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Enzyme extraction of cupuassu (*Theobroma grandiflorum* S.) fat seeds

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SUMMARY: Enzyme-assisted extraction is considered an environmentally friendly technique. Cellulase, pectinase and protease were tested for cupuassu seeds fat extraction. The best fat efficiency (81.66%) was obtained for the solute:solvent 1:5 (m:w), orbital shaker at 120 rpm, 60 °C, for 8 hours and enzyme concentrations (cellulase, pectinase and protease) of 1.0%. The fat was characterized for physicochemical properties, fatty acid profile, phenolic compounds, antioxidant activities and oxidative stability. The fat showed good thermal stability (14.26 h) and high contents of monounsaturated (42.42%) and saturated (43.47%) fatty acids with higher concentrations of oleic and stearic acids, respectively, and a high content of phenolic compounds (141.84 µg EAG·g^{-1}) in the fat, and in the aqueous extract (926.47 µg EAG·g^{-1}). The results indicated that the cupuassu seed fat obtained by enzymatic extraction showed superior properties to cupuassu fat obtained by cold pressing, in addition to generating an aqueous fraction which is rich in bioactive compounds that can be used as ingredients in the food and pharmaceutical sectors.

***KEYWORDS*:** *Amazon seeds; By-products properties; Green extraction;* Theobroma grandiflorum.

RESUMEN: ***Extracción enzimática de semillas grasas de cupuaçu (*Theobroma grandiflorum *S.).*** La extracción asistida por enzimas se considera una técnica respetuosa con el medio ambiente. Se probaron celulasa, pectinasa y proteasa para la extracción de grasa de semillas de cupuaçu. La mejor eficiencia grasa (81,66%) se obtuvo para la relación soluto:solvente 1:5 (m:w), agitador orbital a 120 rpm, 60 °C, durante 8 horas y concentraciones de enzimas (celulasa, pectinasa y proteasa) 1,0%. La grasa se caracterizó por sus propiedades fisicoquímicas, perfil de ácidos grasos, compuestos fenólicos, actividades antioxidantes y estabilidad oxidativa. La grasa mostró buena estabilidad térmica (14,26 h) y alto contenido de ácidos grasos monoinsaturados (42,42%) y saturados (43,47%) con mayores concentraciones de ácido oleico y esteárico, respectivamente, y alto contenido de compuestos fenólicos (141,84 µg EAG·g^{-1}) en la grasa y en el extracto acuoso (926,47 µg EAG·g^{-1}). Los resultados indicaron que la grasa de semilla de cupuaçu obtenida por extracción enzimática mostró propiedades superiores a la grasa de cupuaçu obtenida por prensado en frío, además de generar una fracción acuosa rica en compuestos bioactivos que puede ser utilizada como ingrediente en los sectores alimentario y farmacéutico.

PALABRAS CLAVE: *Extracción verde; Propiedades de los subproductos; Semillas Amazónicas;* Theobroma grandiflorum.

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1. INTRODUCTION

The cupuassu tree is found in the forests of the Amazon region, mainly in the states of Pará, Amazonas, Rondônia and Acre. Its fruit is composed of approximately 38% pulp, 17% seeds, 2% placenta and 43% bark (Pereira *et al*., 2018). Cupuassu pulp is appreciated for its characteristic exotic flavor and odor; the seeds are rich in lipids, representing 60% of its total dry weight and promising sources of phenolic compounds (Contreras-Calderón *et al*., 2011).

The conventional processes used for extracting oils and fats from seeds and pulps are pressing, solvent extraction or a combination of both. Other methods such as extraction by super-critical fluids, microwaves, enzymatic and ultrasonic extraction are used, but they present obstacles to large-scale production in efficiency which is compatible with conventional methods (Tang *et al*., 2011). The cupuassu seed fat solid is traditionally extracted by the cold pressing process, with a characteristic composition of fatty acids, and its main use is in the preparation of chocolates as a substitute for cocoa butter (Bezerra *et al*., 2017).

Enzyme fat extraction is considered an environmentally-friendly technology, which employs enzymes which hydrolyze the constituents of the material's cell walls, according to the structural nature of each raw material, where the lipid fraction is associated, making the structure more permeable and exposing the fat. One of the advantages of this process is the use of mild temperatures which reduce the degradation of thermo-sensitive compounds such as pigments and antioxidants, ensuring higher quality to the final product, meeting the demand for quality and safety based on alternative technologies, eliminating the use of solvents and substantially reducing energy consumption compared to conventional methods (Yusoff *et al*., 2017).

The enzymes used for the extraction of oils and fats frequently reported in the literature are protease, α-amylase, cellulase and pectinase, which act on the main constituents of the cell wall and are directly responsible for the oil efficiency of the extraction. In addition to the enzyme, parameters such as pH, temperature, particle size and agitation can contribute to the extraction efficiency. Several researchers have used the enzymatic extraction process to obtain oils and fats in various vegetable matrices (Silva *et al*., 2019). In some of these studies they obtained oil efficiency greater than 80% (Teixeira *et al*., 2013; Hu *et al.,* 2019).

Cupuassu seeds constitute waste material from the production of fruit pulp, and could be used for fat extraction, in view of the greater demand for oils and fats, and considering that the characterization of the quality attributes of cupuassu fat obtained by aqueous-enzymatic extraction has not yet been reported, the present study aimed to use a more sustainable technological process to obtain fat from cupuassu with physicochemical characteristics, technological properties and nutritional quality superior to those obtained by traditional methods.

2. MATERIALS AND METHODS

2.1. Characterization of raw material

The cupuassu seeds (20 kg) from the 2018 harvest were donated by the *Cooperativa dos Fruticultores de Abaetetuba* (COFRUTA), located in the city of Abaetetuba-Pa, and transported to the Food Analysis Laboratory of the Federal University of Pará (Belém – Pará, Brazil).

Fresh seeds were characterized for moisture, lipids, antioxidant capacity (ABTS) and total phenolics.

2.1.1. Moisture and lipids

Moisture content (method no. 925.10) and total lipids (method no. 922.06) were determined according to the official methodologies of AOAC (2002). The value of total lipids found in the lipid extraction with solvent was considered as the maximum extraction efficiency for the purpose of comparison with the value of lipids found in aqueous enzymatic extraction.

2.1.2. Antioxidant activity (ABTS)

The quantification of antioxidant activity was performed by the ABTS radical method as described by Re *et al*. (1999). Results were expressed as μMol of Trolox equivalents per g of sample.

2.1.3. Total phenolic compounds

The concentration of total phenolic compounds was determined by the Folin-Ciocalteu method described by Singleton and Rossi (1965). The colorimetric method is based on the ability to reduce

phosphomolybdic and phosphotungstic acid by the hydroxyl groups of phenols, producing a blue color. Results were expressed as µg of gallic acid equivalent/g of sample (µg EAG g^{-1}).

2.2. Enzymes

The enzymes Celluclast® 1.5L, (700 U g^{-1} of EGU activity), Pectinex® Ultra SP-L (3800 U g^{-1} of PGNU activity) and Alcalase® 2.4L FG (2.4 U g^{-1} of AU-A activity), were kindly donated by a commercial representative of Novozymes® enzymes (Bento Gonçalves, Rio Grande do Sul, Brazil)

2.3. Enzymatic aqueous extraction

2.3.1. Pre-treatment of raw material

To improve the efficiency in the extraction of fat from the cupuassu seed, the skin was removed and then the almond underwent three pre-treatments: drying in an oven with air circulation (Ethik Technology®, 400-2ND, Vargem Grande Paulista, São Paulo, Brazil) (60 °C;18 hours), autoclaving (121 °C; 1 minute), autoclaving (121 °C; 1 minute) and dry (60 ºC; 18 hours) and fresh. The pre-treatment which showed the highest fat extraction efficiency was autoclaved and drying, and thus was the one used in studies on enzymatic extraction conditions. (Figure 1).

After pre-treatment, the dried almonds were ground in a domestic blender (Walita) and sieved in a Tyler series sieves with 20-mesh granulometry (selected based on previous laboratory work) to standardize the particle size and facilitate the extraction process. The crushed product was packed in transparent plastic bags with a capacity of 300 g and stored at room temperature (25 ºC) until the tests were carried out.

2.3.2. Enzymatic aqueous extraction process

The enzymatic extraction process was performed according to the method proposed by Teixeira *et al*. (2013). The conditions used for the extraction study were selected according to published works (Rosenthal *et al*., 2001), solute:solvent 1:5 (m:w), orbital shaker (Lucadema, Brazil) at 120 rpm, 60 °C, for 8 hours and enzyme concentrations (cellulase, pectinase and protease) 1.0% according to the manufacturer's recommendation. After 8 h of extraction the enzymes were inactivated at 80 °C for 5 minutes and the mixture was centrifuged for 20 minutes at 10,000 g, to separate the aqueous phase from the oil phase. The fat extraction efficiency was calculated according to Equation 1.

$$\text{Efficiency } \% = \frac{Wo\,(g)/\,Wp\,(g)}{Wt\,(g/g)}.100 \quad (1)$$

Where: *W*o is the mass of fat extracted by the enzymatic method (g), *W*p is the total mass of the sample used in the enzymatic extraction process (g) and *W*t is the mass of fat present in the sample extracted by solvent (g). After separating the phases of the mixture, the oily (fat) and aqueous (aqueous extract) fractions were characterized.

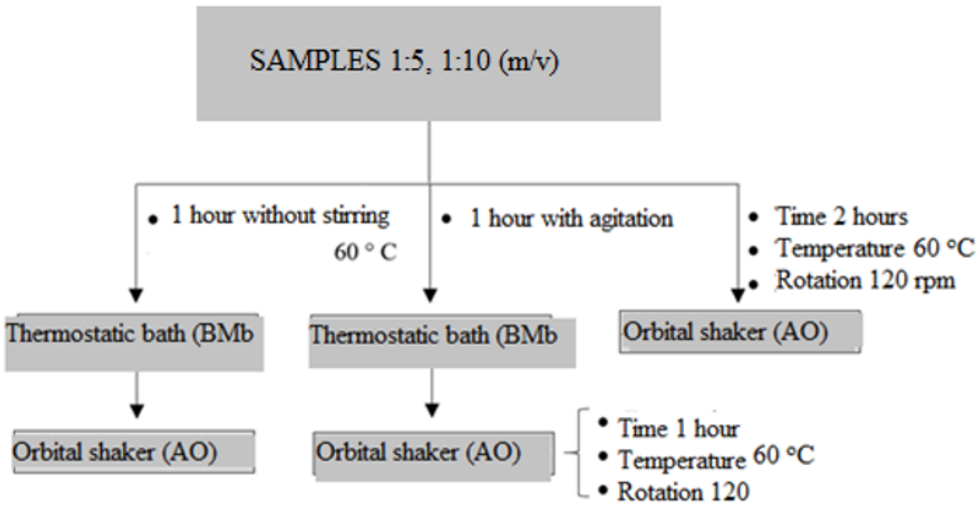

Figure 1. Evaluation of different incubation systems as pre-treatment for the enzymatic aqueous extraction of fat from cupuassu seeds.

2.3.3. Cupuassu seed fat characterization

Fatty acid composition. Fatty acid composition was determined by converting fatty acids into methyl esters (FAMEs) based on the method proposed by Rodrigues *et al.* (2010) and identified using a gas chromatograph (Varian model CP 3380, Texas City, USA) equipped with a flame ionization detector and CP-Sil 88 capillary column (length 60 m, internal diameter 0.25 mm, thickness 0.25 mm). The results were expressed as a relative percentage of the total fatty acids.

Physical chemical Indexes. Acidity was determined according to the Cd 3d-63 method (AOCS, 2004), peroxide value was determined according to the Cd 8-53 method (AOCS, 2004), iodine value was determined by the indirect method Cd 1c-85 (AOCS, 2004), saponification was determined by the indirect method Cd 3b-76 (AOCS 2004).

Oxidative stability. Oxidative stability was determined using the Rancimat equipment (Rancimat Metrohm model 873, USA) according to the method Cd 12b-92 (AOCS, 2004) at a temperature of 130 °C and an air flow of 20 $L \cdot h^{-1}$.

Nutritional parameters. Atherogenicity (*AI*) and thrombogenicity (*TI*) indexes were calculated based on the fatty acid profile, Equations 2 and 3, respectively, according to the methodology proposed by Ulbricht and Southgate (2001).

$$AI = \frac{C12{:}0 + 4\ x\ C14{:}0 + C16{:}0}{\Sigma MUFA + \Sigma FA\omega 6 + \Sigma FA\omega 3} \quad (2)$$

$$TI = \frac{C14{:}0 + C16{:}0 + C18{:}0}{(0.5\ x\ \Sigma MUFA) + (0.5\ x\ \Sigma FA\omega 6) + (3\ x\ \Sigma FA\omega 3) + (\Sigma FA\omega 3 / \Sigma FA\omega 6)} \quad (3)$$

Where C12:0, C14:0, C16:0, and C18:0 are relative percentage masses of lauric, myristic, palmitic, and stearic acids, respectively; *MUFA* is the relative percentage mass of monounsaturated fatty acids; *FAω6* and *FAω3* are the relative percentage mass of omega-3 fatty acids and omega-6 fatty acids, respectively.

Solid fat content. Solid fat content was determined by nuclear magnetic resonance (NMR) (Bruker pc120 Minispec, German) by the direct method, at temperatures of 10, 20, 25, 30, 35, 40 and 45 °C, according to the Cd 16b-93 method (AOCS, 2004).

Thermal analysis. The evaluation of the thermal decomposition profile, TG and DTG, of the fat was carried out by thermogravimetric analysis, under the following conditions: heating rate of 10 $°C \cdot min^{-1}$, temperature range from 27 to 600 °C and flow of 50 mL nitrogen$\cdot min^{-1}$.

Fourier transform infrared spectrophotometry (FTIR). For the ATR-FTIR analyses, Shimadzu Corporation IR Prestige 21 Cat. No. 206-73600-36-Kyoto-Japan was used. All spectra were obtained in the range of 4000 – 600 cm^{-1}, with a resolution of 4 cm^{-1} and 32 scans. Origin Pro v8.0 software was used to graphically plot the extracted spectra.

Table 1. Preliminary tests for cupuassu seed fat extraction efficiency (2 h extraction time, cellulase enzyme)

		Efficiency (%) ± SD			
Conditions	**Dilution**	**Dry**	**Autoclaved**	**Autoclaved + Dry**	**Control**
BMa	1:5	28.02b ± 0.38	16.10c ± 0.15	57.44a ± 0.26	11.62d ± 0.65
	1:10	28.18b ± 0.17	11.57c ± 0.13	55.15a ± 0.27	10.33c ± 0.58
BMb	1:5	36.56b ± 0.24	13.81c ± 0.86	63.86a ± 0.10	11.12d ± 0.37
	1:10	28.18b ± 0.17	11.36c ± 0.19	56.93a ± 0.25	10.74c ± 0.38
AO	1:5	42.90b ± 0.15	28.24c ± 0.20	69.95a ± 0.11	17.35d ± 0.25
	1:10	32.77b ± 0.16	12.33c ± 0.14	61.74a ± 0.75	10.11d ± 0.20

Different letters on the same line indicate a statistical difference at 5% significance. SD: Standard deviation; BMa: Thermostatic bain without stirring. BMb: Thermostatic bain with agitation. AO: Orbital shaker. SE: Dry. AU: Autoclaved. AU+SE: Autoclaved + Dry. CO: Control.

Antioxidant activity. Antioxidant activity of the cupuassu seed fat and aqueous extract were determined by the ABTS radical method as described by Re *et al*. (1999). Results were expressed in µMol of Trolox equivalents per g of sample. The concentration of total phenols was determined by the Folin-Ciocalteu method described by Singleton and Rossi (1965).

2.4. Statistical analysis

All analyses were performed in triplicate. The results were statistically evaluated by analysis of variance (ANOVA) and Tukey's test for comparison of means, at the level of 5% probability, using the STATISTICA 12.0® software.

3. RESULTS AND DISCUSSIONS

3.1. Physicochemical characteristics of cupuassu seed fats

The cupuassu seeds studied in this work had a high moisture content (68.25 ± 0.49%) and the lipid content was 45.03 ± 0.58% (d.b.), consistent with data found in the literature (Silva et *al*., 2018). The concentration of phenolic compounds and the antioxidant capacity observed in fresh cupuassu seeds was 1,503.07 ± 22.38 µg EAG·g^{-1} and 320.42 ± 9.48 µmol Trolox·g^{-1} of sample, respectively. The content of phenolic compounds in cupuassu seeds was close to that reported for cocoa beans (1,441 µg EAG·g^{-1}) in the study conducted by Hu *et al*. (2016). Matrices rich in lipid content and bioactive compounds were present, in addition to other benefits, such as the potential antioxidant capacity, a characteristic directly related to the stability and quality of vegetable oils or fats in general (Hu *et al*., 2016).

3.2. Cupuassu seed oil aqueous enzymatic extraction

Based on preliminary tests, the test with the highest extraction yield (69.95 ± 0.11) was the one in which the raw material was subjected to autoclaving followed by drying, confirming that the heat applied during this step helps to break the wall of the plant cell and facilitates the extraction of fat. Along with this, the most advantageous incubation condition was found when the orbital shaker was used. Efficiency values were maximized when using the lowest solution dilution value (1:5).

Under these previously defined conditions (Table 1), the results of oil extraction efficiency at different times for the three enzymes studied are shown in Table 2. It was observed that as the time increased, the greater the fat extraction efficiency was, until it reached 6 hours, and was kept constant for up to 8 hours of extraction. This behavior was expected, as the longer the enzyme-substrate contact time, the greater the fat extraction (Rosenthal *et al*., 2001). The fat extraction efficiency values found in the processes for the studied enzymes ranged from 31.61 to 81.66%, indicating that the the enzymatic process may be a viable alternative for obtaining fat from cupuassu seeds, resulting in a product obtained from an environmentally-friendly process which is suitable for the Amazon biome.

The protease enzyme was the one that obtained the highest values for fat extraction efficiency from cupuassu seeds (81.66%), an expected behavior, because protein is one of the main components of this seed (15.9%, d.b) (Silva *et al*., 2018). The efficiency values found under this condition are comparable to those obtained by traditional methods, indicating once again the feasibility of its use in commercial

Table 2. Kinetics of enzymatic extraction (cellulase, pectinase and protease) from cupuassu seed fat.

Enzymes	Enzyme extraction efficiency (%)*				
	Time (h)				
	0	2	4	6	8
Protease	36.83 ± 0.28e	58.24 ± 0.23d	69.70 ± 0.41c	80.86 ± 0.21b	81.66 ± 0.16a
Pectinase	33.07 ± 0.53e	53.72 ± 0.27d	64.36 ± 0.33c	70.03 ± 0.23b	75.07 ± 0.32a
Cellulase	31.61 ± 0.51e	54.65 ± 0.18d	56.21 ± 0.17c	68.50 ± 0.19b	77.60 ± 0.29a

*: Calculated as a function of fat content determined by solvent extraction - Soxhlet (45.03 ± 0.58%) (Equation 1). The result presented is the triplicate mean ± standard deviation. Means followed by different letters in the same row are significantly different by Tukey's test ($p \leq 0.05$.

processes (Teixeira *et al*., 2013; Hu *et al*., 2019). It is important to highlight that the use of enzymes, in addition to minimizing environmental impacts, produces oils/fats in milder conditions and with their original characteristics.

Similar oil efficiency results were found by Teixeira *et al*. (2013) in the study of enzymatic extraction from palm pulp (82.35%), using different combinations of enzyme blends (pectinase, cellulase and tannase) and by Santos *et al*. (2022), with the enzymatic extraction of oil from tucumã-i-da-várzea pulp, with pectinase, reaching an extraction efficiency of 81.51%.

Compared to the conventional processes of extraction of oils and fats usually employed, such as pressing and solvent extraction, which have efficiency of between 50 and 80%, the enzymatic extraction proved to be effective in the recovery of fat from the cupuassu seed, and also with the advantage of not presenting a risk of contamination with chemical residues and the use of relatively low extraction temperature, which is considered to be important for the quality of the final product (Daukšas *et al*., 2012; Jiao *et al*., 2014; Hu *et al*., 2019).

In addition to the advantages presented in relation to the extraction process is the quality of the fat obtained, another advantage presented in the aqueous extraction is the generation of the aqueous extract, a by-product which is rich in antioxidants, and can expand the possibilities of use in the industry for the development of new products and for adding value to the products obtained from the Amazon's oilseeds.

3.3. Cupuassu seed fat characterization

Cupuassu seed fat samples obtained from the extraction of 8 hours, were characterized for three enzymes.

3.3.1. Physicochemical characterization

Table 3 presents the characterization of cupuassu fat obtained by the enzymatic process (cellulase,

Table 3. Physicochemical composition and properties of cupuassu seed fat enzymatic extraction (cellulase, pectinase and protease) (CFE).

Fatty acids (%)	Celulase	Pectinase	Protease	Bezerra *et al.* (2017)*
Palmitic acid (C16:0)	8.02 ± 0.13^{a}	7.92 ± 0.20^{a}	7.44 ± 0.11^{a}	7.46 ± 0.36^{a}
Stearic acid (C18:0)	33.34 ± 0.25^{b}	36.62 ± 0.41^{a}	33.92 ± 0.34^{b}	31.45 ± 0.35^{c}
Oleic acid (C18:1, ω-9)	41.84 ± 0.36^{ab}	40.78 ± 0.28^{b}	42.5 ± 0.24^{a}	41.91 ± 0.91^{ab}
Linoleic acid (C18:2, ω-6)	3.56 ± 0.14^{a}	3.71 ± 0.30^{a}	3.58 ± 0.01^{a}	2.95 ± 0.09^{b}
Linolenic acid (C18:3, ω-3)	10.49 ± 0.31^{b}	10.98 ± 0.19^{ab}	10.59 ± 0.05^{b}	11.59 ± 0.32^{a}
Araquidic acid (C20:0)	0.47 ± 0.01^{a}	0.45 ± 0.00^{b}	0.45 ± 0.00^{b}	-
Behenic acid (C22:0)	1.53 ± 0.01^{a}	1.64 ± 0.02^{a}	1.67 ± 0.02^{a}	1.68 ± 0.16^{a}
∑ SFA	43.28	46.60	43.42	40.59
∑ MUFA	41.92	40.80	42.41	41.91
∑ PUFA	14.06	14.70	14.17	14.54
AI	0.14	0.14	0.13	0.13
TI	0.72	0.76	0.71	0.64
Iodine index (g I_2 ·100 g^{-1})	69.68	70.26	70.38	64.1
Saponification index (mg KOH·g^{-1})	190.61	190.69	190.61	191.12
Acidity index (mg KOH·g^{-1})	1.26 ± 0.05^{b}	1.14 ± 0.18^{b}	1.07 ± 0.03^{b}	11.46 ± 0.87^{a}
Peroxide value (meq O_2·kg^{-1})	5.69 ± 0.21^{b}	5.77 ± 0.19^{b}	5.46 ± 0.11^{b}	14.28 ± 0.95^{a}
Oxidactive stability (h)	14.15 ± 0.29^{a}	14.20 ± 0.11^{a}	14.26 ± 0.17^{a}	2.38 ± 0.17^{b}
Bioactive compounds				
Oil phase				Fresh seed
ABTS (µmol Trolox·g^{-1})	18.29 ± 1.40^{b}	18.74 ± 1.74^{b}	22.09 ± 1.08^{b}	320.42 ± 22.38^{a}
TPC (µg EAG·g^{-1})	134.50 ± 12.57^{b}	125.54 ± 10.36^{b}	141.84 ± 12.57^{b}	1,503.07 ± 9.48^{a}
Aqueous phase				
ABTS (µmol Trolox·g^{-1})	106.26 ± 4.26^{b}	110.27 ± 3.94^{b}	122.76 ± 4.70^{b}	320.42 ± 22.38^{a}
TPC (µg EAG·g^{-1})	897.06 ± 15.31^{b}	838.24 ± 15.72^{c}	926.47 ± 22.61^{b}	1,503.07 ± 9.48^{a}

The result presented is the mean of triplicate ± standard deviation. *SFA*: saturated fatty acids; *MUFA*: monounsaturated fatty acids; *PUFA*: polyunsaturated fatty acids; *AI*: atherogenicity index; *TI*: thrombogenicity index.
Means followed by different letters in the same row are significantly different by Tukey's test ($p \leq 0.05$).

pectinase and protease) (CFE) and of the commercial fat (CCF) obtained from the literature, in work carried out with cupuassu fat obtained by cold extraction. The main fatty acids found in cupuassu seed fat extracted by the enzymatic method were oleic acid (42.35%), stearic acid (33.72%) and linolenic acid (10.59%), a composition that gives greater plasticity to cupuassu seed fat. These fatty acids and most of the others are in accordance with the profile found by Bezerra *et al*. (2017).

Enzymes can influence the concentration of fatty acids, which can be attributed to the action on different tissues of the cupuassu seed according to the way the enzyme works, as well as to the bonds of fats in the seed. However, despite these differences, the general fat profile is not significantly altered for all enzymes. Cupuassu fat extracted with pectinase showed the highest concentration of stearic acid ($p < 0.05$), and the lowest concentration of oleic acid, which presented the highest concentration in the fat extracted by protease.

Cupuassu seed fat has higher levels of unsaturated fatty acids in relation to cocoa and murumuru fats, and can be considered high quality nutritional fat due to the high content of monounsaturated fatty acids, such as oleic acid which play an important role in the prevention of retinal and cardiovascular diseases and in the formation of nerve cells, among other benefits (Rodrigues *et al*., 2010; Bhattacharjee *et al*., 2020).

Oleic acid (ω-9) is the most important in the group of monounsaturated fatty acids, and is present in high concentrations in cupuassu seed fat, and traditionally found in vegetable oils, such as olive, canola, avocado and oil seeds, nuts, walnuts and almonds, which makes it another source of lipids in the diet (Garcia-Aloy *et al*., 2019).

In addition, cupuassu fat extracted by enzymes presented high saturated fatty acids (42.43%), which has a beneficial effect on thermal stability, suggesting that this fat may be useful in the food industry as a frying fat. These characteristics show the versatility of cupuassu seed fat for the food industry (production of cupulate, fat for frying, source of ω-9) in addition to its consolidated use by the cosmetic industry (Connor, 2000; Rodrigues *et al*., 2010).

The iodine index of cupuassu seed fat was 70.38 g $I_2 \cdot 100\ g^{-1}$, close to the value found by Bezerra *et al.* (2017) of 64.1 g $I_2 \cdot 100\ g^{-1}$. These values are in agreement with the fatty acid compositions, raw materials with high contents of unsaturated fatty acids (Dubois *et al*., 2007; García-González *et al*., 2013).

The Codex Alimentarius Commission (1999) established the limits for maximum acidity and peroxide values for cold-pressed and unrefined oils and fats, as 4.0 mg $KOH \cdot g^{-1}$ and 15 meq $O_2 \cdot kg^{-1}$, respectively. Ther acidity index (1.07 mg $KOH \cdot g^{-1}$) and peroxide value (5.46 meq $O_2 \cdot kg^{-1}$), of cupuassu seed fat showed values within the established standard indicating that the extraction method used as well as the fat storage was adequate.

Oxidative stability is an important property of oils and fats and is expressed as the time required for the formation of oxidation by-products which are detected under different conditions (expressed by the induction period - *IP*) (Pardauil *et al*., 2011). Cupuassu seed fat extracted by the enzymatic process presented high oxidative stability (14.26 h), indicating that it has a high temperature resistance which is compatible with refined fats such as palm (Teixeira *et al*., 2013) and coconut (Mohammed *et al*., 2021). In addition to the fatty acid profile, oxidative stability may also be related to the concentration of antioxidants present in the fat (Pardauil *et al*., 2011).

In general, the oxidative stability of vegetable oils such as cotton (1.50 h), canola (1.85 h), soybean (1.51 h) and sunflower oils (0.88 h) is lower due to the predominance of unsaturated fatty acids in their composition (Anwar *et al.*, 2003).

3.3.2. Nutritional quality

Atherogenicity (*AI*) and thrombogenicity (*TI*) indexes are directly related to the potential to stimulate platelet aggregation. Lipids with lower *AI* and *TI* values have a greater potential to prevent coronary heart disease; values lower than 1.0 and 0.5, respectively, are recommended in terms of human health (Fernandes *et al*., 2014). Cupuassu fat has a value for *AI* (0.13) which indicates that these samples have high levels and equivalents of anti-thrombogenic fatty acids and *TI* results presented a value of 0.71, higher than indicated for this parameter, due to the saturated fatty acid composition of these products, especially stearic acid (Table 3).

Bezerra *et al*. (2017), analyzed oils and fats from some Amazonian raw materials, including cupuassu seed fat, and found *AI* and *TI* indexes, which ranged from 0.02 to 1.03 and 0.14 to 2.01, respectively.

Santos *et al*. (2022), found values of *AI* (0.09) and *TI* (0.6) in tucumã-i-da-várzea pulp oil. The results indicate the nutritional potential of these Amazonian oil and fat sources.

3.3.3. Antioxidant activity

Cupuassu seed fat showed potential antioxidant activity of 22.09 and 122.76 µmol Trolox·g^{-1} and total phenol values of 141.84 µg EAG·g^{-1} (Table 3) and in aqueous phase (926.47 µg EAG·g^{-1}).

These results suggest that the fat obtained by enzymatic extraction has technological properties which are superior to the fat extracted by the conventional method, and can be better explored and absorbed by the industrial sector of the pharmaceutical, cosmetic and food areas, in addition to representing a sustainable friendly technique that collaborates in the preservation of the environment and encourages the use of residues from the agro-industry.

The determination of phenolic compounds in oils and fats is considered necessary, as it is one of the important indicators of oil quality. These compounds are responsible for the ability to scavenge free radicals and lipid peroxidation.

Phenolic compounds are water soluble, that is, soluble in water, a fact that can be proven by the concentration of phenolics found in the oil phase (fat) 141.84 µg EAG·g^{-1} and in the aqueous phase (extract) 926.57 µg EAG·g^{-1} (Hayouni *et al*., 2007). The enzymatic extraction process makes it possible to potentiate the use of antioxidant compounds, since the fat-soluble compounds present in the raw material remain in the fat and the water-soluble compounds are extracted by water, thus producing two extracts. Teixeira *et al*. (2013), characterized palm oil in terms of the content of phenolic compounds and found values between 14.76 and 26.43 µg EAG·g^{-1} oil. Jiao *et al*. (2014), used the enzymatic aqueous extraction to obtain pumpkin seed oil and found that this extraction (128.8 µg EAG·g^{-1}oil) had a greater effect on phenolic compounds than the Soxhlet extraction (73.3 µg EAG·g^{-1} oil), confirming the efficiency of this extraction technique for the preservation of bioactive compounds.

According with the physicochemical properties of the cupuassu seed fat obtained by the enzymatic extraction process, it can be verified that for all properties, the values found are close (fatty acid profile and *AI* and *TI*) or higher (oxidative stability, acidity, peroxide) than presented by commercial fat, with emphasis on the quality and antioxidant compounds.

3.3.4. ATR-FTIR

The ATR-FTIR spectra, obtained in the region from 4,000 to 500 cm^{-1}, of the cupuassu seed fat samples (extracted by cellulase, pectinase and protease) are shown in Figure 2. Table 4 shows the wave number (cm^{-1}) of the peaks identified in the spectra for the cupuassu fat samples extracted with

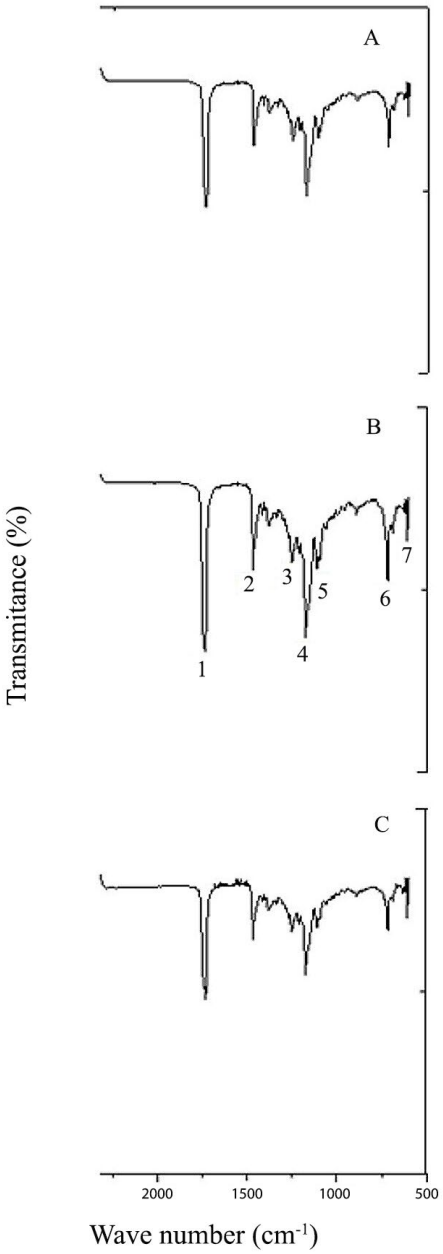

Figure 2. Infrared vibrational spectra of cupuassu seed fat extracted by enzymatic process (CFE), celulase (A), pectinase (B), and protease (C).

Table 4. Wave number (cm^{-1}) of the peaks identified in the FTIR spectra of cupuassu seed fat samples extracted by cellulase, pectinase and protease.

Identification (Band)	Cellulase	Pectinase	Protease
	cm^{-1}		
1	2918	2914	2920
2	2851	2849	2850
3	1734	1744	1736
4	1468	1470	1466
5	1179	1177	1179
6	717	717	719
7	609	608	608

cellulase, pectinase and protease, where it can be observed that there is great similarity between the extracted fats.

The spectra obtained by FTIR show vibrational modes and combinations of functional groups of fatty acids present in the chemical composition of the cupuassu seed fat, with higher intensity absorption bands in the region of 3,000 to 2,800 cm^{-1}, which can be attributed to vibrations of axial deformation of the *CH* bonds of the methyl (CH_3), methylene (CH_2) groups and of the double bonds (*=C–H*) .

The bands with intermediate intensity, which appear in the region of 1,500 to 1,300 cm^{-1}, originate from the angular deformation vibrations of the *C-H* bonds of the methyl and methylene groups. The band that appears approximately in the region of 1730 cm^{-1} is related to the axial deformation vibrations of the carbonyl group (*C=O*) present in the constituent ester groups of triacylglycerides. In the region from 1,300 to 900 cm^{-1}, which contains part of the "fingerprint" region of the compounds, are the absorption bands referring to the axial deformation vibrations of the *CO* bond of the constituent esters of the triacylglycerides (García-González *et al.,* 2013). Finally, the peak at 719 and 609 cm^{-1} can be attributed to the benzene ring.

It can be observed that enzymatic extraction with the three enzymes tested did not cause changes in the chemical characteristics of cupuassu seed fat. The properties found in this work for cupuassu seed fat support future work because of the industrial interest in this material and because the results found offer important data for the elaboration of programs to control the composition of Amazonian fats as ingredients.

3.3.5. Solid fat content

In addition to physicochemical properties, other specific attributes of fats define their applications, such as physical properties, and among them, solid fat content is one of the most important. An analysis of the solid fat content (*SFC*) at 20 °C provides information about the resistance to oil exidation of a lipid matrix, which should not be below 10% (Bezerra *et al*., 2017).

Table 5 illustrates the solid fat content of cupuassu fat extracted by the enzyme process (CFE), cupussu fat extracted with solvent and cocoa fat (CF) (Zarringhalami, 2021), at temperatures of 10, 15, 20, 25, 30, 35, 40 and 45 °C.

The information from the solid fat content curve as a function of temperature is used to predict the applicability of a fat. It is responsible for many product characteristics such as margarines, shortenings and spreads, including their general appearance, ease of packaging, oil exudation, sensory and melting properties and consistency (Rao *et al*., 2001).

Table 5. Solid fat content in cupuassu seed fat extracted by the aqueous enzymatic process and for cacao fat, as a function of temperature.

Samples	Content of solid fat (%)						
	°C						
	10	20	25	30	35	40	45
CFE	68.28 ± 0.31^{b}	49.11 ± 0.38^{b}	33.06 ± 0.46^{b}	2.33 ± 0.08^{b}	0.11± 0.00^{b}	0.27± 0.00	0.07± 0.00
CCF	63.12 ± 0.28^{c}	39.85 ± 0.36^{c}	19.23 ± 0.31^{c}	1.08 ± 0.19^{c}	0.02 ± 0.00^{c}	0.00	0.00
CF*	94.80 ± 0.10^{a}	78.60 ± 0.30^{a}	26.90 ± 0.30^{a}	8.49 ± 0.02^{a}	0.76± 0.03^{a}	0.00	0.00

CF: Cocoa fat; * Zarringhalami (2021). The result presented is the mean of triplicate ± standard deviation. Means followed by different letters in the same column, are significantly different by Tukey's test ($p \leq 0.05$).

It can be observed that the enzyme-extracted cupuassu fat differs statistically from the commercial cupuassu fat, which is significantly higher ($p < 0.05$) in terms of solid contents at all temperatures tested. This is another important difference between the extraction methods and an additional proof that the enzymatic process preserves the identity of the extracted fat as well as the oxidative stability, the peroxide index and iodine value, which showed differences between the commercial sample and the one extracted in an enzymatic process. Although the fatty acid general profile did not show differences, the cupuassu fat obtained by the enzymatic process is different from the same commercial fat.

Cupuassu fat has been used in the preparation of cupulate. The solid contents in cocoa fat (Table 5) exceed that of the cupuassu fat sample extracted by enzymes up to 35 ºC, while at 40 and 45 ºC, it resembles commercial cupuassu fat. Although the solid contents in cocoa fat show this behavior, studies have been conducted using blends and modifications in order to achieve the ideal properties in these fats (Zarringhalami *et al*., 2021), which contributes to improving the properties of fats and expands opportunities for applications of these materials.

3.3.6. Thermal analysis

The thermogravimetric profile indicates the effect of temperature on fat degradation. To evaluate the thermal behavior in a nitrogen atmosphere, the fat was heated to a temperature of 600 °C at a heating rate of 10 °C·min^{-1} (Figure 3).

The *TG* analyses indicated stability in the sample up to 345 °C, with great mass loss at around 440 °C, until complete decomposition at around 480 °C. This loss increases with the rise in temperature, which is higher than the operating temperature normally used in most activities involved in food preparation. This event can be better visualized by the decay of the baseline, as shown by the *DTG* curve, confirming the decomposition of the samples at temperatures above 345 °C.

Cupuassu seed fat was degraded at very high temperatures, due to the fact that it has a relatively high proportion of saturated compounds, making it a thermally stable fat, which is the second indication of fat in frying with the generation of few degradation compounds, which may be associated with safety in consumption and for the environment in the cooking process, considering the stability at the temperatures practiced, which are below that determined as the degradation temperature of this fat.

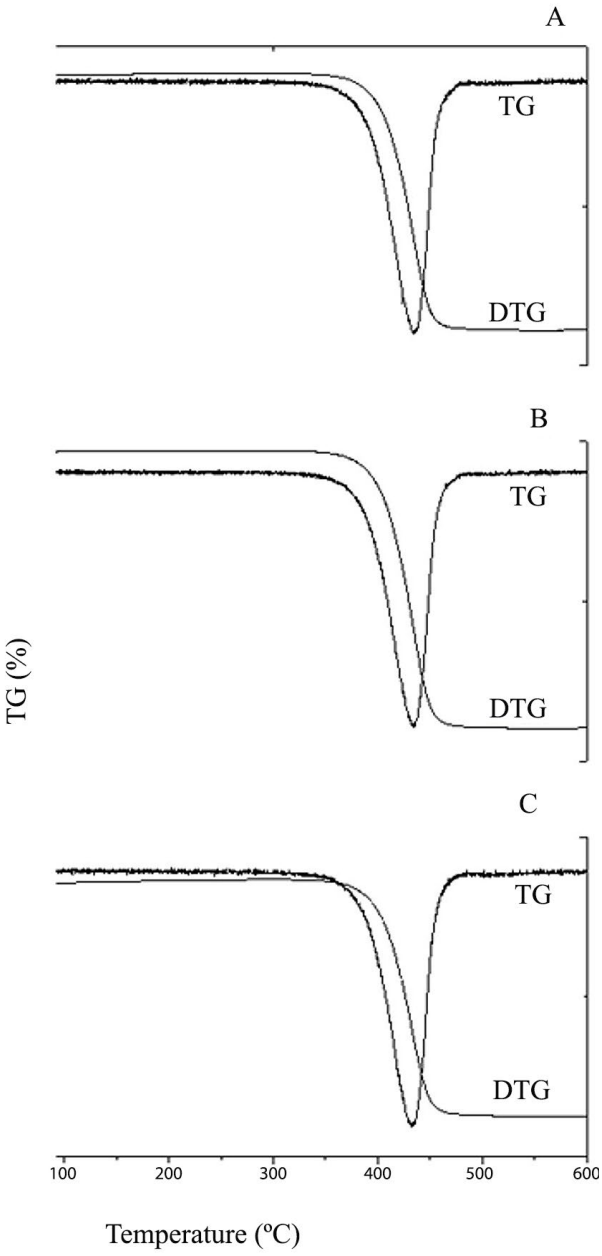

FIGURE 3. TG – DTG curves of cupuassu seed fat samples extracted by celulase (A), pectinase (B), and protease (C), in a nitrogen atmosphere, heated to 600 °C at a heating rate of 10 °C min^{-1}.

The cupuassu fats extracted by different enzymes showed similar thermal behavior (Table 6) regarding the *on-set* and *end-set* of temperature. However, regarding the mass loss and residue, the cupuassu fat sample obtained by protease showed the lowest value for mass loss and the highest value for waste. This

Table 6. Thermal behavior by thermogravimetry of cupuassu fat samples extracted by cellulase, pectinase, and protease.

Enzymes	Temperature *On-set* (ºC)	Temperature *End-set* (ºC)	Lost of mass (%)	Residue (%)
Cellulase	405.94	450.73	97.14	2.86
Pectinase	405.55	450.28	98.21	1.79
Protease	406.77	449.16	95.95	4.05

response may be related to the characteristics of the fat, as confirmed by the data shown in Table 3, with slightly lower concentrations of monounsaturated fatty acids.

These results suggest the need for future works with more detailed analyses regarding the specific characteristics of cupuassu seed fat extracted with enzymes and by pressing which can contribute to indicate the viability of the enzymatic process, as well as studies for the valorization of the aqueous extract obtained as a by-product with antioxidant properties.

4. CONCLUSION

The enzymatic aqueous extraction process presented results for fat extraction efficiency similar to those obtained by the conventional extraction method.

The extracted fat showed better quality parameters (fat solid content, acidity, iodine index and oxidative stability) and a higher concentration of bioactive compounds than the fat obtained by the pressing extraction method currently adopted by the industry.

The cupuassu seed fat extracted by the aqueous enzymatic process showed several important properties that justify its potential for application in the food industry.

The results presented in this work contribute to the understanding of the enzymatic extraction process as an alternative to the conventional methodology for extracting fat from cupuassu seeds.

The aqueous enzymatic extraction process produced a superior quality fat, in addition to the aqueous extract, a by-product that presents prospects with bioactive properties, and is, therefore, an environmentally-friendly and sustainable technology.

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Conflict of Interest

The authors declare no conflict of interest.

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