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Article

Brazil Nut Oil Extraction Using Subcritical *n*-Propane: Advantages and Chemical Composition

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In order to provide an effective and environmentally correct alternative for oil extraction, subcritical *n*-propane was used under different temperature and pressure conditions to obtain Brazil nut oil. The composition of the oil was determined and compared to the oils obtained by conventional methods. The result of the extraction yield obtained using subcritical fluid extraction (SFE) at 60 °C, 6 MPa and granulometry < 1.40 mm (63.13%) presented minor differences to the yield obtained by Soxhlet extraction (SE) (68.44%) and Bligh and Dyer extraction (BD) (59.54%). The composition in fatty acids was similar regardless of the method of extraction used. Oxidized triacylglycerols (TAGs) were found in the oils extracted by SE and BD while they were not detected in the oils extracted by SFE. The quantity of bioactive compounds was higher in the oils obtained by SFE. Thus, the SFE using *n*-propane preserves the nutritional characteristics and lipophilic components of the oil, besides improving the availability of bioactive compounds from an effective "green extraction" without the use of toxic solvents.

Keywords: subcritical *n*-propane, Soxhlet extraction, Bligh and Dyer extraction, green extraction, bioactive compounds

Introduction

Bertholletia excelsa is a large tree, measuring up to 50 m tall and 2 m in diameter. Its fruit is spherical, with a hard, outer shell, and the Brazil nut, a true nut native to the Amazon rainforest, is found inside.[1]

Brazil nut composition may contain about 70% lipids, in addition to carbohydrates (10-12%), proteins (15%), potassium (660 mg 100 g^{-1}), calcium (160 mg 100 g^{-1}), and vitamins (A, B6, B12, C and D). They contain a high index of monounsaturated fatty acids, especially oleic acid (18:1n-9), an important fatty acid that aids in prevention of chronic diseases such as diabetes.[1-4] Brazil nut oil is often used in the cosmetic industry because of its sweetness and agreeable scent. It is commercialized for use as a hydrating oil for skin and hair. In addition, its smooth and agreeable flavor is similar to that of olive oil, so its use in gastronomy has been growing in recent years.[1]

The extraction of this oil is typically done through cold pressing, which does not degrade the bioactive compounds, however, the yield is usually small (30-40%). Therefore, a second extraction is usually performed, using apolar organic solvents, often with elevated temperatures, to increase the effectiveness of the technique. However, fatty acids that

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feature only one unsaturation, such as the oleic acid so abundantly present in Brazil nut oil, are very susceptible to lipid oxidation by high temperatures or light, causing disagreeable flavors and odors.[1] Another disadvantage related to extraction using organic solvents, such as hexane, methanol, ethers and chloroform, is that they are toxic. Whenever these solvents are used, adequate treatment becomes necessary to eliminate the solvents present in the extraction, as well as treatment of the extraction waste.[5,6]

In order to minimize the use of toxic solvents and to prioritize the quality of the oil obtained, research utilizing supercritical (temperature and pressure above the critical points) and subcritical fluid extraction (temperature or pressure above the critical points) (SFE) has been developed for various matrices. The large majority of extractions are performed using carbon dioxide (CO_2) as the extraction agent, due to its lack of toxicity and inflammability, and low critical temperature and pressure.[7] However, scientific researches show that the solubility of CO_2 in vegetable oils is not as satisfactory as solubility of *n*-propane, when similar flows are used. For this reason, the use of *n*-propane is favored for extraction of true nut oils, with less time of duration and similar conditions.[5,8-10] The critical temperature of *n*-propane is elevated, and therefore this solvent tends to be used in the subcritical state (or compressed, or pressurized), with pressures above and temperatures below critical points.[11,12]

Another variable that has been studied is the granulometry of the extraction sample, because it is believed that the smaller the size of the particle, the larger the fluid penetration in the matrix, allowing a better solvation and, consequently, more effective extractions in terms of yield.[13]

Therefore, the objective of this work was to evaluate the ability of *n*-propane to extract Brazil nut oil, to observe the influence of pressure, temperature and particle size on the extraction yield of these oils and to compare chemical composition of the oils extracted by conventional extraction methods (hot and cold with organic solvents) in terms of fatty acids, phytosterols, tocopherols and triacylglycerols.

Experimental

Samples

Three kilograms of Brazil nuts *in natura* were purchased from rural producers in Campo Grande, MS (Brazil). The nuts were shelled, ground quickly in a multiprocessor, and separated into two groups with different granulometries: particles with granulometry above 12 mesh (< 1.40 mm) and between 9-12 mesh (1.40-2.00 mm), using Tyler series sieves (WS Tyler, USA). Next, the samples were placed in polyethylene bags, vacuum sealed, and frozen at −18 °C until the time of analysis.

Extraction of lipids with hot mixture of ethers

For determination of total lipids (TL) using Soxhlet extraction (SE) method, the experiments were conducted according to Soxhlet,[14] AOAC 920.39 method, using a Soxhlet extractor (New Ethics, Brazil). The sample (3.00 ± 0.01) g was initially extracted with 40 mL of a petroleum ether and ethyl ether (Merck Millipore) (1:1 v/v) until reaching the boiling point for 30 min, then remaining in reflux for an additional 930 min. The extraction system was turned off and the solvent evaporated in an air circulation oven. To determine lipid yield, an analytical balance was used. The extractions were performed in triplicate.

Cold lipid extraction

The Bligh and Dyer method (BD) was applied according to the methodology described.[15] Briefly, (3.00 ± 0.01) g of the samples from each size group were submitted to the extraction using a chloroform-methanol-water solution mixture (2:2:1.8 v/v/v) with the addition of 12.00 mL of water. The total lipids were determined by gravimetric analysis. The extractions were performed in triplicate.

Subcritical fluid extraction (SFE)

The nut samples (30.00 ± 0.01) g were placed in the extractor with an internal extraction bed volume of 53.4 cm^3 for extraction with *n*-propane (99.5%, White Martins, Maringá, Brazil). The pressurization of *n*-propane was done using a pump-type syringe (model 500D, Teledyne ISCO) with a thermostatic bath at 10 °C.[16] A flow of 2 mL min^{-1} of *n*-propane through a micrometric valve (Autoclave Engineers) associated with a thermoregulator (Tholz, model CTM-04E) was used to perform the extraction. The process lasted 60 min, with periodic weighing on the analytical balance (model APX-200, Denver Instrument) to determine the kinetics and yields of the extractions. Different conditions of temperature, pressure and particle size were evaluated using the 2^3 factorial design (three factors at two levels) with two replications *per* point (Table 1). The order of extractions was random, and TL was the response.

Analysis and quantification of fatty acids

To conduct the separation and determination of fatty acids by gas chromatography-flame ionization detector (GC-FID),

Table 1. Factors and levels for the 2^3 factorial design

Factor	Symbol	Type	Level	
			−1	+1
Temperature / ºC	T	numeric	30	60
Pressure / MPa	P	numeric	6	12
Particle size / mm	S	numeric	< 1.40	1.40-2.00

the fatty acids of the TL of the samples were methylated, according to the method described by Hartman and Lago.[17] The methyl esters of the fatty acids were analyzed in a Thermo gas chromatograph, Trace Ultra 3300 model, in accordance with Sargi *et al.*[18] Sigma standards (Sigma-Aldrich Co., Brazil) were used as comparison parameters with retention times for identification of the fatty acids. The calculation of the areas of the peaks was performed by ChromQuest 5.0 software, and the quantification of these in mg g^{-1} of TL was performed in relation to the compound methyl tricosanoate (standard) using equation 1.[19,20]

$$FA = \frac{A_X W_{IS} CF_X}{A_{IS} W_X CF_{AE}} \quad (1)$$

where fatty acid (FA) is expressed as mg g^{-1} of TL, A_X is the peak area (FA), A_{IS} is the peak area of the internal standard (IS) methyl ester of tricosanoic acid (23:0), W_{IS} is the IS weight (mg) added to the sample (in mg), W_X is the sample weight (in mg), CF_X is the theoretical correction factor, and CF_{AE} is the conversion factor necessary to express results as mg of FA rather than as methyl esters. The results were converted from FA mg g^{-1} of TL.

Composition of triacylglycerols

The triacylglycerols (TAG) composition was obtained using easy ambient sonic-spray ionization mass spectrometry technique (EASI-MS) in the positive acquisition mode. The analyses were performed according to the methodology described by Zanqui *et al.*[21] using a quadrupole mass spectrometer (LCMS-2010 EV, Shimadzu, Japan), and the main compounds of the oils were differentiated using the specific *m/z* values for the TAG ions.[22,23] The EASI(+) was operated with methanol and nitrogen gas (N_2) as nebulizing gas using the flows at 20 and 2 μL min^{-1}, respectively, where the surface-entrance angle of the mass spectrometer in relation to the EASI source was fixed at 30°. One drop of Brazil nut oil (1 μL) was deposited on Kraft paper, and the MS data were collected at a range of *m/z* 100-1000 over a period of 30 s. The blank of the measurements also was acquired, and the final MS was obtained by the subtraction the sample less the blank. The software LabSolution 3.7 (Shimadzu, Japan) was utilized for data processing.

Analysis and quantification of bioactive compounds

The Brazil nut oils were derivatized according to Beveridge *et al.*,[24] using the BSTFA derivatization ((trimethylsilyl)trifluoroacetamide). Phytosterols, tocopherols, and other bioactive compounds were simultaneously analyzed as described by Du and Ahn.[25] They were determined using a Thermo gas chromatograph, Focus GC model (Thermo-Finnigan) with a DB-5 fused-silica capillary column connected to a mass spectrometer with electron ionization (EI), DSQ II model (Thermo-Finnigan). The Xcalibur 2.0 software performed the identification of the compounds, and the quantification was determined using the internal standard 5-α-cholestane (Sigma).[26]

Statistical analysis

The data were submitted to variance analysis (ANOVA) by Tukey's test and Student's *t*-test at 5% significance and principal components analysis (PCA), using the software Statistica 8.0.[27] For analysis of the main effects and the effects of interactions resulting from 2^3 factorial design, the software Design Expert 7.1.3[28] was used, where it was possible to determine the effect of the independent variables on the response.

Results and Discussion

Total lipids

The results of the extraction yields (oil mass in relation to mass of the sample) of the experiments performed with Brazil nuts for TL are summarized in Table 2. Statistical analyses were performed on two distinct groups of extraction yields. The first group compares only the experiments performed with *n*-propane (1 to 8); the variations are indicated in Table 2 in lowercase letters. The second group (1 to 12) compares all the experiments and are indicated with uppercase letters.

Firstly, only the results obtained by SFE are evaluated. The experiments 1, 2, 3 and 4 performed using particles with 1.40-2.00 mm resulted in yields much smaller than those obtained in the experiments 5, 6, 7 and 8, which were performed with samples with particles with granulometry < 1.40 mm, which means that the minor size of particles is responsible to highest amounts of TL. There are two classifications of lipids that may be extracted by

Table 2. Factors and levels evaluated using 2^3 factorial design from SFE method and Brazil nut lipids extraction yields from SFE, BD and SE methods

Experiment	Temperature / °C	Pressure / MPa	Particle size / mm	TL / %
1 (SFE)	30	6	1.40-2.00	$38.50^{fE} \pm 0.15$
2 (SFE)	60	6	1.40-2.00	$41.29^{deDE} \pm 0.54$
3 (SFE)	60	12	1.40-2.00	$42.57^{dDE} \pm 0.31$
4 (SFE)	30	12	1.40-2.00	$40.05^{eE} \pm 0.71$
5 (SFE)	30	6	< 1.40	$56.69^{cB} \pm 0.16$
6 (SFE)	60	6	< 1.40	$63.13^{aAB} \pm 0.03$
7 (SFE)	30	12	< 1.40	$57.66^{cAB} \pm 0.44$
8 (SFE)	60	12	< 1.40	$61.33^{bAB} \pm 0.15$
9 (BD)	–	–	< 1.40	$59.54^{AB} \pm 0.80$
10 (BD)	–	–	1.40-2.00	$44.38^{DE} \pm 0.41$
11 (SE)	–	–	< 1.40	$68.44^{A} \pm 0.43$
12 (SE)	–	–	1.40-2.00	$47.68^{D} \pm 1.08$

TL: total lipids; SFE: subcritical fluid extraction; BD: extraction performed with Bligh and Dyer method; SE: extraction performed with Soxhlet method. Values followed by different letters in the same column indicate significant difference according to the Tukey's test ($p < 0.05$). Lowercase letters are relative to the variation within 2^3 factorial design (SFE extractions); uppercase letters are relative to the variation among all the extractions (SFE, BD and SE extractions).

SFE, called "easy access oil" and "difficult access oil".[9] The former is the oil that is available on the surface of the particle, in such a way that the fluid easily penetrates and solubilizes the oil that will be extracted. "Difficult access oil" is located in a more internal part of the particle, therefore, there is more necessity for fluid penetration to extract this oil. This behavior are in agreement with previous studies, which showed that the smaller the particle used in the extractions, the higher is the fluid penetration, facilitating the extraction of the most easily accessed oil (convective stage), improving the capacity for extraction of more difficult access oil (diffusive stage).[9,13]

The 2^3 factorial design preceded to study SFE conditions have showed us that the largest yield was obtained in experiment number 6, resulting in 63.13% using the largest temperature studied in the design (60 °C), the lowest pressure (6 MPa) and the lowest granulometry (< 1.40 mm).

Santos *et al.*[4] extracted Brazil nut lipids with supercritical CO_2 over a two-hour period, varying the temperature and pressure of the solvent, and obtained from 22.67 to 67.20% of lipids in the sample, similar values to those determined in this study, as well as other studies that indicate that Brazil nuts usually contain up to 65% lipids.[29]

Figure 1 shows graphs drawn from the kinetics of the extraction, relating the average extraction yields as a function of time for the extractions conducted by SFE.

The difference in extraction yields for nuts with 1.40-2.00 mm (1 and 4) and < 1.40 mm (5 and 8) granulometry is also evident upon observing the graphs

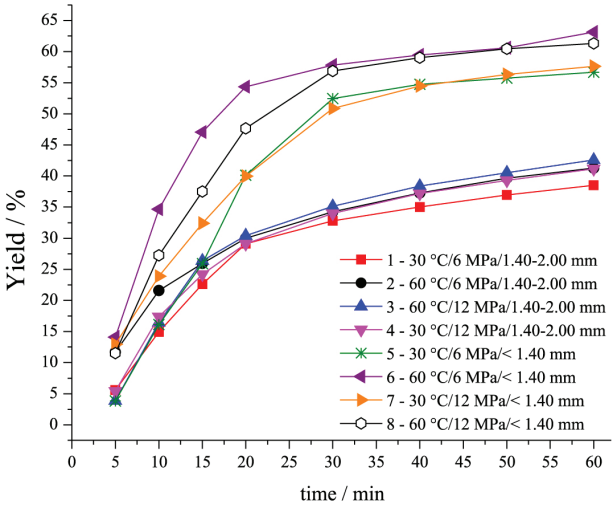

Figure 1. Kinetics of the extraction, relating the average extraction yields with time.

of extraction kinetics. After 50 min, for extractions with granulometry < 1.40 mm, the variation in mass is very small. In addition, the extraction is stabilized possibly by the finalization of the extraction by the convection mechanism occurring only by diffusion, which justifies not prolonging extraction time. The extraction 6 stands out in the first 30 min of the extraction, indicating that the factors used favor the extraction of easy access oil. Results obtained by variance analysis for the response of the lipid extraction yield according to factorial 2^3 design for Brazil nuts are shown in Table 3. The model fits a linear regression described by equation 2, in which T corresponds to the variable temperature, P to the variable

pressure, and S to the size of the particle, Y to response, β_0, constant, β_1, β_2, β_3, β_{12}, β_{13}, β_{23} and β_{123} are the constant terms of regression model.

$$Y = \beta_0 + \beta_1 T + \beta_2 P + \beta_3 S + \beta_{12} PT + \beta_{13} TS + \beta_{23} PS + \beta_{123} TPS + \varepsilon \quad (2)$$

The determination coefficient R^2 for a statistical model should be the closest of the unit. This indicates that the sum total of the residuals of the regression are very small. In this model, $R^2 = 0.9993$, such that 99.33% of the total variation around the average is explained by the regression, with only 0.07% of residuals left. The coefficient of variation was 0.76%, and adjusted R^2 was 0.9986, adequate values that give confidence to the model.

The reference value for the *F*-test at the level of 95%, for this linear model is 5.59%.[30] Table 3 shows that the three variables and their interactions were significant, with values above the reference values. The *p*-values obtained were also satisfactory, below 0.5, as desired. Among the three variables, the one that most contributed to the model was the size of the particle, which, when smaller, favored the extraction yields increase, followed by temperature and interactions between these variables. Figure 2 describes the surface of the response obtained for this model, fixing the particle size in < 1.40 mm (Figure 2a) and 1.40-2.00 mm (Figure 2b).

It is observed that as temperature increases, and pressure decreases, the extraction yield is greater, however, the interaction between the variables also contributed to the model generated, according to the expression of the model (for TL) in terms of temperature (T), pressure (P) and particle size (S) and response EY (extraction yield) given by equation 3.

$$EY = 50.16 + 1.93T + 0.25P - 9.55S - 0.38TP - 0.60TS + 0.46PS + 0.31TPS \quad (3)$$

Comparing the experiment 6 (SFE, 60 °C, 6 MPa and granulometry < 1.40 mm), which presented higher yield among those conducted by SFE method, with experiment 11 (SE with granulometry < 1.40 mm) by Student's *t*-test, the SE method presented result (68.44%) higher than experiment 6 (63.13%) of SFE (60 °C, 6 MPa and particle

Table 3. Data of variance analysis for extraction yields of Brazil nut oils using 2^3 factorial design

Term	Sum of squares	Degree of freedom	Mean square	*F*-value	*p*-value
Model	1533.05	7	219.01	1524.05	< 0.0001
T	59.44	1	59.44	413.67	< 0.0001
P	0.99	1	0.99	6.89	0.0304
S	1459.62	1	1459.62	10157.43	< 0.0001
TP	2.30	1	2.30	15.97	0.0040
TS	5.78	1	5.78	40.25	0.0002
PS	3.35	1	3.35	23.30	0.0013
TPS	1.56	1	1.56	10.87	0.0109
Residuals	1.15	8	0.14	–	–
Total	1534.20	15	–	–	–

T: temperature; P: pressure; S: particle size.

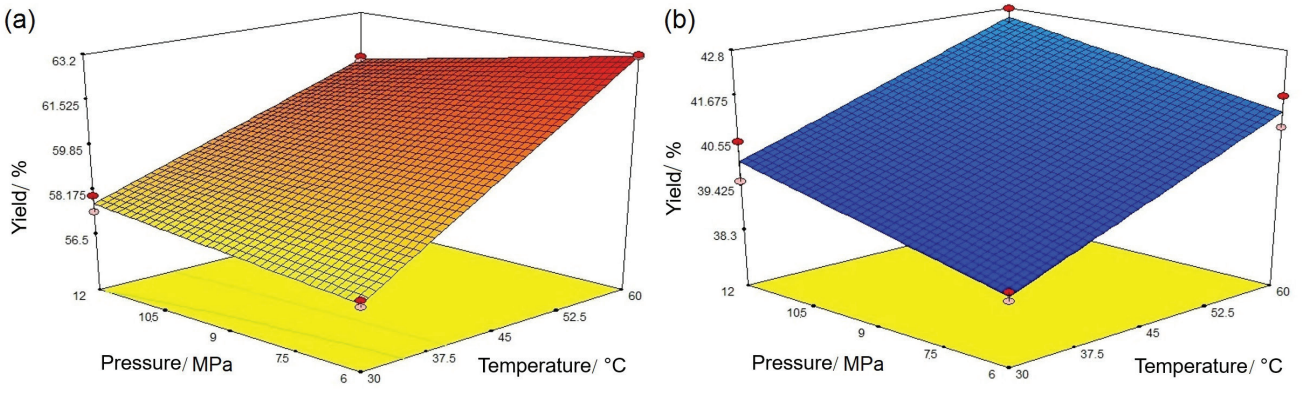

Figure 2. Surface of the response fixing the particle size in (a) < 1.40 mm and (b) 1.40-2.00 mm.

size < 1.40 mm), however, conducted in only 60 min, without the use of toxic solvents, potentially substituting, with great advantages, the use of noxious solvents. The experiments 6 (SFE, 60 °C / 6 MPa), 7 (SFE, 30 °C / 12 MPa), 8 (SFE, 60 °C / 12 MPa), 9 (BD) and 11 (SE) were all conducted with particles with granulometry < 1.40 mm, with minor variations between each other, showing that the use of SFE is as effective as the traditional methods, without the use of noxious solvents.

Composition of fatty acids

The quantification of fatty acids (FA) and the total saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) are shown in Table 4. Between the extractions performed using conventional methods (9 and 11 by SE and BD, respectively), only the oils obtained from samples with granulometry < 1.40 mm were used, because they produced larger extraction yields.

Twelve fatty acids were identified in all the Brazil nut oils by SFE, SE and BD methods. The major fatty acids were linoleic (LA, 18:2n-6), oleic (O, 18:1n-9), palmitic (P, 16:0), and stearic (S, 18:0). The linoleic acid corresponded to 40% of the total fatty acids, varying between 390 to 410 mg g^{-1} of total lipids (TL), followed by oleic acid with about 275 mg g^{-1} of TL. The profile and values obtained in the quantification of fatty acids in Brazil nut oils were in accordance with other studies.[2,4,29]

Santos *et al.*[4] extracted lipids using pressing, petroleum ether, hexane and subcritical CO_2 and identified six fatty acids, coinciding with the major fatty acids obtained in this study.

Statistical variations were observed for all the fatty acids (Table 4) and can also be evaluated by observing the sum totals of the classes of fatty acids. Brazil nut oil lipid composition, regardless of the extraction method used, is about 40% PUFA and 25% SFA, indicating that the use of SFE does not compromise the fatty acid composition of Brazil nuts, and furthermore, is not toxic to the environment or to human health. Studies show that the ingestion of polyunsaturated fatty acids and oleic acid can aid in the prevention of various diseases, and Brazil nut oil can be considered a source of these fatty acids.[31,32]

Composition of triacylglycerols from EASI-MS

The EASI(+)-MS analyses were performed on all samples extracted with subcritical *n*-propane and on experiments by BD and SE methods with granulometry < 1.40 mm (9 and 11). The main TAG extracted from the MS profile with the most abundant ions is summarized in Table 5 with their

Table 4. Quantification and sum total of fatty acids for Brazil nut oil

		Experimental conditions									
		1 (SFE)	2 (SFE)	3 (SFE)	4 (SFE)	5 (SFE)	6 (SFE)	7 (SFE)	8 (SFE)	9 (BD)	11 (SE)
Temperature / °C		30	60	60	30	30	60	30	60	–	–
Pressure / MPa		6	6	12	12	6	6	12	12	–	–
Particle size / mm		1.40-2.00	1.40-2.00	1.40-2.00	1.40-2.00	< 1.40	< 1.40	< 1.40	< 1.40	< 1.40	< 1.40
t_R / min	FA	FA / (mg g^{-1} TL)									
8.7	14:0	$0.48^{ab} \pm 0.02$	$0.46^{ab} \pm 0.01$	$0.49^{ab} \pm 0.01$	$0.50^{a} \pm 0.03$	$0.43^{b} \pm 0.03$	$0.50^{a} \pm 0.01$	$0.47^{ab} \pm 0.01$	$0.50^{a} \pm 0.01$	$0.52^{a} \pm 0.01$	$0.50^{a} \pm 0.01$
11.2	16:0	$136.97^{b} \pm 0.62$	$135.66^{b} \pm 0.74$	$138.54^{ab} \pm 1.02$	$143.99^{a} \pm 5.54$	$137.47^{ab} \pm 0.55$	$139.22^{ab} \pm 0.74$	$136.35^{b} \pm 0.60$	$137.39^{ab} \pm 0.65$	$139.26^{ab} \pm 0.53$	$138.11^{ab} \pm 0.49$
11.8	16:1n-7	$2.94^{ab} \pm 0.08$	$2.88^{ab} \pm 0.04$	$2.95^{ab} \pm 0.03$	$2.96^{ab} \pm 0.10$	$2.76^{b} \pm 0.11$	$3.03^{a} \pm 0.05$	$2.95^{ab} \pm 0.01$	$3.02^{a} \pm 0.06$	$2.96^{ab} \pm 0.03$	$3.08^{a} \pm 0.02$
12.8	17:0	$0.72^{ab} \pm 0.02$	$0.74^{ab} \pm 0.01$	$0.75^{ab} \pm 0.01$	$0.74^{ab} \pm 0.04$	$0.74^{ab} \pm 0.03$	$0.72^{b} \pm 0.03$	$0.71^{b} \pm 0.01$	$0.74^{ab} \pm 0.02$	$0.80^{a} \pm 0.01$	$0.72^{b} \pm 0.01$
14.6	18:0	$109.99^{b} \pm 5.09$	$111.66^{ab} \pm 1.59$	$111.44^{ab} \pm 1.45$	$112.07^{ab} \pm 1.87$	$119.83^{a} \pm 4.09$	$110.16^{b} \pm 2.90$	$112.98^{ab} \pm 0.56$	$108.76^{b} \pm 2.08$	$110.39^{b} \pm 0.27$	$107.95^{b} \pm 0.16$
15.3	18:1n-9 *cis*	$276.21^{b} \pm 2.60$	$276.79^{ab} \pm 0.40$	$277.71^{ab} \pm 0.95$	$274.87^{b} \pm 3.47$	$282.84^{a} \pm 2.64$	$277.84^{ab} \pm 1.56$	$278.92^{ab} \pm 0.21$	$274.54^{b} \pm 1.59$	$278.57^{ab} \pm 0.39$	$275.75^{b} \pm 0.70$
15.5	18:1n-7	$8.01^{ab} \pm 0.01$	$8.17^{a} \pm 0.03$	$7.92^{ab} \pm 0.12$	$7.83^{ab} \pm 0.27$	$7.96^{ab} \pm 0.06$	$7.96^{ab} \pm 0.07$	$7.94^{ab} \pm 0.13$	$7.76^{b} \pm 0.07$	$8.09^{ab} \pm 0.06$	$7.80^{ab} \pm 0.01$
16.8	18:2n-6	$407.27^{a} \pm 7.72$	$406.16^{a} \pm 3.39$	$407.79^{a} \pm 2.00$	$399.36^{ab} \pm 4.11$	$389.75^{b} \pm 5.30$	$408.02^{a} \pm 4.28$	$404.51^{a} \pm 2.63$	$410.99^{a} \pm 4.28$	$402.87^{ab} \pm 1.48$	$407.12^{b} \pm 0.89$
18.4	18:3n-3	$0.83^{bc} \pm 0.01$	$0.78^{c} \pm 0.02$	$0.77^{c} \pm 0.03$	$0.68^{c} \pm 0.06$	$0.71^{c} \pm 0.02$	$0.80^{c} \pm 0.01$	$0.80^{c} \pm 0.03$	$0.85^{bc} \pm 0.05$	$1.04^{b} \pm 0.16$	$1.29^{a} \pm 0.05$
19.1	20:0	$2.62^{a} \pm 0.19$	$2.76^{a} \pm 0.07$	$2.65^{a} \pm 0.03$	$2.55^{a} \pm 0.30$	$2.93^{a} \pm 0.20$	$2.53^{a} \pm 0.14$	$2.74^{a} \pm 0.02$	$2.62^{a} \pm 0.13$	$2.67^{a} \pm 0.01$	$2.51^{a} \pm 0.01$
19.8	20:1n-9	$0.49^{b} \pm 0.03$	$0.52^{b} \pm 0.01$	$1.11^{ab} \pm 0.45$	$2.87^{a} \pm 1.63$	$0.47^{b} \pm 0.11$	$0.45^{b} \pm 0.03$	$0.40^{b} \pm 0.02$	$0.40^{b} \pm 0.05$	$0.58^{b} \pm 0.01$	$0.45^{b} \pm 0.02$
22.7	22:0	$0.41^{b} \pm 0.04$	$0.44^{b} \pm 0.02$	$0.42^{b} \pm 0.01$	$0.38^{b} \pm 0.07$	$0.45^{b} \pm 0.04$	$0.41^{b} \pm 0.05$	$0.43^{b} \pm 0.01$	$0.45^{b} \pm 0.01$	$0.64^{a} \pm 0.01$	$0.42^{b} \pm 0.02$
		Sum of FA / (mg g^{-1} TL)									
SFA		$251.19^{bc} \pm 5.14$	$251.71^{bc} \pm 1.75$	$254.29^{abc} \pm 1.77$	$260.23^{ab} \pm 5.86$	$261.86^{a} \pm 4.13$	$253.54^{abc} \pm 3.00$	$253.69^{abc} \pm 0.82$	$250.47^{bc} \pm 2.18$	$254.28^{abc} \pm 0.60$	$250.22^{c} \pm 0.52$
MUFA		$279.65^{b} \pm 2.60$	$280.20^{b} \pm 0.41$	$281.77^{ab} \pm 1.05$	$280.70^{ab} \pm 3.83$	$286.07^{a} \pm 2.65$	$281.32^{ab} \pm 1.57$	$282.27^{ab} \pm 0.21$	$277.96^{b} \pm 1.59$	$282.11^{ab} \pm 0.39$	$279.28^{b} \pm 0.70$
PUFA		$408.09^{a} \pm 7.72$	$406.94^{a} \pm 3.39$	$408.56^{a} \pm 2.00$	$400.04^{ab} \pm 4.11$	$390.45^{b} \pm 5.30$	$408.82^{a} \pm 4.28$	$405.31^{a} \pm 2.64$	$411.83^{a} \pm 2.28$	$403.91^{ab} \pm 1.49$	$408.41^{a} \pm 0.89$

Mean ± standard deviation. Different letters on the same line indicate significant difference according to the Tukey's test ($p < 0.05$). SFE: subcritical fluid extraction; BD: extraction performed with Bligh e Dyer method; SE: extraction performed with Soxhlet method; t_R: retention time; FA: fatty acids; TL: total lipids; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

respective *m/z* (mass spectra in Figure S1, Supplementary Information (SI) section).

The TAGs were detected mainly as adduct sodium ions [TAG + Na^+], and in much lower intensities than the adduct potassium ions [TAG + K^+] at *m/z* range of 800-950. For all samples the most abundant ions were found to *m/z* 881, 903 and 907, which are related to P-O-O, O-L-L and O-O-O, respectively, in agreement with the main FA quantified in the GC-FID analysis (Table 4). The MS profile for the TAG composition of Brazil nut oil was very similar to another MS profiles of Buriti oils from Amazon forest,[33] unusual oils from *C. flexuosa*, *S. guianensis* and *P. caimito* oils of the northeast of Brazil,[34] and soybean and olive oils[35] described in previous studies.

Table 5 shows some oxidized TAG ions with *m/z* between 940 and 970 could be detected, just in the oil extracted by SE. Some hydroperoxide TAG were also found in the range of *m/z* 933-971, in the oils extracted by SE and BD. The Brazil nut oils extracted by BD and SE showed oxidation products of relevant abundances. The ingestion of products of lipid oxidation can cause health problems, such as high cholesterol, atherosclerosis, and higher risk of developing cancer.[36] Oils extracted by SFE did not indicate oxidized TAGs in relevant relative abundances, then, we conclude that the oils extracted using SFE is "cleaner" in relation to the other evaluated oils.

Bioactive compounds

To evaluate the extraction capacity of bioactive compounds in Brazil nut oil by SFE, the oils were compared to that obtained in experiment 11 by Soxhlet method. Five bioactive compounds were identified and quantified in the samples of Brazil nut oil: squalene, tocopherol, stigmasterol, sitosterol, and amyrin (Table 6). The quantity of squalene, tocopherols, and phytosterols was lower in the oil extracted by Soxhlet in relation to the oils extracted by SFE. These substances may have degraded due to light exposure during the SE extraction, because they are photosensitive, or they were not extracted because they have less affinity for the solvents utilized.

Experiment 8, performed at 60 °C, 12 MPa, and using particles with granulometry < 1.40 mm, stands out as being the most capable of extracting the largest amount of squalene, tocopherols, and stigmasterol, among the other experiments conducted with Brazil nuts. The quantity of tocopherols obtained in experiment 8 was five times larger than the quantity obtained by SE method, showing that the new method can be efficiently applied to the Brazil nut oil extraction. The total values of phytosterols determined in this study are in accordance with Costa *et al.*,[37] who examined various fruits and nuts of the northern and northeastern regions of Brazil, among them, Brazil nuts, and found a total of phytosterols that varied between 47 and 148 mg 100 g^{-1} of TL.

Olive oils, valued for their composition, have between 0.2 and 0.7% squalene,[38] and this study shows that Brazil nut oil contains about 0.3% squalene in its composition. Xiang *et al.*[39] studied various olive oils and determined that the quantity of sitosterol can vary between 27.9 and 84.6 mg 100 g^{-1} of TL, values similar to those quantified for Brazil nut oils extracted by SFE. The quantity of sitosterol is yet another relevant factor in the bioactivity of this oil compared to olive oil, since consumption of squalene

Table 5. Attribution of the principle ions detected by EASI(+)-MS for Brazil nut oil

Composition in TAG	Experiments 1-8 (SFE), 9 (BD) and 11 (SE)		Experiments 9 (BD) and 11 (SE)	
	[TAG + $Na]^+$ (*m/z*)	[TAG + $K]^+$ (*m/z*)	[Hydroperoxide + $Na]^+$ (*m/z*)	[Hydroperoxide + $K]^+$ (*m/z*)
P-LA-LA	877	893	941	957
P-O-LA	879	895	943	959
P-O-O	881	897	–	961
P-S-O	883	899	–	–
LA-LA-LA	901	917	933	949
O-LA-LA	903	919	–	–
O-O-LA	905	921	937	–
O-O-O	907	923	971	–
S-O-O	909	925	–	–
S-S-S	913	–	–	–

EASI-MS: easy ambient sonic-spray ionization mass spectrometry; TAG: triacylglycerol; SFE: subcritical fluid extraction; BD: extraction performed with Bligh e Dyer method; SE: extraction performed with Soxhlet method; P: palmitic acid; S: stearic acid; O: oleic acid; LA: linoleic acid.

Table 6. Quantification of phytosterols, tocopherols, and other bioactive compounds in Brazil nut oil, extracted by different methods

Test	Experimental condition			Bioactive compound / (mg 100 g^{-1} TL)				
	Temperature / °C	Pressure / MPa	Particle size / mm	Squalene	(γ + δ) tocopherols	Stigmasterol	Sitosterol	(α + β) amyrin
1	30	6	1.40-2.00	$296.63^{bc} \pm 19.26$	$15.96^{bc} \pm 1.92$	$4.57^{b} \pm 0.90$	$49.52^{c} \pm 3.85$	$12.20^{b} \pm 1.85$
2	60	6	1.40-2.00	$330.22^{ab} \pm 28.69$	$20.58^{a} \pm 2.16$	$5.40^{ab} \pm 1.05$	$55.55^{abc} \pm 5.43$	$11.09^{b} \pm 0.36$
3	60	12	1.40-2.00	$325.75^{ab} \pm 8.28$	$17.91^{ab} \pm 1.00$	$4.31^{b} \pm 0.20$	$65.06^{a} \pm 5.80$	$7.34^{cd} \pm 0.66$
4	30	12	1.40-2.00	$323.77^{ab} \pm 3.26$	$17.71^{abc} \pm 1.57$	$4.39^{b} \pm 0.03$	$53.81^{bc} \pm 4.39$	$5.35^{d} \pm 0.79$
5	30	6	< 1.40	$315.21^{ab} \pm 6.73$	$13.94^{c} \pm 0.56$	$4.18^{b} \pm 0.38$	$48.08^{c} \pm 0.54$	$8.23^{c} \pm 1.36$
6	60	6	< 1.40	$333.96^{ab} \pm 22.76$	$15.93^{bc} \pm 1.42$	$7.33^{a} \pm 1.85$	$52.27^{bc} \pm 2.32$	$6.79^{cd} \pm 0.44$
7	30	12	< 1.40	$332.48^{ab} \pm 5.54$	$14.18^{bc} \pm 0.33$	$5.92^{ab} \pm 0.34$	$56.36^{abc} \pm 1.46$	$12.22^{b} \pm 1.02$
8	60	12	< 1.40	$353.69^{a} \pm 12.04$	$21.53^{a} \pm 1.38$	$7.42^{a} \pm 1.80$	$62.57^{ab} \pm 4.22$	$12.33^{b} \pm 0.90$
11	SE		< 1.40	$269.94^{c} \pm 16.73$	$4.73^{d} \pm 0.59$	ND	$35.92^{d} \pm 2.63$	$18.49^{a} \pm 0.85$

Mean ± standard deviation. Different letters in the same column indicate significant different ($p < 0.05$) according to the Tukey's test. TL: total lipids; SE: extraction performed by Soxhlet method; ND: not detected.

and sitosterol aids in the prevention of various diseases.[40] Thus, in terms of oil composition, Brazil nut oil may be an alternative to replace olive oil.

Through the principal components analysis (PCA), two principal components (PC) were obtained, which explained approximately 50% of the results (PC1 = 35.84% and PC2 = 12.31%). The other PCs produced progressively smaller eigenvalues (P < 1) (for example PC3 = 10.19%), however, PC1 and PC2 were, in fact, the PC that best describe the data. In the graph of PC1 × PC2 (Figure 3a), two groups were formed, in which it can be observed that the SE extraction method was capable of extracting more amyrin. The same can be observed in PC1 × PC3 (Figure 3b) and in Figure S2 (SI section). The experiments using *n*-propane indicate similarity for the rest of the bioactive compounds, among them, phytosterols and tocopherols, indicating the relevance and of use of this method in the extraction of bioactive compounds. In Figure 3, number 11 indicates SE extraction with granulometry < 1.40 mm and the other numbers indicate the experimental conditions by SFE: 1: 30 °C, 6 MPa, 1.40-2.00 mm; 2: 60 °C, 6 MPa, 1.40-2.00 mm; 3: 60 °C, 12 MPa, 1.40-2.00 mm; 4: 30 °C, 12 MPa, 1.40-2.00 mm; 5: 30 °C, 6 MPa, < 1.40 mm; 6: 60 °C, 6 MPa, < 1.40 mm; 7: 30 °C, 12 MPa, < 1.40 mm; 8: 60 °C, 12 MPa, < 1.40 mm.

Conclusions

The three evaluated extraction methods (SE, BD, and SFE) were effective in the extraction of total lipids of Brazil nuts. Furthermore, the SFE using subcritical *n*-propane brings many advantages in the extraction process, such as the non-use of toxic solvents, the reduction in extraction time, and the maintenance of the main chemical constituents (in terms of fatty acids, triacylglycerols, and phytosterols) of the oils in order to keep the main nutritional properties, when it was compared with the oils obtained from conventional extraction methods. SFE was able to extract more bioactive compounds than SE method. Through regression analysis, it was observed that the three variables evaluated in the SFE extraction process, temperature, pressure, and sample granulometry, play a significant role in determining the yield of the process. In conclusion, it is possible to extract Brazil nut lipids using SFE with subcritical *n*-propane in various conditions, providing a product with preserved nutritional characteristics without the use of toxic solvents and in a shorter time compared to the conventional method.

Supplementary Information

Supplementary information is available free of charge at http://jbcs.sbq.org.br as PDF file.

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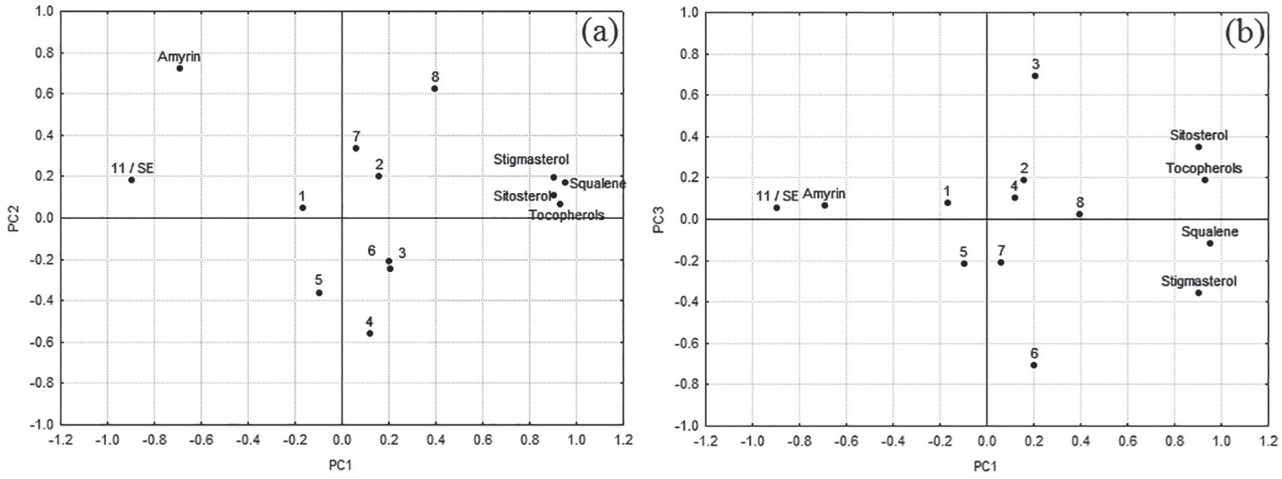

Figure 3. PCA applied to the bioactive compounds of Brazil nut oil to (a) PC1 × PC2 and (b) PC1 × PC3.

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