Article

Journal of the Brazilian Chemical Society

https://dx.doi.org/10.21577/0103-5053.20230124

J. Braz. Chem. Soc. **2024**, *35*, 2, e-20230124, 1-15

Effects of Pectinase Treatment on the Optimization and Extraction of Pigments from Bacupari, Tucumã, and Peach Palm Using Response Surface Methodology

Pedro H. S. Miranda,[a,b] Rômulo A. Morais,[a,b]* Hermanny M. S. Sousa,[a,b] Larissa S. Gualberto,[a,b] Barbara C. B. Freitas,[b] Eduardo R. Asquieri,[c] Glêndara A. S. Martins,[b] Eduardo V. B. V. Boas[d] and Clarissa Damiani[a,e]

[a]*Programa de Pós-Graduação em Ciência de Alimentos, Departamento de Ciência e Tecnologia de Alimentos, Universidade Federal do Tocantins (UFT), 77001-090 Palmas-TO, Brazil*

[b]*Laboratório de Cinética e Modelagem de Processos, Departamento de Ciência e Tecnologia de Alimentos, Universidade Federal do Tocantins (UFT), 77001-090 Palmas-TO, Brazil*

[c]*Faculdade de Farmácia, Universidade Federal de Goiás (UFG), 74690-900 Goiânia-GO, Brazil*

[d]*Departamento de Ciência dos Alimentos, Universidade Federal de Lavras (UFLA), 37200-900 Lavras-MG, Brazil*

[e]*Departamento de Engenharia de Alimentos, Universidade Federal de Goiás (UFG), 74690-900 Goiânia-GO, Brazil*

This work aims to optimize the process of extracting pigments from the peel of Amazonian fruits (bacupari, peach palm, and tucumã) using the enzyme pectinase. First, pigment extraction was carried out using different solvents (ethanol and acetone 80%, ethanol and acetone 50%, and water), temperatures (44, 40, 35, and 26 °C), and wavelengths (400, 450, and 490 nm). In the second stage, the best parameters found in the first stage were applied in a statistic with a response surface varying time (60, 120, and 180 min) and enzyme concentration (40, 80, and 120 U). The best results for extraction for bacupari and peach palm were acetone at 50% and 45 °C and ethanol at 50% and 35 °C for tucumã. In the second stage, the optimum point found was 1.5 h and 100 U concentration, and 2.5 h and 90 U concentration resulted in better extractions for bacupari, peach palm, and tucumã.

Keywords: exotic fruits, extraction of pigments, carotenoids, use of waste, stability, storage

Introduction

The Amazon region, recognized for its vast area of tropical forests, is home to some of the most outstanding biodiversity in the world, with a rich collection of more than 12,000 plant species, many of them probably not yet cataloged.[1,2] Among the various fruits present in the Amazon region and native to this biome, the fruits of Bacupari (*Garcinia gardneriana*), Pupunheira (*Bactris gasipaes*), and Tucumã (*Astrocaryum vulgare*) stand out. The three fruits are popular in the cultural region and have the potential for production on an industrial scale, and several food products are already made from their pulps, such as ice cream, sweets, jellies, and fermented drinks, among others.[3-5] In addition, Amazon fruit peels are considered agro-industrial waste because they are discarded in the environment, containing significant amounts of natural pigments and bioactive compounds, which can be used as raw material for food (nutraceuticals) or dyes. Approximately 84% of the total weight of peach palm is estimated to be waste, and about 40% of the total weight of tucumã is also waste. However, it is notable that the residues of these raw materials are applied in the generation of energy (biodiesel) manufacture of food products, among others. Therefore, the physical-chemical characterization of Amazonian fruit peels and the quantification of their bioactive compounds are of great importance for the knowledge of nutritional and nutraceutical values and, from a commercial point of view, to add value and quality to the final product.[6]

Garcinia gardneriana, popularly known as "*bacupari*", "*yellow mangosteen*," or "*bacupari-mirim*", is one of the native species of Brazil, belonging to the Clusiaceae

*e-mail: romuloitallo2505@gmail.com
Editor handled this article: Eduardo Carasek

family, whose fruit is rich in bioactive compounds and has anti-inflammatory properties.[7] The peach palm (*Bactris gasipaes* Kunth), known as peach palm, is a native representative of the Americas and has a wide variety of races and ecotypes. When peach palm fruits are mature, they have a fibrous epicarp that can be red, orange, or yellow.[4,8] Tucumã (*Astrocaryum vulgare*), in turn, is a palm tree native to the Amazon region, whose fruit is divided into three portions (epicarp, mesocarp, and endocarp). The epicarp and mesocarp show color variation; however, the skin and pulp are generally yellow-orange.[3,5,9]

The pigments, or food colorings, confer color to food to raise its attractiveness to consumers. They have been used for a long time by the food, cosmetic and pharmaceutical industries. The consumers' demand for natural colors has boosted the clean label revolution and the green chemistry in place of artificial colors.[10,11] Natural pigments are present in plants, seeds, fruit, peels, and roots, contain some color responsible for light absorption, and are non-toxic and non-allergenic. The pigments are divided into chlorophylls, carotenoids, anthocyanins, and betalain compounds, accounting for most of the natural colors in the fruits. The factors that affect the extraction of the natural pigments are the studied matrix, particle size, and the method and operation conditions used for the extraction.[12-14]

There are different methods for extracting these pigments; among many found methods, there is the extraction through solvent and enzymes. Conventional extraction methods with organic solvents have many disadvantages, such as prolonged exposure, generation of hazardous volatile organic compounds, low extraction efficiency, and degradation of thermosetting compounds.[15-17] However, amidst the new, utilized techniques, the extraction with enzymes grants a selective and superior natural color extraction, reducing solvents and energy consumption. In addition, the enzyme-assisted extraction (EAE) technology offers great promise in isolating pigments, should its selection and optimized operational conditions be adequate.[11,18] Several authors report the extraction of pigments from food residues such as bark and seeds, for example, Shen *et al.*[19] verified enzyme-assisted alkaline extraction and pigment identification from jujube bark (*Ziziphus jujuba* Mill.). On the other hand, Qi *et al.*[20] verified the action of the enzyme polyphenol oxidase in the extraction of persimmon peel pigments (*Diospyros kaki*).

This way, the objective of this work was to characterize the Amazonian fruit peels and to optimize the pigment extraction conditions, assessing the extraction temperature, solvent, time, and the application of the pectinase enzyme, varying different conditions to hydrolyze the epicarp fiber, assisting the pigment liberation in potentializing the extraction efficiency. Moreover, we also aimed to assess the stability of the extracted pigments, with and without enzyme, varying some conditions such as light exposure, temperature, and storage time.

Experimental

Chemicals

DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2'-azino-bis-(3-ethylbenzenothiazoline-6-sulfonic acid), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), Folin-Ciocalteu reagent, copper sulfate, potassium sulfate, potassium persulfate, petroleum ether, aluminum chloride, sodium molybdate, ferric chloride, sodium carbonate, sodium phosphate, tripyridyl triazine (TPTZ), ethanol, acetone, glacial acetic acid, oxalic acid, sulfuric acid, standards were obtained from Sigma- Aldrich (São Paulo, Brazil). All chemical reagents were analytical grade.

Plant material

Peach palm, tucumã, and bacupari were collected during the 2019 and 2020 seasons from plants that naturally occurred in the Pará State, Brazil (8°15'29'' S, 49°16'11'' W). First, the fruit was harvested ripe and free from deformity or physical injuries. Next, they were conditioned and sanitized in the Kinetics and Process Modeling Laboratory at the Federal University of Tocantins (Palmas, Brazil). After sanitization, the fruits were pulped manually to separate the peel fraction. Next, the different parts of the fruits were packed in low-density polyethylene bags, protected from light. Then, the samples were stored at −18 ± 2 °C in a domestic refrigerator (Bosch, Inteligent, Freezer 32) until further analysis.

Proximal characterization and physical-chemical characteristics

The chemical composition of peel fruits was determined according to the Association of Official Analytical Chemists[21] for moisture (925.09), ash (923.03), lipids (920.85) using hexane for six hours, proteins (920.87), fiber (991.43), carbohydrates by difference, pH (981.12) and titratable acidity (942.15B). The results were expressed in g *per* one hundred g (g 100 g^{-1}). The total energy value was estimated, considering the conversion factors of 4 kcal g^{-1} for proteins and carbohydrates and 9 kcal g^{-1} for lipids and expressed in kcal 100 g^{-1} of the sample. The soluble solids content was analyzed through digital refractometer reading

(AKSO model RHBO-90), with its results described in °Brix. Water activity was done at room temperature using the Aqualab (AQUALAB CX-2) device with the devices' cuvette. We used the dew point determination technique to assess the water activity of a product, placing about one g of the sample in the device.

Antinutritional factors

Hydrocyanic acid in the natura peels of the Amazonian fruits was assessed using the Guignard test. The plum seed was used to compare cyanogen's presence since it presents cyanogenic glycosides and hydrocyanic acid precursors; the results were expressed as the presence or absence of cyanogenic compounds.[22] Trypsin inhibitor contents were determined in the dry peel samples based on extracting three extracts (basic, neutral, and acid). The phytic acid content was determined in the dry peels using DEAE-Cellulose resin (ion-exchange resin).[23] The total tannin content was estimated using the method of Price *et al*.,[24] with adjustments made by Barcia *et al*.[25] To extract condensed tannins, 1 g of dry bark was used, to which 50 mL of methanol were adding, stirring for one hour, followed by filtration. Then, 1 mL of the extract was removed, and 5 mL of vanillin: HCl (1:1) solution (1% vanillin in methanol; 4% hydrochloric acid in methanol) were adding, allowing it to react for 15 min, and reading at 500 nm in a spectrophotometer (Rayleigh, UV-1800). The results were expressed in mg of catechin *per* 100 g of sample (mg CA 100 g^{-1})

Determination of pigments

Total anthocyanin content was estimated, initially, according to Lees and Francis[26] method, with adaptations performed by Barcia *et al*.[25] For the extraction of the anthocyanin compounds, 1 g of *in natura* fruit peel was weighed, then 25 mL of acidified ethanol solution with 1.5 M HCl was added in the proportion (85:15), incubated for one hour at room temperature. After this procedure, the reading was performed in a spectrophotometer at a wavelength of 532 nm (Rayleigh, UV-1800). The results were expressed in mg of cyanidin-3-glycoside 100 g^{-1}. Next, the determination of total carotenoids was performed in natura sample, according to Higby,[27] using 10 g of sample, adding 40 mL of alcohol/hexane extractor solution (3:1), and allowing it to rest for 30 min. Finally, the sodium sulfate solution was sprayed onto a cotton swab in a funnel, where the extracting solution was filtered. The readings were performed at 450 nm (Rayleigh, UV-1800) with results expressed in mg 100 g^{-1}.

Bioactive compounds and antioxidant potential

The extraction of the bioactive compounds and antioxidant potential was adapted according to Rufino *et al*.[28] First, 5 g of *in natura* sample were weighed in a 100 mL beaker, adding 40 mL of ethanol 80%, homogenized, and allowed to rest for 60 min at room temperature. Then, it was centrifuged at 15.000 rpm (Brand DAIKI 80-2B) for 15 min, and the supernatant was transferred to a 100 mL volumetric flask. 40 mL of ethanol 80% were added and allowed to rest for 60 min at room temperature. After that, it was centrifuged again at 15.000 rpm for 15 min. Finally, the supernatant was transferred to the volumetric flask containing the first supernatant and completed the volume to 100 mL with distilled water. These extracts were used to carry out assays of antioxidant potential and bioactive compounds.

The extracts' total phenolic compounds (TPC) were determined using the Folin-Ciocalteau method, according to the methodology described by Singleton and Rossi.[29] For the oxidation reaction, 25 µL of Folin-Ciocalteu (2.0 M) were added, followed by 200 µL of ultrapure water and 25 µL of the obtained extracts. After 5 min, 25 µL of 10% sodium carbonate (Na_2CO_3) were added to the complex. The mixture was allowed to stand in the dark at room temperature for 60 min. Absorbance was measured at 725 nm in a spectrophotometer (Rayleigh, UV-1800). The results were expressed in the gallic acid equivalent *per* 100 g sample (mg GAE 100 g^{-1}). The vitamin C content of the whole fruit was determined by the colorimetric method with 2,4-dinitrophenylhydrazine (2,4-DNPH), according to Strohecker and Henning.[30] Absorbance was measured at 520 nm (Rayleigh, UV-1800), and the results were expressed in mg of ascorbic acid 100 g^{-1} (mg AA 100 g^{-1}).

The scavenging capacity of DPPH was estimated using the method proposed by Brand-Williams *et al*.[31] and adapted by Rufino *et al*.[28] The fruit extracts at different concentrations (0.1 mL) were reacted with 3.9 mL of the DPPH radical. Absorbance was measured at 515 nm (Rayleigh, UV-1800), and the results were expressed as EC_{50} (concentration at which the extract produces 50% of its maximum effect) (g peel g^{-1} DPPH). Iron-reducing antioxidant power (FRAP) was determined according to Benzie and Strain,[32] the FRAP solution was prepared by adding TPTZ solution (10 mmol L^{-1}) diluted with HCl, add of ferric chloride hexahydrate ($FeCl_3.6H_2O$) (20 mmol L^{-1}) and sodium acetate buffer (pH 3.6), respectively. In a tube, 90 µL of the obtained extracts were added, along with 270 µL of distilled water and 2.7 mL of FRAP reagent. The absorbance of the solutions was measured at 595 nm (Rayleigh, UV-1800). The results were expressed in micromoles of ferrous sulfate *per* g of sample (µM $Fe_2SO_4\,g^{-1}$).

Pigment extraction

The enzyme-assisted extraction (EAE) was performed in the peach palm, tucumã, and bacupari fruit peels. The extraction study was divided into two stages where the first one corresponded to preliminary tests to define which was the best solvent, temperature, and wavelength. The pectinase enzyme concentration effect and extraction time were studied in the second stage. Finally, the pigment extraction was performed on the dry fruit peels using the pectinase enzyme (*Aspergillus niger*) (P4716-5KU, Sigma-Aldrich, São Paulo, Brazil). The peels were dried for extraction at 70 °C until the samples hit constant weight.

Optimization and extraction of fruit pigments

First stage: preliminary testes

The first stage consisted of a factorial experiment 5 × 4 × 3. Five types of solvents (water, acetone 70%, ethanol 80%, acetone 50%, and ethanol 50%), four temperatures (35, 40, 45, and 26 °C), and three wavelengths (400, 450, and 490 nm, Rayleigh, UV-1800), were tested. The factors that presented the highest yield in the pigment extraction and the highest absorbance read in the spectrum were submitted for further analysis. Extracting pigments with solvents in the preliminary test was performed according to Swer *et al*.[33] with some modifications in triplicate and two repetitions. First, 2.5 g of dry peels of the three fruits were weighed separately, and 100 mL of each solvent was added; after, it was taken to the magnetic agitator for 2 min (DIST, DI-03) and allowed to rest for 30 min in a water bath in three levels of temperature (35, 40 and 45 °C) (FISATOM, 230 V, 60 Hz). Soon after, it was centrifuged at 15.000 rpm for 10 min (DAIKI 80-2B). Finally, the reading was performed in a spectrophotometer in three wavelengths (400, 450, and 490 nm) (Rayleigh, UV-1800).

Second stage: optimization of extraction with pectinase enzyme

After the best solvent, temperature, and wavelength were defined for the EAE, the experiment followed the 3 × 3 factorial (enzymatic concentration × extraction time) run on the response surface in Statistica 10 software.[34] The first analyzed factor as an independent factor was the "enzyme concentration" (40, 80, and 120 units of pectinase enzyme); the second factor, "time," was 60, 120, and 180 min as shown in Table 1, where the maximum and minimum limits are defined. The response surface methodology (RSM) in this work was applied to improve the optimal yield conditions of pigment extraction in bacupari, peach palm, and tucumã. The project provides ten sets of test conditions for each fruit, with each factor with three levels of high, average, and low. The factorial scheme was made with axial points and repetition at the central point. Levels of variables, or independent factors, are listed in Table 1. For the pigments extraction process assisted by the enzyme pectinase, 1.25 g of dry samples were weighed, and 50 mL of acetone (50%) were added in the tucumã peel, and ethanol (50%) in the bacupari and peach palm peel; after that, pectinase enzyme was added using a pipette, the mix was taken to the magnetic agitator for 2 min and allowed to rest for 60, 120, and 180 min in a water bath at 45 °C temperature (peach palm and bacupari dry peel), and 35 °C for tucumã dry peel. Soon after, it was centrifuged at 15000 rpm for 10 min, and the reading was done in a spectrophotometer at 400 nm.

Table 1. The independent variables, with their applied levels, for the pigment extraction in the fruit peels of bacupari, peach palm, and tucumã

Factor	Level of factor		
	–1	0	1
time (x) / min	60	120	180
Enzyme concentration (y) / U	40	80	120

Stability of the extracted pigments with enzyme

The extract that presented the best extraction yield was submitted to the stability test in adverse situations. The assessed factors were: light (absence and presence); temperature (frozen, refrigerated, and room temperature of 26 °C); storage time in days (0, 15, 30, 45). The stability test was performed on the three peels with and without the enzyme to verify whether the enzyme influenced the stability of the pigments or not. The samples stored away from light were wrapped in aluminum foil. The others were subjected to the direct incidence of light in chambers composed of light emitting diode (LED) lamps throughout the storage period.

Statistical analysis

Preliminary test results were assessed using the Tukey's test at 5% ($p < 0.05$) by multiple comparisons of means and analysis of variance to indicate the significant effect of solvent, temperature, and wavelength variables in the extraction of pigments on the peels. Next, the results of the second stage were subjected to the response surface methodology (RSM), and the data analysis was conducted by Statistica 10.0 software (StatSoft Inc., Tulsa, OK, USA).[34] Finally, the stability test was run on OriginPro 2022 software (OriginLab Corporation, Northampton, MA,

USA)[35] to check for statistical differences in the method used and linear regression to check time behavior during the 45 days.

Results and Discussion

Physical and chemical characterization

The fruit epicarp that presented the lowest moisture content ($p < 0.05$) was tucumã (33.07%), followed by peach palm (63.94%) and bacupari (77.87%) (Table 2). Tucumã peel moisture was similar to the study of Santos *et al*.[36] in the same fraction of the fruit (31.20%). The moisture content is an important attribute to be determined in foods because it is through these contents that it is possible to estimate their lifespan and apply adequate technological processes.[37] In the bacupari case, which showed a higher moisture content, it will have a lesser lifespan compared to tucumã peel, which presented a lower moisture level.

Peach palm peels (7.41%) and tucumã (7.90%) showed the best lipid contents when compared to bacupari (0.32%) (Table 2). However, the lipid content found for tucumã peel was lower than the one found by Santos *et al*.[38] in the same fraction (12%) and by Santos *et al*.[36] in tucumã pulp (18.28%). Considering tucumã peel as a coproduct/residue with approximately 8% of lipids shows, according to Santos *et al*.[39] significant content of unsaturated fatty acids, especially oleic acid (C18:1), the oil extraction from the peel, or even its usage as flour in the diet is promising. Lipids, in addition to representing an essential group of macronutrients for the human diet, also influence the flavor and texture of foods.[40] Thus, tucumã peel oil can serve as a technological adjunct in the development of products. A prior study[41] showed that the oil extracted from tucumã is composed especially of unsaturated fatty acids (74.40%) and saturated (25.60%), ω3, 6, and 9.

As for the total carbohydrates, the peel that presented the highest content was tucumã (39.46%), followed by peach palm (21.78%) and bacupari (11.42%) (Table 2). The value found in tucumã peel was close to that found in Silva *et al*.[42] research in the same matrix (42%). In tucumã pulp carbohydrate contents were 31.46%, lower than the one found in this study (39.46%).[38] Total carbohydrate-rich fruits are essential in the human diet. According to the World Health Organization,[43] a daily recommendation is a diet comprising 55 to 75% carbohydrates. Regarding the protein contents, all assessed peels had low indices (Table 2) when compared to Rambutan's (*Nephelium lappaceum*), a fruit from the Amazonian region that presents around 12.40% of protein.[44] Protein contents are also different from each other ($p < 0.05$) (Table 2). The protein percentage in peach palm peel was lower than the results of Carvalho *et al*.[45] in the fruit pulp (4.23%). This occurs because most peels are not a protein source, but the pulps are.

The most caloric peel was tucumã (239 kcal 100 g^{-1}) (Table 2), representing 11.95% of the recommended daily calory ingestion for an adult person. This one can be considered a high value for fruit due to the lipid percentage in its composition (7.9%) and the amount of carbohydrates present in the sample (39.46 %). The number of calories in tucumã peel was lower than the ones found in the pulp of umari (*Poraqueiba sericea*) 257.20 kcal 100 g^{-1}, and pajurá (*Couepia bracteosa*) 169.73 kcal 100 g^{-1},[46] however, similar to sour bacuri peels (*Garcinia madruno*) 248.45 kcal 100 g^{-1}, according to Berto *et al*.[46]

Table 2. Physical and chemical characterization of bacupari, peach palm, and tucumã fruit peels harvested in the Brazilian North Region in 2019/2020. Results are expressed in a wet base

Analysis	Bacupari	Peach palm	Tucumã
Moisture / (g 100 g^{-1})	77.87 ± 1.60	63.94 ± 5.18	33.07 ± 0.45
Lipids / (g 100 g^{-1})	0.32 ± 0.00	7.41 ± 1.96	7.90 ± 0.71
Ash / (g 100 g^{-1})	2.42 ± 0.48	0.27 ± 0.10	3.81 ± 0.06
Protein / (g 100 g^{-1})	1.37 ± 0.10	3.22 ± 0.10	2.56 ± 0.22
Fiber / (g 100 g^{-1})	6.60 ± 0.05	3.38 ± 0.43	13.20 ± 1.36
Total carbohydrates / (g 100 g^{-1})	11.42 ± 0.22	21.78 ± 0.89	39.46 ± 0.51
Caloric value / (kcal 100 g^{-1})	54.04 ± 2.12	166.69 ± 3.61	239.18 ± 4.57
Hydrogen potential (pH)	4.61 ± 0.06	5.5 ± 0.03	6.01 ± 0.04
Total titratable acidity[a]	2.84 ± 0.33	2.20± 0.13	1.05 ± 0.2
Water activity	0.93 ± 0.01	0.91 ± 0.03	0.89 ± 0.01
Soluble solids / °Brix	10.16 ± 0.41	8.00 ± 0.00	13.04 ± 0.05

Values expressed as mean ± standard deviation (n = 6); means followed by the same lowercase letter on the same row do not differ statistically by Tukey's at 5% probability ($p \leq 0.05$). [a]Values expressed in g citric acid 100 g^{-1}; carbohydrates calculated by difference.

It is possible to observe that the highest pH was observed on the bacupari fruit peel (4.61), which the total titratable acidity being 2.84%. Peach palm peels showed pH 5.5, a value very close to that found in the peel flour of the same fruit (5.68)[45] and total titratable acidity of 2.20% in peach palm pulp flour (2.40%).[45] Tucumã peel showed a lower level of total titratable acidity (1.05%), however, with higher contents than the ones found by Silva *et al*.[42] in the peel of the same fruit (0.43%). The peels of the three assessed fruits can be considered food of low acidity, according to Mostafidi *et al.*[47] data for showing a pH above 4.5.

Regarding water activity (Aw), the peel that showed the lowest value was tucumã (0.89), followed by peach palm (0.91) and bacupari (0.93) (Table 2). These Aw values make the peels of those fruits prone, when *in natura*, to the attack of microorganisms such as fungi and yeasts, which can grow in Aw higher than 0.62, and to bacteria, whose growth is facilitated in Aw higher than 0.86.[42] Therefore, the drying of the peels must be considered an essential factor for better conservation and food safety. The soluble solids contents (°Brix) are commonly associated with the soluble sugars content and organic acids from a portion of food.[48] The fruit peel that presented the lowest soluble solids content was peach palm (8 °Brix), followed by bacupari (10.16 °Brix) and tucumã (13.04 °Brix) (Table 2). This data corroborates with the contents of total carbohydrates, that is, in bacupari peel, practically all carbohydrates are composed of soluble sugars; on the other hand, in the peach palm and tucumã peels, not just soluble sugars can be found, but also insoluble carbohydrates such as fibers.

Antinutritional compounds

None of the three assessed fruit peels (bacupari, tucumã, peach palm) showed cyanogenic compounds, phytates, and trypsin inhibitors. However, there was the presence of total tannins in the peels of tucumã (263.69 mg tannic acid 100 g^{-1}), bacupari (224.27 mg tannic acid 100 g^{-1}), and peach palm (159.11 mg tannic acid 100 g^{-1}) (Table 3). Report found in the literature[49] affirm that tannins are present in the fruits of the two cultivars *chemlali* and *neb jmel* of *Olea europaea* L., with 50.55 and 82.44 mg of catechin equivalents 100 g^{-1}, respectively.[49] Tannins reduce food digestibility in the body, precipitating digestive enzymes, and because of that, they are classified as antinutritional compounds.[50] On the other hand, they present some benefits, such as antibacterial effects, and can promote increased antioxidant activity.[51,52] However, the value of tannins found in the studied fruit peels (Table 3) was low when compared to grape peels (665 mg 100 g^{-1} of samples) in certain kinds of grapes.[53]

The absence of phytates in the studied fruit peels is positive since this component has the power to form insoluble complexes with metal, which reduces the absorption of minerals such as zinc, magnesium, iron, and calcium in humans and animals. None of the fruit peels showed trypsin inhibitors, a substance widely found in soy, containing 94 U mg^{-1} of trypsin inhibitors.[14,54] The protease inhibitors inhibit the activity of trypsin and chymotrypsin digestive enzymes in the gastrointestinal tract. The cyanogenic compounds were found in neither of the analyzed Amazonian fruit peels. These substances are composed of fractions of sugar and aglycone of the α-hydroxy nitrile type, and they are found in many plants, such as cassava.[55,56]

Table 3. Bioactive compounds, antioxidant potential, antinutritional factors, and pigments from bacupari, peach palm, and tucumã fruit peels harvested in northern Brazil in 2019/2020

Fruit	Bacupari	Peach palm	Tucumã
Total tannins / (mg tannic acid 100 g^{-1})	224.27 ± 8.25	159.11 ± 12.07	263.69 ± 7.01
Phytates	absent	absent	absent
Trypsin	absent	absent	absent
Cyanogenic	absent	absent	absent
Total carotenoids / (mg 100 g^{-1})	0.45 ± 0.15	34.74 ± 1.43	22.88 ± 1.62
Anthocyanins[a]	0.33 ± 0.03	0.40 ± 0.01	0.36 ± 0.02
Total phenolics / (mg GAE 100 g^{-1} of sample)	84.21 ± 9.53	89.14 ± 4.06	108.52 ± 1.39
Vitamin C / (mg of ascorbic acid 100 g^{-1})	51.95 ± 0.91	51.47 ± 0.52	106.22 ± 1.52
DPPH / (g of fruit g^{-1} DPPH)	715.96 ± 1.8	1994.95± 61.47	1402.43 ± 2.68
FRAP / (μM Fe_2SO_4 g^{-1})	97.34 ± 0.09	81.88 ± 1.5	87.56 ± 1.21

[a]Values expressed in mg of cyanidin 3-glycosidic 100 g^{-1}. GAE: gallic acid equivalent; DPPH: 2,2-difenil-1-picrilhidrazil; FRAP: ferric reducing antioxidant power; Fe_2SO_4: iron sulfate(III).

Determination of pigments present in fruits

All studied fruit peels showed low anthocyanin content; none showed significant chlorophyll values (Table 3). The highest rate of anthocyanins was found in peach palm and tucumã, which presented 0.4 and 0.36 g of cyanidin 3-glycoside 100 g^{-1} of samples (Table 3). In a study by Santos *et al.*[39] with peach palm and tucumã, low concentrations of anthocyanin (1 mg cyanidin 3-glycoside 100 g^{-1} of sample and 4 mg of cyanidin 3-glycoside 100 g^{-1} of peel sample) were also found. The fact that the fruit has low levels of anthocyanins is due to this pigment being present in blue and purple vegetables,[28] and peach palm, tucumã, and bacupari are yellow and dark orange, colors that are observed and related to carotenoids (Table 3). Peach palm and tucumã present higher carotenoid contents in the peels, with 34.74 mg 100 g^{-1} of a sample and 22.88 mg of carotenoids 100 g^{-1} of a sample, respectively (Table 3).

The carotenoid value found in peach palm and tucumã peels was higher than that reported in the literature by Davies *et al.*,[57] which found contents of 33.69 and 18.06 mg 100 g^{-1} for peach palm and tucumã peels, respectively. This variation in the pigment content is natural, especially in exotic fruits. A study performed by Carvalho *et al.*[45] showed values that varied from 0.80 to 12.40 mg 100 g^{-1} when assessing 21 genotypes of peach palm pulp. In the United States, the recommendation for the ingestion of carotenoids is 12 mg day^{-1} adding all carotenoids (α-carotene, β-carotene, β-cryptoxanthin, lutein, zeaxanthin, and lycopene), this way, by only ingesting 100 g of either peach palm, or tucumã would be enough to fulfill the daily need for carotenoids.[9]

Potential antioxidant

The peel with the highest total phenolics content was tucumã with 108.52 mg GAE 100 g^{-1} (Table 3). The peels of the fruits showed satisfactory levels of phenolic compounds when compared to data from Borges *et al.*,[58] who studied different varieties of açaí (*Euterpe oleracea*) and found total phenolic values ranging from 132.60 to 391.20 mg GAE 100 g^{-1}. According to Faria *et al.*,[59] murici (*Byrsonima crassifolia*), a typical yellow fruit from the Legal Amazon and the Brazilian Cerrado, has phenolic compound values of 134 mg GAE 100 g^{-1}, a little higher than the one found in the peel of tucumã (108.52 mg GAE 100 g^{-1}). Tucumã peel, among the three fruits, was the one that showed the highest vitamin C level with 106.22 mg of ascorbic acid 100 g^{-1}, a much higher value than the ones found in the other two fruit. Tucumã, according to literature,[38] is a fruit rich in ascorbic acid, and data from it showed 58 mg 100 g^{-1} of ascorbic acid in its pulp, however, the peel showed a much higher value than the pulp.

The three fruit peels showed elevated antioxidant activity. According to the DPPH and FRAP methodology, the fruit peels with the most *in vitro* antioxidant activity were bacupari (EC_{50} of 715.96 g g^{-1} DPPH and 97.34 uM Fe_2SO_4 g^{-1}, respectively) (Table 3). The low DPPH and FRAP values found in the assessed fruits could be related to the interference of carotenoids, pigments that are quite present in the fruit peels. Since peach palms presented the highest carotenoid content (Table 3), this pigment may have interfered with the DPPH and FRAP methodology. The tucumã peel showed minor antioxidant activity than the tucumã pulp studied by Araujo *et al.*[44] (EC_{50} of 3343 g g^{-1} DPPH). A higher activity value was found when comparing tucumã to curcuma (*Curcuma longa*), with EC_{50} of 228 g g^{-1} DPPH.[60] The antioxidant tucumã properties (pulp and peel) have been associated with the β-carotene composition.[41]

Optimization and extraction of fruit pigments

Regarding the best extraction solvent, it is possible to notice that the best results were obtained with acetone 50% for peach palm and bacupari and ethanol 50% for tucumã peel. A study about pigment extraction found the best pigment extraction rate in a proportion of water and ethanol of 50:50 (v/v), the same found in this study. This is due to the water-miscible properties that carotenoids have, such as epoxy (violaxanthin and neoxanthin), hydroxyl (lutein and zeaxanthin), keto (canthaxanthin) or keto with hydroxy groups (astaxanthin).[61-63] Yet, pure water had lower efficiency in the extraction of these pigments. Therefore, the most efficient solvents were the ones that contained a higher percentage of water in them.

The best temperature found was 45 °C for peach palm and tucumã and 35 °C for tucumã. This factor affects the cellular structure of the pigments because, when connected to different proteins, they form globular, crystalline, fibrillar, membranous, or tubular structures, allowing the liberation or retention of pigments in the vegetal matrix.[64] The increase in the pigment extraction, associated with the increase in temperature, could be due to the higher diffusivity coefficient of the solvent in the matrix, which allows the solubility of the pigments and the increase of the concentration gradient between the solvent and the matrix, facilitating the transference of the total pigments to the solvent medium.[65,66] The wavelength that showed the highest efficiency was 400 nm in all samples.

The effects of extraction time (60, 120, and 180 min) and enzyme concentration proportion in the extractor solution (40, 80, and 120 units) were investigated to determine their influence on the extraction of pigments from the three fruits of the Amazon region. The experiment conditions were optimized in previous tests, in which the best solvent and temperature range used in the process were defined. As for bacupari and peach palm, temperatures of 45 °C and 50% ethanol solvent (50% ethanol and 50% water) were used, while for tucumã peel, 50% acetone solvent (50% acetone and 50 % water) and temperature of 35 °C were used. As a result, it was verified that the variation of time and enzyme concentration relation significantly affected the pigment extraction efficiency. Table 4 shows the results of the analysis of variance for the pigment extraction in the three fruits (bacupari, peach palm, and tucumã), whose method effectively assesses the quality and significance of mathematical models.[67] When the p-value < 0.05, the adjusted regression equation was considered to have statistical significance. At the same time, the degree of fit of the model was assessed through the determination coefficient (R^2).

The coefficients with confidence interval values ($\alpha = 0.05$), higher than the parameter value, were considered non-significant; significant values were assessed with a confidence level of 95%. Results showed that two independent variables, concentration and time, affected ($p < 0.05$) the absorbance found in the pigment extraction. The most significant effect for the extraction of pigments in bacupari peel was the linear effect of the enzyme concentration due to having the lowest p-value found; the second variable was linear time, which also presented a significant difference, lower than the concentration results, though. Analyzing data from peach palm peel, the only important factor was the linear effect of concentration in the pigment extraction process due to the p-value being lower than 0.05. As for the tucumã peel, the enzyme's linear time effect and linear concentration effect were

Table 4. Analysis of variance of the factorial experiment of pigment extraction in the peel of bacupari, peach palm, and tucumã

Bacupari	SQ	GL	QM	*F*-value	*p*-value	Significance
Time (L)	0.007860	1	0.007860	32.3133	0.010790	significant
Time (Q)	0.000017	1	0.000017	0.0699	0.808547	not significant
Concentration (L)	0.060000	1	0.060000	246.6590	0.000561	significant
Concentration (Q)	0.000001	1	0.000001	0.0057	0.944524	not significant
1L by 2L	0.005100	1	0.005100	20.9674	0.019552	significant
Error	0.000730	3	0.000243			
Total	0.073709	8				
Coefficient		R^2: 0.99			Adj. R^2: 0.97	
Peach palm	SQ	GL	QM	*F*-value	*p*-value	Significance
Time (L)	0.003053	1	0.003053	1.22585	0.349013	not significant
Time (Q)	0.000796	1	0.000796	0.31949	0.611428	not significant
Concentration (L)	0.080852	1	0.080852	32.46900	0.010718	significant
Concentration (Q)	0.002182	1	0.002182	0.87613	0.418314	not significant
1L by 2L	0.000000	1	0.000000	0.00010	0.992635	not significant
Error	0.007470	3	0.002490			
Total	0.094352	8				
Coefficient		R^2: 0.92			Adj. R^2: 0.78	
Tucumã	SQ	GL	QM	*F*-value	*p*-value	Significance
Time (L)	0.021620	1	0.021620	75.26203	0.003223	significant
Time (Q)	0.000007	1	0.000007	0.02558	0.883100	not significant
Concentration (L)	0.015234	1	0.015234	53.03236	0.005345	significant
Concentration (Q)	0.000265	1	0.000265	0.92076	0.408064	not significant
1L by 2L	0.000083	1	0.000083	0.28722	0.629197	not significant
Error	0.000862	3	0.000287			
Total	0.038070	8				
Coefficient		R^2: 0.97			Adj. R^2: 0.93	

SQ: sum of squares; GL: degrees of freedom; QM: mean squares; L: linear; Q: quadratic; R^2: determination coefficient; R^2 adj: adjusted R squared.

significant ($p < 0.05$) in the process, indicating that both factors increased the pigment extraction rate in the tucumã peel. For the tucumã peel, independent variables of time and concentration, both in the linear model, showed a significant effect in the process of extraction, and time was the factor with the most relevance in the process due to the *p*-value being lower than the *p*-value of concentration, thus time is the most influent factor in the pigment extraction process of tucumã peel.

Regarding the difference between the fruits, all showed differences in the linear model and none in the quadratic model. All models showed high R^2 and the highest was in bacupari peel (0.99), followed by tucumã (0.97), and peach palm (0.92), so the results indicated good concordance between experimental and predicted data since the R^2 is at least 0.80 for a perfect adjustment of the model. These data showed a high degree of adjustment between the model and experimental data, and it is appropriate to use this model to describe the relationship between the increase in pigment extraction with the combination of extraction conditions.[68] The surface response graph was used to evaluate the response and the independent variables. The significance of all terms in the polynomial equation was considered statistically different when $p < 0.05$. Based on multiple linear regression equations, tridimensional response surface graphs were established in Figure 1 to explain the extraction process and the interactive influences of both factors (time and enzyme concentration).

It is possible to observe in Figure 1a that when the used enzyme concentration and time were elevated, the absorbance value also gradually increased for bacupari peel. This occurs because time and enzyme concentration had relevance in the extraction process. Analyzing Figure 1, it is possible to notice that when there is an increase in the concentration, there is a more inclined curvature, showing that the concentration was more relevant in the process. On the other hand, in the peach palm peel, it is possible to notice that, when there is a variation in time, this was little influenced in the extraction rate (Figure 1b) because there is no elevated curvature, a result that is by that shown in Table 4. This occurs because time has not influenced the pigment extraction in the peach palm peel. Observing Figure 1c, it can be seen that as the time and the enzyme concentration increase, higher is the pigment extraction rate in the tucumã peel.

Regarding the line in the 3D graph of the response surface, if the board in the elevation of the independent variable is plain, it means this variable has weak influence

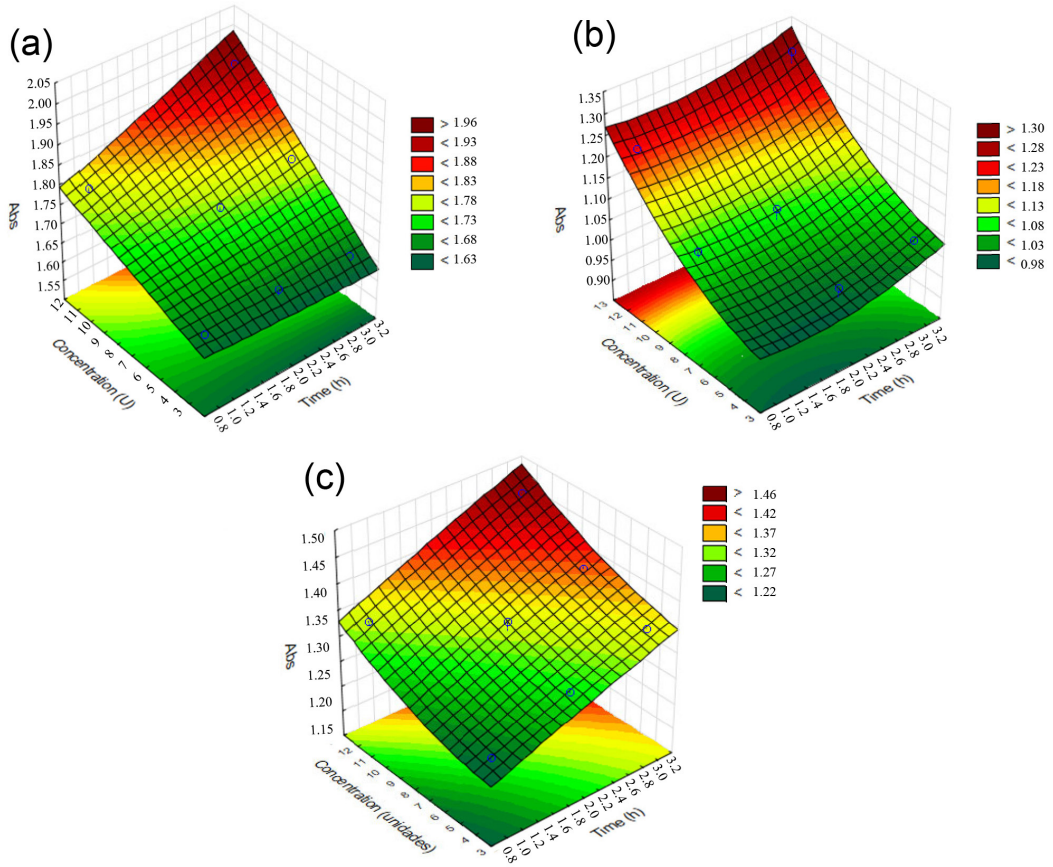

Figure 1. Graph of the response surface of the extraction of pigments from bacupari (a), peach palm (b), and tucumã (c) peel.

over the response value (efficiency), however, if the board is inclined, it indicates a strong influence.[19] By comparing the *p*-values, it can be concluded that the influence of the independent variables was classified as follows: time effect > concentration effect for the extraction of pigments in the tucumã peel; as for the peach palm and bacupari peels, the concentration effect > time effect. In a study by Shen *et al*.[19] about response surface extraction, the authors also found that the enzyme concentration influenced more than time in sterol extraction process of soy.

Using RSM allowed us to assess the quadratic and the variable interaction effects. The parametric values of the different variables can facilitate determining the optimal conditions for each response.[47] The best points found in the extraction were run in the tool within Statistica 10.0. The ideal extraction parameters were as follows: in the bacupari peel, the ideal extraction point was 1 h, and the concentration was 60 U; in the peach palm peel, the optimal point was 1.5 h, and the concentration was 100 U; in tucumã peel, time was 2.5 h and concentration 90 U. The results with the use of enzymes depend on various factors, thus, the parameters involved in the process need to be adequately balanced to increase extraction efficiency.[69] It is possible to notice with the data found in Figure 1 that as enzyme concentration and time increase, the pigment extraction rate also increases, and the response variable (absorbance) elevates. This occurs because the enzymes act to break the plant cell wall, thus facilitating the process of pigment extraction as higher as time and concentration are, the higher the activity of these enzymes, increasing the efficiency of the process.[69]

After finding the best extraction point, a test with enzyme and another without enzyme was run to see the percentage increase from the optimized method without enzyme. Pigment extraction in the bacupari peel without enzyme showed 1.157 nm of absorbance and with enzyme was 1.678 nm (45% increase); as for peach palm peel, the result of the extraction without enzyme was 0.633 nm and with it 1.275 nm (101.50% increase); tucumã peel, without enzyme 1.217 nm and with it 1.850 nm (52% increase). In a study performed by Strati *et al*.,[70] the pectinase enzyme effectively increased the total efficiency of carotenoids and lycopene by 50% in the pigment extraction of tomatoes. Thus, enzyme-assisted pigment extraction is superior to extractions with conventional methodologies such as solvents, and in this study, the pectinase enzyme acted with an increase of up to 101% higher.

Pigment stability

Assessing the stability of pigments extracted from the food matrix during either processing or storage is essential to determine the shelf value regarding the visual quality and feasibility of using natural dyes because pigments can degrade by different factors.[71] In this study, it was possible to notice that some aspects, such as temperature and storage time influenced the loss of color of the pigments over time. Figure 2 shows pigment stability in the peels of bacupari, peach palm, and tucumã with time variation over 45 days. Tests were carried out with the extraction with enzyme and without enzyme to verify if this influenced the results of the pigment stability. Analyzing Figure 2, it is possible to notice the test that most preserved its pigments was the frozen sample (BC4) and the refrigerated (BC3). On the other hand, the samples that most degraded over the test were BC1 and BE1. Both were at room temperature and exposed to light, and the pigments of the ones exposed to light suffered the most degradation.

The sample exposed to light had the greatest abrasion over time, with the greatest loss of color. This occurs because light is the primary pigment degradation factor.[72] The letters in Figures 2A, 2B, and 2C represent statistical differences with time variation. In sample BC4 (purple line), all letters are the same, showing it did not present any statistical difference with time variation, revealing that time did not result in any loss of coloring of the pigments, and kept stable in the freezer at −18 °C without light incidence. Although, according to Figures 2A, 2B, and 2C, all pigments suffered degradation caused by light, in many ways, pigments can be degraded by direct light exposure, including photodegradation and photoisomerization.[73]

There are many different ways in which molecules can interact with light. For example, when a molecule absorbs light, its energy can be transferred in heat or a chemical reaction. The latter is called a photochemical reaction because it is a reaction triggered by light, that is, a photon is absorbed to excite a molecule electronically.[72] For this type of reaction to occur, two requirements must be fulfilled: the photon energy must be sufficiently high to be absorbed, and the energy must be high enough to break and form bonds. Carotenoids are examples of food components that can degrade through this reaction. This is because carotenoids are very light-sensitive.[74] For bacupari (Figure 2A), time influenced the sample with and without enzyme about light exposure. It is possible to notice that the greatest loss of color was in the samples exposed to light (samples BC1 and BE1). Using the enzyme accelerated the pigment degradation process and had the curve a little more inclined when compared to the sample without enzyme since the enzyme is an acceleration factor in pigment degradation.[75] In the enzymatic process, it is possible to notice that degradation occurred faster in the first 15 days, and after this period, the pigments kept stable with a lower loss in color over time.

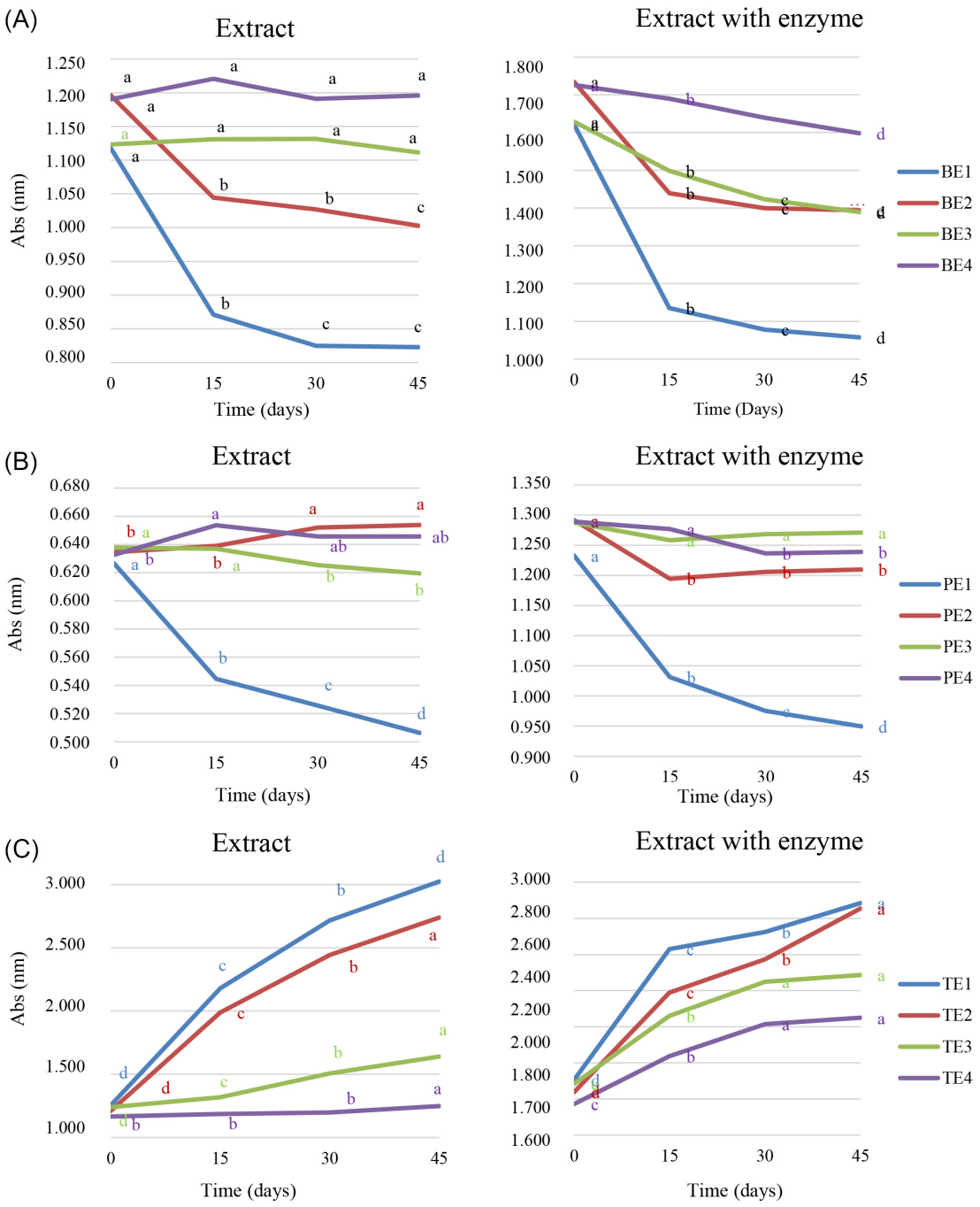

Figure 2. Pigment stability graphs in the bacupari (A), peach palm (B), and tucumã (C) peel, extracted with and without enzyme (control). BE1: bacupari with an enzyme with light exposure; BE2: bacupari with enzyme no light exposure; BE3: bacupari with enzyme refrigerated at 4 °C; BE4: bacupari with the enzyme in the freezer at −18 °C. PE1: peach palm with both enzyme and light exposure; PE2; peach palm with enzyme and without light exposure; PE3: peach palm with enzyme and refrigerated at 4 °C; PE4: peach palm with enzyme and in a freezer at −18 °C. TE1: tucumã with both enzyme and light exposure; TE2: tucumã with enzyme and without light exposure; TE3: Tucumã with enzyme and refrigerated at 4 °C; TE4: tucumã with the enzyme in the freezer at −18 °C. Different letters in the same line show there was a statistical difference with time variation.

The absorbance dropped drastically during storage for all samples from the peel extract of bacupari and peach palms exposed to light (BC1, BE1, PE1, and PC1). Figures 2B and 2C show that due to the degradation of the extract at light exposure, one of the most present pigments in the extract is the carotenoids, so the loss of carotenoids was significant. According to Rodriguez-Concepcion *et al.*,[76] the main factors that degrade the pigments are light exposure and temperature, which are the most important factors in carotenoid isomerization. In addition, due to the presence of a polyunsaturated hydrocarbon chain and a comprehensive system of conjugated double bonds, carotenoids become

easily susceptible to isomerization, oxidation, and chemical degradation. This especially occurs when exposed to heat, light, free radicals, acid conditions, and oxygen. This exposure causes the degradation of the compound through isomerization reactions and oxidation that form intermediate compounds with lower biological activity.[76]

Figures 2A, 2B, and 2C show that temperature intensifies pigment degradation. The extracts with pigments kept in the environment at a temperature of −18 °C (BC4) show they are quite stable because the degradation indices varied just a little in 45 days compared to the pigments exposed to room temperature (BC2). According to Mestry *et al.*,[77] lower temperature promotes higher stability of pigments from fruit and vegetable extracts because higher temperature denatures the pigments. Analyzing the data in Figure 2B, it is possible to see that samples PC4 and PE4 showed little alterations over the 45 days, either for the sample or for the enzyme. This behavior for peach palm samples is related to non-exposure to light and the use of low temperatures. All results for tucumã peel (Figure 2C) were influenced by the time factor ($p > 0.05$), except for sample TC4, which was the sample at −18 °C without enzyme.

In Figure 2, it is possible to see both graphs have very similar behaviors, however, in the tucumã peel, stability was a bit different from the one found in the peel of the other two fruits. In tucumã peel, it is possible to see a darkening in the extract during the whole extraction process, increasing the absorbance found in all analyzed points. The sample that presented the highest darkening was the one that was at room temperature and exposed to light; meanwhile, the one that had the lowest enzymatic darkening was sample TC1 TE1. It is possible to notice that in sample TC4 without enzyme, the pigment remained stable, and in sample TE4 with enzyme, there still was a slight enzymatic darkening in the process, even though the sample was at −18 °C.

The darkening of the extract with the pigments and the increase of the absorbance with the increase of time can be related to the role of reactive carbonyl species (RCS) derived from the reactions and degradation of the ascorbic acid in the formation of the brown color, causing the darkening of the extract. Tucumã is a fruit rich in ascorbic acid. In previously published studies,[38,78] tucumã showed 58 mg 100 g^{-1} of ascorbic acid. The degradation of ascorbic acid and the reaction of Maillard were identified as the main ways of non-enzymatic reactions responsible for the darkening in citric fruit extract.[79]

Therefore, the darkening of the tucumã extract over time may have occurred due to the ascorbic acid being defined as the main contributor to the darkening of citric extracts, as its degradation follows a linear tendency, significantly correlating with the formation of the brown color. More than one mechanism might be involved in forming the darkening of pigment extracts: the reactions between amino acids and reducing sugars (Maillard reactions) and the aerobic and anaerobic degradation of ascorbic acidity.[78,80] The best treatments to keep the pigment stability of the three fruit peels were the refrigerated environment at 4 °C and the freezer at −18 °C, both without light and at low temperatures. The treatment that most degraded the pigments of the three fruit peels was light exposure and room temperature for 45 days, as Figures 2A, 2B, and 2C illustrates.

Antioxidant potential

It is possible to see that in all extracts of the three assessed fruits, the samples with enzymes showed higher antioxidant activity ($p < 0.05$) (Figure 3). According to Rashid *et al.*,[81] the antioxidant activity with enzymes is higher because pectinase hydrolyzes the cellular wall and liberates important components such as antioxidants and bioactive compounds.

Correlating both used methodologies to measure the antioxidant activity, the fruits that showed higher antioxidant activity in the extract with enzyme were bacupari (483.44 EC_{50} g g^{-1} DPPH and 166.75 uM Fe_2SO_4 g^{-1}, respectively) > tucumã (810.01 EC_{50} g g^{-1} DPPH and 148.14 uM Fe_2SO_4 g^{-1}) > peach palm (1537.63 g g^{-1} DPPH and 119.45 uM Fe_2SO_4 g^{-1}). The samples without enzymes present the same order of antioxidant activity. However, the DPPH free radical scavenging capacity of the extracts obtained with enzyme significantly varied ($p < 0.05$) compared to the extract without enzyme, showing that DPPH with enzyme presented a higher antioxidant activity. According to the results found in the bacupari fruit, the DPPH value in the extract with enzyme was around 38% lower than in the with enzyme one (Figure 3); in the peach palm, this value was 16%, and in bacupari, it was 34% lower.

In the FRAP methodology, the antioxidant activity in the bacupari sample with enzyme was approximately 60% higher than the samples without enzyme; in the peach palm, this value was 20%, and in tucumã 52% higher (Figure 3). The highest radical scavenging potential of enzyme-treated samples can be attributed to the increased release of bound and free phenolics due to the hydrolytic activity of the applied enzymes.[82] The extract with enzyme showed higher antioxidant activity in the DPPH and FRAP methodology. This is due to the plant cell wall being mainly composed of cellulose, hemicellulose, and pectin, which are the main barriers to the extraction of bioactive. The pectinase enzyme degrades these cell wall components and allows the release

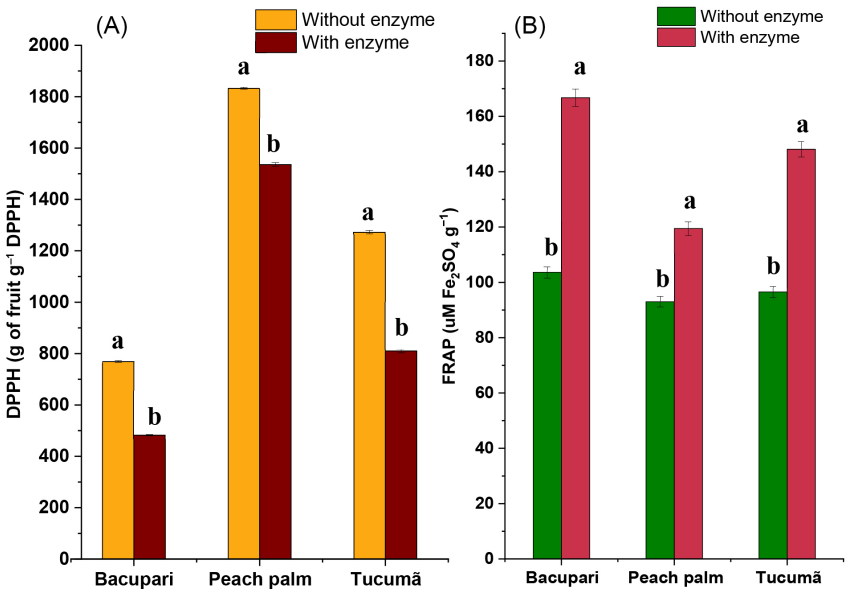

Figure 3. Antioxidant activity ((A) DPPH and (B) FRAP) with and without enzyme in the extract of the bacupari and peach palm.

of the compound of interest. The interaction between the enzyme and the substrate decides the extension of the hydrolysis of these barriers. Ultimately, this can directly correlate with the amount of released bioactive, increasing the antioxidant activity.[83]

Conclusions

The fruit peels are rich in carotenoids, and peach palm is the fruit that presented the highest value (34.74 mg 100 g^{-1}). The best solvent found for the pigment extraction, with the response variable being the absorbance of the peels, was the 50% ethanol solvent for bacupari and peach palm and 50% acetone for tucumã. The temperature influenced the extraction process being the best one, 35 °C for tucumã and 45 °C for bacupari and peach palm. Another determinant factor in the extraction is the enzyme concentration and time. The enzyme can act in the extraction increase, and the results showed 101% in the increase of the pigment extraction for peach palm, using 100 units of enzyme; bacupari 45% with six units, and tucumã a higher than 52% increase with 90 units of the enzyme. The extracted pigments were not very stable to light exposure and remained more stable in the dark, refrigerated at 4 °C, or in the freezer at −18 °C. In addition, the enzymes increased the antioxidant potential of the fruits by up to 60% and the antioxidant activity of the fruit peel extracts. Therefore, fruit peels are rich in nutrients and with a high load of carotenoids and antioxidant activities. Solvents, temperature, and the amount of enzymes used influenced pigment extraction. Excellent data were found to optimize pigment extraction with enzymes in the Amazonian fruit peels.

Acknowledgments

The authors acknowledge Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil) and PROCAD-AM: (1707/2018) for the financial support, Process number: (88881.200497/2018-01), and Federal University of Tocantins (UFT, Brazil) for the financial support through Introduction to Research scholarships.

Author Contributions

Pedro Henrique S. Miranda was responsible for conceptualization, methodology, validation, formal analysis, original draft, investigation; Rômulo A. Morais for methodology, validation, original draft, investigation, review and editing; Hermanny M. S. Sousa for methodology, validation; Barbara Catarina B. de Freitas for validation, original draft, investigation, review and editing; Larissa S. Gualberto for methodology, validation; Glêndara Aparecida S. Martins for formal analysis, conceptualization, resources, supervision; Eduardo R. Asquieri for conceptualization, resources, supervision; Eduardo V. B. Vilas Boas for conceptualization, resources, supervision; project administration; Clarissa Damiani for conceptualization, resources, supervision, project administration, review and editing.

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Submitted: May 13, 2023
Publishde online: July 28, 2023