not the other compounds reported in the present study, whose identification may be helpful to understand better oxidative reactions and its effects in BNO during ambient storage.

Tabela 5. Volatile aroma compounds formation in cold-pressed Brazil nut oil stored in clear and brown glass bottles

Compounds	Treatment	Time zero	1 month	2 months	3 months	4 months	5 months
Hexanal*	Clear	118 (12.7)^{c}	141 (11.4)^{bcA}	160 (9.2)^{bcA}	204 (38.7)^{bA}	177 (30.1)^{bA}	315 (43.1)^{aA}
	Brown	118 (12.7)^{b}	130 (17.9)^{Ba}	124 (11.7)^{bB}	161 (10.7)^{aA}	159 (4.6)^{aA}	164 (13.3)^{aB}
Trans-2	Clear	22 (2.3)^{d}	28 (1.7)^{dA}	39 (0.3)^{cA}	44 (6.2)^{bcA}	55 (5.3)^{bA}	82 (6.0)^{aA}
-heptenal*	Brown	22 (2.3)^{c}	28 (2.1)^{bcA}	26 (2.3)^{cB}	35 (5.1)^{abA}	36 (2.9)^{abB}	50 (1.0)^{aB}
Trans-2	Clear	8 (0.9)^{c}	8 (0.7)^{cA}	10 (0.1)^{bcA}	10 (1.3)^{bcA}	11 (0.4)^{abA}	14 (0.2)^{aA}
-octenal	Brown	8 (0.9)^{b}	9 (0.1)^{abA}	8 (0.5)^{bB}	10 (0.6)^{aA}	10 (0.7)^{aA}	10 (0.4)^{abB}
Nonanal	Clear	10 (0.9)^{c}	9 (0.7)^{cA}	11 (0.1)^{bcA}	11 (1.5)^{bcA}	13 (0.5)^{abA}	15 (0.6)^{aA}
	Brown	10 (0.9)^{cd}	10 (0.7)^{bcdA}	9 (0.3)^{dB}	12 (1.4)^{abcA}	12 (0.5)^{abA}	13 (0.8)^{aB}
3-Octen-2-one	Clear	18 (1.2)^{b}	16 (1.2)^{bA}	18 (0.5)^{bA}	18 (2.0)^{bA}	19 (0.8)^{bA}	24 (1.1)^{aA}
	Brown	18 (1.2)^{bc}	18 (1.3)^{bcA}	16 (1.0)^{cB}	20 (1.5)^{abcA}	21 (1.1)^{abA}	23 (3.2)^{aA}
2-Nonanone	Clear	3 (0.2)^{b}	3 (0.1)^{bA}	3 (0.1)^{bA}	3 (0.2)^{bA}	3 (0.3)^{bA}	9 (0.8)^{aA}
	Brown	3 (0.2)^{a}	3 (0.2)^{aA}	3 (0.2)^{aA}	3 (0.1)^{aA}	3 (0.2)^{aA}	4 (0.4)^{aB}
1-Heptanol	Clear	2 (0.2)^{c}	2 (0.2)^{cA}	2 (0.2)^{bcA}	2 (0.5)^{bcA}	3 (0.1)^{bA}	4 (0.1)^{aA}
	Brown	2 (0.2)^{bc}	2 (0.2)^{bcA}	2 (0.2)^{cB}	2 (0.3)^{abA}	2 (0.2)^{abB}	3 (0.2)^{aA}
1-Octen-3-ol	Clear	24 (1.8)^{cd}	24 (1.4)^{dA}	29 (0.2)^{bcA}	30 (2.9)^{bA}	34 (2.4)^{bA}	46 (2.4)^{aA}
	Brown	24 (1.8)^{b}	25 (1.9)^{bA}	23 (1.5)^{bB}	28 (2.3)^{abA}	29 (1.3)^{abB}	35 (4.4)^{aB}

Results expressed as peak area. Volatile aroma compounds tentatively identified based on both mass spectra and linear retention index. *Identification confirmed by comparing mass spectrum and retention time of samples with those of samples spiked with commercial standards. Results expressed as mean and standard deviation (in parenthesis) of triplicates. Superscript lower-case letters within the same row mean statistically significant differences within treatments (one-way ANOVA, Tukey, $p < 0.05$). Superscript upper-case letters within the same column mean statistically significant difference between treatments for each compound within storage time (2-sample t test, $p < 0.05$).

Hexanal was the major VAC found, considering its peak area. It was also a prominent peak in GC chromatograms of Brazil nut essence in another study [26]. This saturated aldehyde, formed during linoleic acid autoxidation [27], has been used to assess oxidative stability of Brazil nut oil and other nut oils [11], and its content correlated with sensory parameters in Brazil nuts stored at 80 ºC [12]. Hexanal formation for the oil stored in brown glass remained low up to the second month, after which it increased and remained stable until the fifth month of storage. In contrast, for the oil stored in clear glass, hexanal formation continuously increased and was almost twofold higher than for the oil stored in brown glass after five months of storage.

The formation of *trans*-2-heptenal increased and after five months doubled for the oil stored in brown glass and was four times higher for the oil stored in clear glass. 2-Heptenal is an important product formed during autoxidation and photoxidation of linoleic acid [27,28], and was identified as an important lipid oxidation product formed during rapeseed oil storage [29]. Another volatile compound related to linoleic acid photoxidation, 1-octen-3-ol [30], was tentatively identified and its formation increased significantly more for the oil stored in clear glass, which has lower protection against light exposure.

The other tentatively identified VACs showed smaller peak areas in BNO. 2-nonanone formation kept low up to the fourth month and then increased in both treatments. This VAC showed to have a peanutty/fruity/slightly vinegary aroma and is likely to contribute to overall aroma profile of Brazil nuts [26]. Formation of nonanal, 1-heptanol, *trans*-2-octenal and 3-octen-2-one increased more for the oil stored in clear glass, and among them, *trans*-2-octenal and 3-octen-2-one are formed during linoleic acid autoxidation [27]. On the other hand, nonanal and 1-heptanol are formed during oleic acid oxidation [30], which may indicate the oxidative

degradation of this monounsaturated fatty acid, although it could not be observed by fatty acid composition analysis in the present study (Table 4). In another study [11], the content of nonanal showed to be a suitable parameter to monitor oxidative changes in stripped Brazil nut oil under accelerated storage conditions. Nevertheless, some compounds (*trans*-2-heptenal, 1-octen-3-ol, *trans*-2-octenal and nonanal) are estimated to have lower odor thresholds than hexanal [27,31,32], and thereby their increased formation may contribute to flavor deterioration of BNO.

3.3.4. Tocopherols

The stability of naturally occurring tocopherols in foods may be influenced by factors such as fatty acid profile [33] and storage conditions [5]. In the present study, alpha- and gamma-tocopherol were detected and their depletion over time monitored (Figure 4).

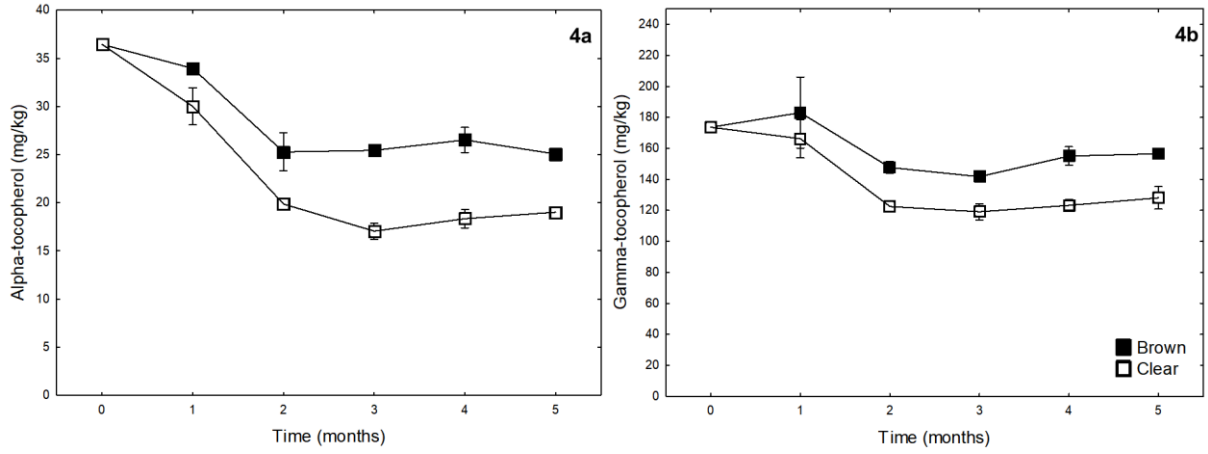

Figura 4. Alpha- (4a) and gamma-tocopherol (4b) content in cold-pressed Brazil nut oil stored in clear and brown glass bottles Results expressed as mean (n = 2) and bars represent standard deviation.

Initial contents found (36.4 μg/mL for alpha- and 173.7 μg/mL for gamma-tocopherol) are consistent with those reported elsewhere [12]. For both treatments, alpha-tocopherol content dropped significantly during the first two months and then remained stable (Figure 4a). By the end of storage, contents of alpha-tocopherol decreased 48% in clear glass and 31% in brown glass. Gamma-tocopherol content remained stable during the first month and decreased significantly from the first to the second month, remaining stable up to the fifth month (Figure 4b). After five months, gamma-tocopherol content decreased 26% in clear glass and 11% in brown glass. In agreement, tocopherol contents were better preserved in soybean oil stored in brown glass, which shows greater barrier to cold fluorescent light under ambient conditions [5]. Furthermore, the higher depletion rate suggests the alpha analog may have higher ability than the gamma analog to donate hydrogen atoms to lipid peroxyl radicals, which is the major antioxidant mechanism of tocopherols in lipid containing foods [9]. In agreement, alpha-tocopherol has previously been found to be the most sensitive tocopherol analog in Brazil nuts stored at 80 ºC [12], and in several other vegetable oils [34].

3.3.5. Tendency of radical formation by ESR

ESR spectroscopy is a technique that relies on the exposure of samples to an electromagnetic field at a constant frequency and the absorption of electromagnetic radiation by unpaired electrons from radical species present in the samples [35]. All spectra recorded consisted of three lines (Figure 5a), which is characteristic of the nitroxyl type spin adducts formed in vegetable oils when PBN is the spin trap [36].

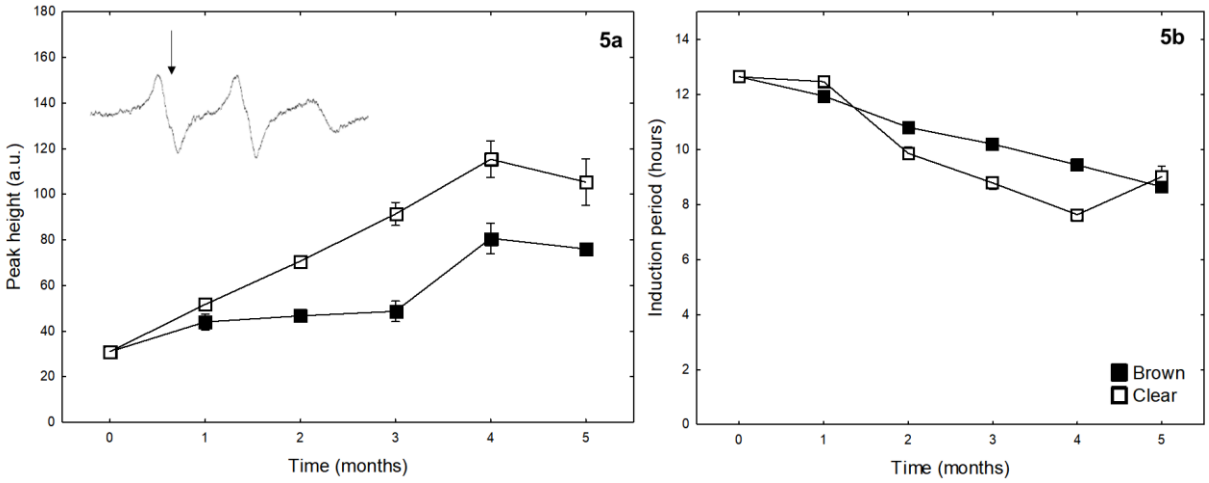

Figura 5. Tendency of radical formation (5a) and induction period determined by the Rancimat method (5b) in cold-pressed Brazil nut oil stored in clear and brown glass bottles

Image on top-left of Figure 5a is an ESR spectra obtained by spin trapping PBN, and vertical arrow indicates the peak used for peak height determination. Results expressed as mean (n = 2 for Rancimat method; n = 3 for tendency of radical formation) and bars represent standard deviation.

In this study, the tendency of radical formation significantly increased for both treatments during the first month of storage (Figure 5a), likely due to the formation of alkyl and peroxyl radicals, which are precursors for hydroperoxide formation and compete with other radical species to be trapped by PBN adducts [8]. Afterwards, the tendency of radical formation continuously increased up to four months for the oil stored in clear glass. In comparison, the tendency of radical formation remained stable from the first to the third month and increased lesser during the fourth month for the oil stored in brown glass. From the fourth to the fifth month, the formation of spin adducts slightly decreased, although not significantly, for both treatments. During the same period, PV and K_{232} decreased for the oil stored in clear glass (Figures 3a and 3b) while formation of off-flavor VACs increased for both treatments (Table 5). Therefore, one hypothesis is that the reaction between PBN and alkyl and peroxyl radicals may be favored, even when their formation tend to fall, and formation of alkoxyl radicals, which are precursors for VACs, rise. Accordingly, Velasco et al. [36] concluded that PBN added in oil samples prior to long-term storage tests trapped mainly peroxyl radicals.

3.3.6. Rancimat method

Initial IP (12.64h) remained relatively stable during the first month of storage, with no statistically significant differences between treatments (Figure 5b). From the second to the fourth month, IP for both treatments continuously decreased, reaching 7.63h for the oil stored in clear glass and 9.44h for the oil stored in brown glass. On the fifth month, however, IP increased for the oil stored in clear glass (9.02h), indicating a false improvement in BNO oxidative stability at that stage, while it kept decreasing for the oil stored in brown glass (8.65h). These results are similar to those found for PV, K_{232} and ESR method and suggest the Rancimat method follow better the formation of primary oxidation products than VACs in BNO under the considered conditions and during the evaluated period of storage.

3.3.7. Linear correlations

Table 6 presents high correlation coefficients between PV, K_{232}, tendency to radical formation and IP by Rancimat, which confirm the results reported in this study.

Depletion rates of both tocopherol analogs correlated with PV, K_{232}, tendency of radical formation ($r < -0.834$, $p < 0.05$) and IP by Rancimat ($r = 0.929$, $p < 0.01$) for the oil stored in clear glass, as shown on Table 6. On the other hand, significant correlations were observed only between alpha-tocopherol and PV ($r = -0.850$, $p < 0.05$) and IP by Rancimat ($r = 0.869$, $p < 0.05$) for the oil stored in brown glass, possibly due to the lower tocopherol loss, especially gamma-tocopherol, for this packaging type. Therefore, the content of alpha-tocopherol may be helpful to indicate the oxidative status of BNO stored under retail conditions.

Tabela 6. Pearson correlation coefficients (p value) between primary lipid oxidation products, tocopherol content, tendency of radical formation and induction period by Rancimat method for cold-pressed Brazil nut oil stored in clear and brown glass bottles for five months

Clear bottles				
	Peroxide Value	K_{232}	ESR analysis	IP by Rancimat
K_{232}	0.973 (p=0.001)	-	-	-
ESR analysis	0.941 (p=0.005)	0.961 (p=0.002)	-	-
IP by Rancimat	-0.972 (p=0.001)	-0.957 (p=0.003)	-0.957 (p=0.003)	-
α-tocopherol	-0.834 (p=0.039)	-0.888 (p=0.018)	-0.903 (p=0.014)	0.929 (p=0.007)
γ+β-tocopherol	-0.949 (p<0.001)	-0.848 (p=0.033)	-0.854 (p=0.031)	0.929 (p=0.007)
Brown bottles				
	Peroxide Value	K_{232}	ESR analysis	IP by Rancimat
K_{232}	0.966 (p=0.002)	-	-	-
ESR analysis	0.882 (p=0.020)	0.947 (p=0.004)	-	-
IP by Rancimat	-0.983 (p<0.001)	-0.976 (p=0.001)	-0.931 (p=0.007)	-
α-tocopherol	-0.850 (p=0.032)	n.s.	n.s.	0.869 (p=0.025)
γ+β-tocopherol	n.s.	n.s.	n.s.	n.s.

IP: induction period. n.s.: not statistically significant ($p < 0.05$).

Regarding volatile secondary oxidation products formation (Table 7), hexanal, *trans*-2-heptenal, nonanal, and 1-octen-3-ol strongly correlated with storage time for both treatments, while 1-heptanol and oct-3-en-2-ne correlated with storage time only for the oil stored in clear glass and for the oil stored in brown glass, respectively. The continuous increase and the high correlation coefficients with storage time suggest these VACs, which are products from oxidation of unsaturated fatty acids, form the off-flavor of deteriorated BNO.

Tabela 7. Pearson correlation coefficients (p value) between volatile aroma compounds, storage time, induction period by Rancimat method, and tendency of radical formation for cold-pressed Brazil nut oil stored in clear and brown glass bottles for five months

Clear bottles			
	Storage time	IP by Rancimat	ESR analysis
Hexanal	0.870 (p=0.024)	n.s.	n.s.
Trans-2-heptenal	0.959 (p=0.003)	n.s.	0.844 (p=0.035)
Trans-2-octenal	n.s.	n.s.	n.s.
Nonanal	0.932 (p=0.007)	n.s.	0.822 (p=0.045)
3-Octen-2-one	n.s.	n.s.	n.s.
2-Nonanone	n.s.	n.s.	n.s.
1-Heptanol	0.866 (p=0.026)	n.s.	n.s.
1-Octen-3-ol	0.908 (p=0.012)	n.s.	n.s.
Brown bottles			
	Storage time	IP by Rancimat	ESR analysis
Hexanal	0.902 (p=0.014)	-0.881 (p=0.020)	0.813 (p=0.049)
Trans-2-heptenal	0.959 (p=0.002)	-0.940 (p=0.005)	0.893 (p=0.017)
Trans-2-octenal	n.s.	n.s.	n.s.
Nonanal	0.872 (p=0.024)	-0.837 (p=0.038)	0.826 (p=0.043)
3-Octen-2-one	0.815 (p=0.048)	n.s.	n.s.
2-Nonanone	n.s.	n.s.	0.821 (p=0.045)
1-Heptanol	n.s.	n.s.	n.s.
1-Octen-3-ol	0.845 (p=0.034)	-0.814 (p=0.049)	n.s.

n.s.: not statistically significant ($p < 0.05$).

Despite results obtained by ESR and Rancimat methods reflected the possible decomposition of hydroperoxides for the oil stored in clear glass bottles during the fifth month of storage, significant correlations between ESR results and *trans*-2-heptenal and nonanal were found ($r > 0.822$, $p < 0.05$). Likewise, for the oil stored in brown glass, ESR results, which also decreased during the fifth month, strongly correlated with hexanal, *trans*-2-heptenal, nonanal and 1-octen-3-ol ($r > 0.813$, $p < 0.05$). Therefore, these high correlation coefficients at the level of confidence of 0.05 may lead to the conclusion that the ESR and Rancimat results reflect the formation of VACs in stored BNO, which was not demonstrated based on performed analyses.

In summary, the measurement of VACs formation showed to be reliable to assess oxidative status of BNO under the considered storage conditions. The employed ESR and Rancimat methods demonstrated to be solvent-free alternative methods to PV and K_{232} to follow the formation of primary oxidation products in BNO stored under retail conditions. When compared with Rancimat method, the employed spin-trapping ESR spectroscopy method required lower amount of samples (0.001 g vs 2 g) and the time of analysis was considerably faster (5h vs ~10h).

3.4. Conclusion

Based on the analyses performed, it was possible to identify VACs, which may play a key role on off-flavor formation in BNO during storage under retail conditions. The packaging barrier against light exposure of brown glass bottles showed to reduce lipid oxidation rates and depletion of tocopherol analogs. Moreover, the tendency of radical formation detected by a spin-trapping ESR spectroscopy method was demonstrated to be a faster, simpler and less sample-demanding alternative to monitor the oxidative status of BNO stored under the considered conditions, in comparison with PV, K_{232} and Rancimat method. However, if the sample history is not known, the simultaneous measurement of off-flavor VACs formation is recommended.

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4. EFFECT OF WATER ACTIVITY ON LIPID OXIDATION AND NONENZYMATIC BROWNING IN BRAZIL NUT FLOUR

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Abstract

Brazil nut flour (BNF), as a byproduct from Brazil nut water-soluble extract production, has high contents of proteins and linoleic acid. The effect of water activity (a_w) on lipid oxidation and nonenzymatic browning reactions (NBR) rates in BNF has been studied. The tendency of radical formation detected by electron spin resonance (ESR) spectroscopy, formation of lipid hydroperoxides, and browning index were measured in samples with initial a_w of 0.101 and 0.196 throughout 18 days of storage at 60 ºC. Results showed that the tendency of radical formation and the formation of lipid hydroperoxides sharply increased and linearly correlated with browning index for BNF with initial a_w of 0.196, but not for BNF with initial a_w of 0.101. Furthermore, volatile aroma compounds (VACs) from the scission of lipid hydroperoxides, as well as VACs from the breakage of brown polymeric compounds formed during NBR were detected by HS-SPME-GC-MS analysis. Hence, there is indication that the lower the initial a_w, the greater the storage stability of BNF. Moreover, lipid oxidation products may have contributed to NBR for BNF with initial a_w of 0.196.

Keywords: Food powders; Lipid oxidation; Maillard reactions; Water activity; Storage

4.1. Introduction

Bertholletia excelsa tree (family of *Lecythidaceae*) is present in natural rainforests from South America and is known due to its seeds, the Brazil nuts. The manual harvesting of Brazil nuts by local communities is one of the most important non-timber productive activities in the Brazilian Amazon (FAO, 2013). Although global production has increased, Brazil nut trade is still small when compared with other nuts, such as walnuts and hazelnuts (FAO, 2017). One way to expand Brazil nut market is by trading value added co-products (Clay & Clement, 1993). The commercialization of Brazil nut co-products is mainly restricted to the cold-pressed oil. However, local communities have long been using Brazil nut kernels (BNK) to prepare a water-soluble extract known as Brazil nut milk (Clay &

Clement, 1993), which is nowadays commercially available. The solid waste from the milk preparation can be dried and ground to be used as a flour for culinary purposes.

The Brazil nut flour obtained by water extraction (BNF) is a homogeneous powder with 50% lipids, 23% proteins, and moisture content lower than 5% (Regitano-d'Arce, 1998). One BNF particularity is the amount of lipids, which is larger than in Brazil nut flours directly obtained by grinding partially defatted solid wastes from pressing, which contain 10-25% oil (Souza & Menezes, 2004; Santos et al., 2013). In addition, the sugar content of BNF is expected to be low, since it represents only 3.3% in BNK composition (USDA, 2015) and part of this amount is removed during water extraction procedures.

Food quality must be kept from production to consumption; thereby storage conditions are key to successful food commercialization. Two major chemical processes leading to organoleptic and nutritional changes of stored food powders such as BNF are lipid oxidation and Maillard reaction, and both are sensitive to a_w (Hedegaard & Skibsted, 2013). While oxidation processes in lipids generate volatile compounds leading to rancidity, Maillard reactions may also alter the color of the dry food and decrease its nutritional value. The general assumption is that a_w between 0.2 and 0.4 are optimal to retard lipid oxidation, and that Maillard reaction products are not formed at a_w of $\leq$ 0.2 (Labuza, Tannemba & Karel, 1970; Labuza, 1971). However, it is not followed for lipid oxidation in food powders such as whole milk powders (Stapelfeldt et al., 1997) and other different types of dry foods (Labuza 1980). In addition, lipid oxidation and Maillard reactions are interrelated (Zamora & Hidalgo, 2011), and studies on model systems suggest that secondary lipid oxidation products may be sources of carbonyl compounds as reactants for Maillard reactions, whether or not reducing sugars are present (Zamora & Hidalgo, 2011; Hedegaard et al., 2014).

BNF storage stability was previously assessed based on fat acidity, which increased from 1.7 to 2.1 KOH/g, indicating fat hydrolysis during a six-month storage study (Regitano-d'Arce, 1998). Furthermore, no studies evaluating the effect of a_w on storage stability of plant-origin lipid-rich and low-sugar food powders, such as BNF, considering both lipid oxidation and NBR were found. Accordingly, the objective of the present study was to investigate the effect of a_w on deteriorative changes in BNF.

4.2. Materials and methods

4.2.1. BNF preparation

Shelled, dried, and fresh BNK (20 kg) was collected from Óbidos, Pará. BNF preparation was conducted as described by Regitano-d'Arce (1998) and represented at Figure 6.

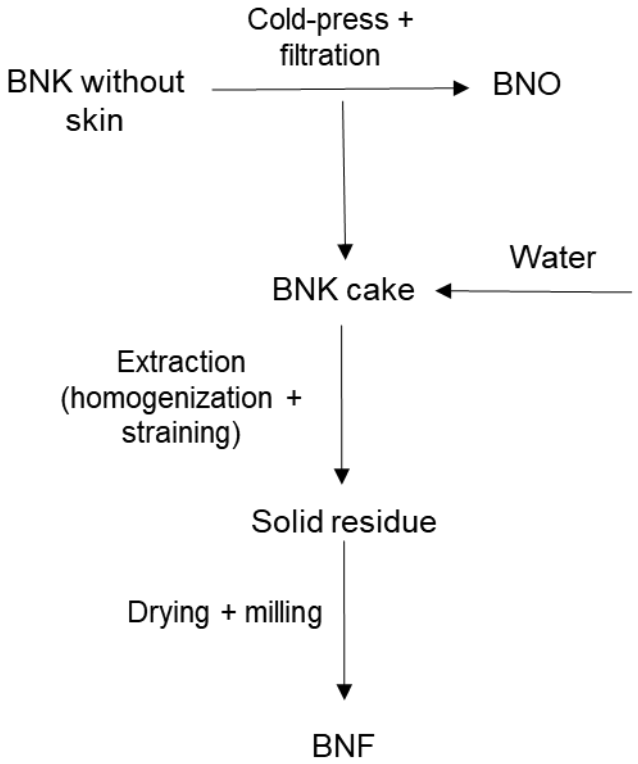

Figura 6. Preparation process of Brazil nut flour obtained by water extraction

BNK Shelled Brazil nut kernels, *BNO* cold-pressed Brazil nut oil, *BNF* Brazil nut flour obtained by water extraction

Two kg of BNK had their brown skins removed and were partially defatted in a Carver hydraulic press (Model B, Sterling Hydraulic, Menomonee Falls, USA) until extraction of around 27% oil in mass. BNK cake was homogenized with distilled water (1:2; w/v) in a food blender for 1 min, strained in sterilized cotton cloth, and sieved through a 149-μm screen. The solid residue retained in the cloth and in the sieve was mixed and dried (4 h in an 80 ºC air-forced oven) and finely ground in a mill (A 11 basic, IKA, Staufen, Germany) (Treatment 1). Half of the obtained BNF (300 g) remained loosely packed in a polyethylene bag at 24 ºC for one month and then underwent freeze-drying for 36 h in order to reduce a_w (Treatment 2).

4.2.2. Storage test

Subsamples (10 g) of both treatments were placed in uncovered identical glass flasks, which were stored in an oven at 60 ºC for 18 days. Two random subsamples of each treatment were collected every two days, vacuum packed and kept at –18 ºC until analysis. Water activity was determined at 25 ºC with an Aqualab CX-2 water activity meter (Decagon Devices, Pullman, USA).

4.2.3. Total oil and protein content and oil extraction

Aiming at characterization, total contents of lipids (925.40) and protein (960.52) were determined according to AOAC methods (Horwitz & Latimer Jr, 2005). Nitrogen conversion factor used was specific for Brazil nut: *N* g /100 g x 5.46 (FAO, 1973).

For analyses in storage test samples, the oil was extracted with hexane (1:5; m:v) 3x and the solvent was evaporated at < 40 ºC.

4.2.4. Fatty acid profile

Fatty acid profile was assessed using a GC-2010 Plus gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector (FID). Separation was achieved on a Rtx-Wax capillary column (30 m x 0.32 mm, 0.25 μm) (Restek, Bellefonte, USA). Nitrogen was the carrier gas (flow rate of 1.2 mL/min), and the volume of injected sample was 1 μL (split 1:20). Initial column oven temperature (60 ºC) was raised to 210 ºC at 20 ºC/min rate and hold for 7 min, and then raised to 240 ºC at 30 ºC/min rate and hold for 12 min. Both injector and FID temperatures were set at 250 ºC. Samples were prepared as described by Hartman and Lago (1973). Methyl esters were identified using peak retention times from a FAME mix GLC-87 standard (Nu-Chek, Elysian, USA) as reference, and quantified by area normalization using methyl tridecanoate (T0627, Sigma-Aldrich, St. Louis, USA) as internal standard.

4.2.5. Tendency of radical formation

The tendency of radical formation was measured by ESR spectroscopy using a MiniScope MS200 ESR spectrometer (Magnettech, Berlin, Germany). Homogenized BNF samples were transferred to 4 mm inner diameter ESR tubes (Wilmad Glass Company, Buena, USA) and placed in the cavity of the instrument. ESR spectrometer settings were center field 3333.69 G, sweep width 1000 G, sweep time 60 s, and modulation amplitude 3 G. Notes were taken for sample mass and height in the ESR tubes to calculate density. Measurements were taken at 22 ºC, in triplicate.

4.2.6. Peroxide value (PV)

PV was determined according to the method described by Shanta and Decker (1994), and adapted by Branco and Castro (2011).

4.2.7. Browning index

Browning index was calculated according to the equation $100(x\text{-}0.31)/0.172$, where $x = X/(X+Y+Z)$ (Buera & Resnik, 1990). Parameters X, Y, and Z were determined using a chromameter (Model CR-400, Konica

Minolta Sensing Inc., Osaka, Japan) previously calibrated toward a white surface. Five determinations were performed for each sample.

4.2.8. Thermal analysis

Glass transition temperature (T_g) was assessed with a previously calibrated differential scanning calorimetry (DSC) system (1 STARe, Mettler Toledo, Columbus, USA). Between 5-15 mg of homogenized samples were transferred into aluminum DSC crucibles of 40 μL (ME 27331, Mettler Toledo, Columbus, USA), which were hermetically sealed. The heating rate was 20 K/min, and T_g was defined as the onset temperature of the endothermic baseline shift. Analysis was conducted in triplicate.

4.2.9. Volatile aroma compounds (VACs)

Both VACs extraction and determination were based on procedures described by de Camargo and others (2016). Extraction: one gram of BNF was placed in 20 mL vial capped with a gas-tight aluminum lid with silicone septum, which was kept submerged into thermostatted water bath at 60 ºC for 20 min. Then, a solid-phase microextraction (SPME) assembly composed of divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS, 50/30 μm) fiber (Supelco, Bellefonte, USA) was inserted through the septum and exposed to the headspace for 10 min. The fiber was inserted into the injector of a GC-2010 gas chromatograph (Shimadzu, Kyoto, Japan) coupled to a mass spectrometer QP 2010 Plus (Shimadzu, Kyoto, Japan). Separation was achieved on a Rtx-5MS capillary column (30 m x 0.25 mm, 0.25 μm) (Restek, Bellefonte, USA). Helium was used as the carrier gas (flow rate of 1 mL/min). Injected sample volume was 1 μL (split 1:40). Spectral data were obtained over a mass range from 40 to 300 m/z. Linear retention index (LRI) were calculated relative to standard *n*-alkane series and compared with literature data (Aaslyng, Elmore & Mottram, 1998; Babushok et al., 2011).

4.2.10. Statistical analysis

Coefficients of variation were ≤ 10%. All data underwent normality and homoscedasticity tests. The two treatments were compared by two-sample-*t* test, and samples within each treatment were compared over time by one-way ANOVA followed by Tukey test. Afterwards, linear models between the parameters were identified by Pearson correlation analysis. Confidence level was 95%. Software used was Minitab® 17 software (Minitab, State College, USA).

4.3. Results and discussion

BNF composition, on dry matter basis, presented 51.14±0.73% lipids and 24.69±0.64% protein, which was comparable with values reported by Regitano-d'Arce (1998), and similar to commercial almond flour, with 58.70% lipids and 22.24% protein (De Pilli et al., 2008). BNF showed to have fatty acid profile similar to BNK (Table 8), with linoleic acid and oleic acid as the major fatty acids, corresponding to 38.75% and 32.40%,

respectively, of the total fatty acid mass. Linoleic acid is a polyunsaturated fatty acid containing one bisallylic hydrogen atom and therefore is prone to be oxidized when in contact with atmospheric oxygen. In addition, BNF contains relevant contents of the saturated palmitic acid (15.71±0.01%) and stearic acid (12.44±0.08%).

Tabela 8. Fatty acid profile of BNK and BNF on time zero

Fatty acids (%)	BNK	BNF
C14:0	n.d.	0.06 ± 0.00
C16:0	15.66 ± 0.07	15.71 ± 0.01
C18:0	11.32 ± 0.07	12.44 ± 0.08
C20:0	0.26 ± 0.00	0.28 ± 0.00
SFA	27.24	28.48
C16:1	0.33 ± 0.00	0.32 ± 0.01
C18:1	32.80 ± 0.08	32.40 ± 0.04
MUFA	33.13	32.73
C18:2	39.62 ± 0.11	38.75 ± 0.06
C18:3	n.d.	0.05 ± 0.00
PUFA	39.62	38.80
UFA	72.76	71.52

Results expressed as mean ± standard deviation (n = 3) of percent mass of total fatty acid mass, *n.d.* not detected, *SFA* saturated fatty acids, *UFA* unsaturated fatty acids, *MUFA* monounsaturated fatty acids, *PUFA* polyunsaturated fatty acids.

Initial a_w of Treatment 1 was 0.196, which is within the range obtained for other BNF batches previously produced in our laboratory (a_w varying from 0.161 to 0.197). Before freeze-drying, the a_w increased from 0.196 to up to 0.326 for Treatment 2, which was lowered to 0.101 after freeze-drying and prior to storage test. During storage, a_w remained almost constant for Treatment 1, likely due to reaching equilibrium between a_w and moisture content. In contrast, a_w tended to decrease, from 0.101 on time zero to 0.076 after 18 days, for Treatment 2 (Figure 7A), which indicates water binding with food components and consequently, reduced content of free water available to undergo chemical reactions. Changes on physical structure were evaluated based on thermal analysis on time zero and after 18 days of storage test. The endothermal step change in heat flow, which indicates BNF texture degradation, occurred at 167.3 ± 5.5 ºC (time zero) and 185.0 ± 2.7 ºC (after 18 days) for Treatment 1, and at 177.3 ± 1.4 ºC (time zero) and 184.2 ± 3.6 ºC (after 18 days) for Treatment 2. The observed T_g were well above the processing and storage temperatures considered and thus, no changes on BNF texture, which could affect deterioration reaction rates, might have taken place for any treatment.

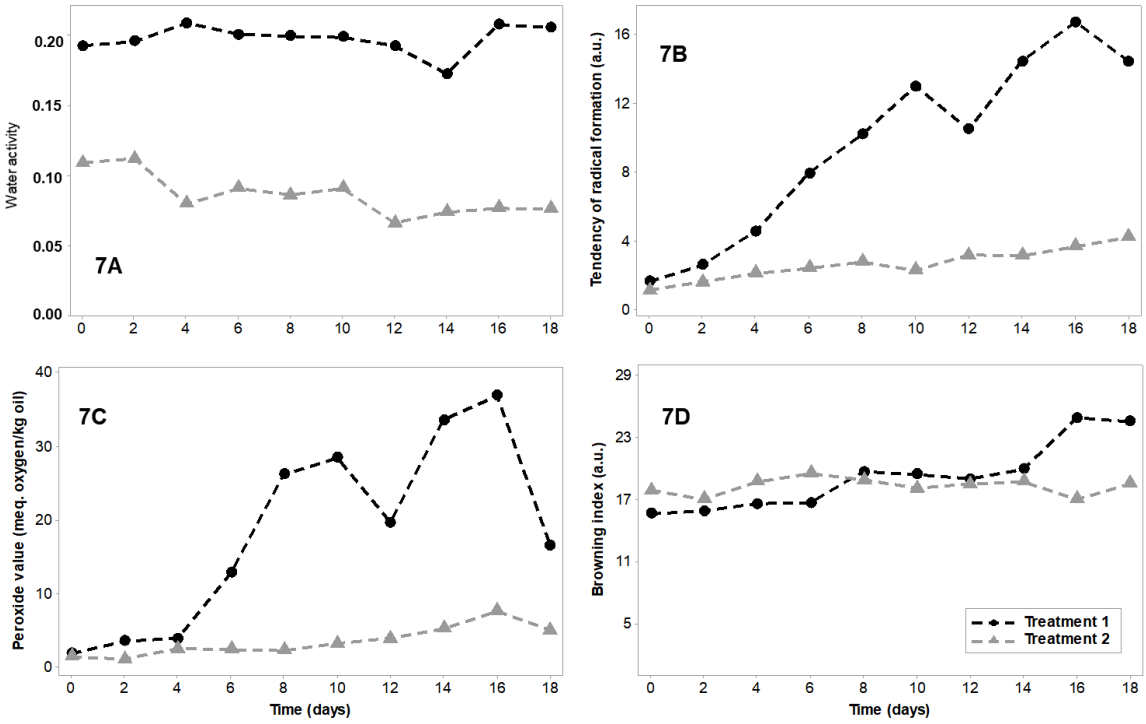

Figura 7. Water activity (7A), tendency of radical formation (7B), peroxide value (7C), and browning index (7D) of Brazil nut flour obtained by water extraction over storage at 60 ºC

Figure 7B shows results for the tendency of radical formation, which was far higher for Treatment 1 than for Treatment 2 over time. Similar tendency was observed for PV (Figure 7C), which indirectly measures primary lipid oxidation products: while Treatment 1 reached 37 meq. O_2/kg oil, Treatment 2 reached 8 meq. O_2/kg oil. These results differ from the general assumption, which postulates a_w < 0.2 may accelerate lipid oxidation supposedly due to the lack of water covering the surface of the oil droplets and preventing it from atmospheric oxygen exposure (Labuza et al., 1971). Similarly, Thomsen et al. (2003) found lower rates of tendency of radical formation and lipid oxidation for an infant formula with a_w values of 0.11 and 0.17 than for the foodstuff with a_w value of 0.31 (Thomsen et al., 2003). Free radicals, which are formed even prior to lipid hydroperoxides and their levels, as detected by ESR spectroscopy, have shown to be a good indicator of early stages of lipid oxidation in several dried products (Hedegaard & Skibsted, 2013). In this study, strong linear correlation was found between the tendency of radical formation and PV for both treatments (Treatment 1: $r = 0.910$, $p < 0.001$; Treatment 2: $r = 0.829$, $p = 0.003$), which suggests both methods may be used to monitor formation of primary lipid oxidation products in BNF. In this context, determination of the tendency of radical formation by ESR spectroscopy presents advantages, such as not destroying samples, being faster than PV and not requiring sample preparation.

The browning index is an adequate tool to measure NBR in foods (Buera and Resnik, 1990). Comparing treatments, Treatment 2 showed to have statistically higher browning index than Treatment 1 on time zero, which indicates the occurrence of NBR within the month under increasing a_w at 24 ºC, from BNF preparation to freeze-drying of Treatment 2 (Figure 2D). However, browning index for Treatment 2 remained rather constant over the whole storage period at 60 ºC with no statistically significant changes over time. In contrast, browning index for Treatment 1 tended to increase significantly during storage test at 60 ºC, showing higher values than Treatment 2 ($p < 0.05$) after 16 and 18 days. Therefore, increased a_w, but not temperature may affect NBR in BNF, likely by

lowering diffusion resistance, and consequently, increasing reactants mobility and reaction products concentration (Labuza, Tannenbaum & Karel, 1970).

Significant correlation coefficients between browning index and tendency of radical formation ($r = 0.888$, $p = 0.001$), and between browning index and PV ($r = 0.716$, $p = 0.020$) were found only for Treatment 1, suggesting a link between NBR and lipid oxidation at initial a_w of 0.196, but not at initial a_w of 0.101 for BNF. This hypothesis is reinforced taking into account the expected low content of reducing sugars in BNF. One proposed mechanism to explain the link between NBR and lipid oxidation is the condensation of lipid carbonyls, such as hexanal, with free amino groups to produce Schiff bases, which polymerize by aldol condensation generating unstable brown polymeric compounds. The brown polymeric compounds break into volatile aroma compounds by scission or dehydration (Zamora & Hidalgo, 2011). Therefore, the interrelation between lipid oxidation and NBR is likely to depend on the mobility of reactants and reaction products concentration, which will increase sufficiently at a_w of ~0.2.

Regarding VACs, they were extracted and determined from samples on time zero and after 18 days of storage (Table 2) and their formation was clearly affected by treatment, since different compounds were formed for each sample.

Tabela 9. Volatile aroma compounds identified in Brazil nut flour obtained by water extraction on time zero and after 18 days storage at 60 ºC

VAC	Criteria	Relative amounts			
		Treatment 1		Treatment 2	
		Time zero	18 days	Time zero	18 days
Aldehydes					
Pentanal	*L*	-	198 ± 11	-	-
Hexanal	*L, LRI, S*	9 ± 0	2293 ± 107	13 ± 1	60 ± 1
Heptanal	*L, LRI*	-	52 ± 1	1 ± 0	-
Trans-2-heptenal	*L, LRI, S*	-	82 ± 3	-	2 ± 0
Octanal	*L, LRI*	-	153 ± 6	3 ± 0	-
Trans-2-octenal	*L, LRI*	-	135 ± 9	4 ± 0	9 ± 1
Nonanal	*L, LRI*	-	179 ± 16	3 ± 0	1 ± 0
Ketone					
2-Heptanone	*L, LRI*	-	28 ± 2	-	-
Alcohols					
1-Pentanol	*L, LRI*	-	41 ± 4	-	8 ± 0
1-Octen-3-ol	*L, LRI*	-	27 ± 1	-	3 ± 0
Trans-2-hexen-1-ol	*L*	-	-	-	1 ± 0
Acids					
Hexanoic acid	*L, LRI*	-	498 ± 30	-	-
Octanoic acid	*L, LRI*	-	27 ± 4	-	-
Heterocyclic compounds					
2{3H}-Furanone,5-	*L*	-	20 ± 1	-	-

ethyldihydro					
2-Butyltetrahydrofuran	*L, LRI*	-	125 ± 10	-	-

Results expressed as mean ± standard deviation of peak area from the chromatogram. Criteria for identification: *L* by matching mass spectrum with Wiley library (similarity ≥ 85%); *LRI* by comparing calculated LRI with those reported in literature (difference ≤ 5%) for similar column types (apolar with stationary phase with 5% phenyl groups); *S* by comparing mass spectrum and retention time with reference standard.

For Treatment 1, only hexanal was detected on time zero, while 15 VACs were detected after 18 days. The content of hexanal increased expressively during this period. Although hexanal is present in fresh Brazil nuts (Clark & Nursten, 1976), it is also an important secondary oxidation product from linoleic acid (Ulrich & Grosch, 1987). Moreover, there is evidence, based on model systems of lipid carbonyls, amino groups and glucose, that hexanal is an important carbonyl source for Schiff bases formation and further generation of brown polymeric compounds (Adams et al., 2009). Furans, which are heterocyclic volatile compounds produced by heating melanoidins or similar brown polymeric compounds (Adams et al., 2009), were also tentatively identified in the present study, corroborating browning index results.

Other VACs detected at the end of Treatment 1 storage were aldehydes, alcohols, ketones, and organic acids, of which several (pentanal, trans-2-heptenal, nonanal, trans-2-octenal, 2-heptanone, and 1-octen-3-ol) are related to lipid oxidation (Ulrich & Grosch, 1987; Adams et al., 2009). Treatment 2 showed other aldehydes and higher amount of hexanal on time zero, when compared with treatment 1, confirming oxidative reactions occurrence before freeze-drying. At the end of storage test, however, hexanal content was far lower, as well as the amounts of the other aldehydes for treatment 1, and therefore, the a_w below 0.1 was more efficient to reduce the rate of flavor deterioration in BNF.

4.4. Conclusions

Results of the present study demonstrate that a_w influences storage stability of BNF and that the lower the a_w the higher the storage stability. Moreover, secondary lipid oxidation products may be major contributors to the formation of Maillard reaction products, depending on initial a_w of BNF. Further studies evaluating other feasible initial a_w and storage temperatures, taking into account also the identification of reducing sugar, free amino groups, and non-volatile brown polymeric compounds should be encouraged in order to elucidate the mechanisms involved in BNF deterioration processes during storage.

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5. GENERAL CONCLUSIONS

The present study focused on the characterization of chemical changes in Brazil nut kernels (BNK), cold-pressed Brazil nut oil (BNO) and Brazil nut flour obtained by water extraction (BNF) and on the study of strategies of control and monitoring these changes during storage.

Different combinations of storage temperatures and packaging atmospheres have affected differently the tendency of lipid radical formation, as detected by spin-trapping electron spin resonance (ESR) spectroscopy and volatile aroma compounds (VACs) in BNK. Refrigeration reduced the tendency of lipid radical formation and of one off-flavor VAC. However, the combination of refrigeration with vacuum packing reduced the formation of hexanal, which is a major off-flavor VAC, as well as of others, and therefore should be recommended for BNK storage.

The measurement of off-flavor VACs formation by HS-SPME-GC-MS was demonstrated to be reliable to follow oxidative changes in BNO stored under retail conditions over time. However, the tendency of radical formation, as detected by the same method used for BNK, showed to be a less sample-demanding and faster solvent-free tool to monitor oxidative status based on primary oxidation products, although the measurement of off-flavor VACs formation is appropriate when sample history is unknown.

Minor variations on water activity (a_w) showed to affect significantly the chemical stability of BNF. Based on the results, it was possible to suggest that the lower the a_w the higher the BNF storage stability. Moreover, secondary lipid oxidation products may be major contributors to the formation of non-volatile brown polymeric compounds and volatile compounds related to Maillard reaction when initial a_w is around 0.2.

As a conclusion, these results are relevant for the academy and for the industry, since they may help to explain better chemical deteriorative processes in Brazil nut and its co-products under different storage conditions and since it was demonstrated that a less sample-demanding, faster and solvent-free analytical method may be used to monitor these changes in BNO.