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Extraction and stability of pigments obtained from pitaya bark flour (*Hylocereus costaricensis*)

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Abstract

Red pitaya fruits have become a source of natural dye, because they are rich in betacyanin, a pigment that imparts a red-violet colour. However, natural dyes can be highly unstable and prone to rapid degradation. In this study, an experiment was initially carried out to determine the best extraction solution with an extraction time of 20 min for each solution at room temperature (50% methanol, water, 95% ethanol and 80% acetone), in different pH ranges (4.0 to 9.0) and wavelength (485, 535 and 700 nm), for the red pitaya bark. The stability of the pigments extracted from pitaya peel meal was analysed taking into account the best extraction solutions by spectrophotometric measurements at 15-day intervals, during a 60-day storage period, in the absence and presence of light and under different storage conditions. Pigments stored in the absence of light at freezing temperatures for less than 15 days showed least degradation. The FTIR spectrum in shows two distinct peaks at 3263.870 and 1636.807, these absorption bands are characteristic of betalain functional groups. The spectrum indicates the presence of hydroxy and amine groups as strong, broad, stretching-mode bands, appearing between 3200 cm^{-1} and 3600 cm^{-1}.

Keywords: peel; pigments; natural dyes; betacyanin.

Practical Application: Pitaya is a fruit rich in bioactive compounds, with antioxidant activity, phenolic compounds among others. Although the fruit is not widely used as a food, it is necessary to make the population aware of its importance and preservation, not only as a source of nutrients, but also for its functional and color potential. Therefore, the extraction of pigments from the pitaya peel can increase the application of the fruit's potentialities, in addition to being a way to add value to products already prepared or even the development of new foods.

1 Introduction

Pitaya *(Hylocereus costaricensis)* is a tropical fruit from the rainforest regions of Mexico and Central and South America. It has a fibre-rich pulp and low calorific content (Xu et al., 2016). The seeds are rich in essential fatty acids and phytosterols, and the peel contains high amounts of pectin, fibres, and antioxidant compounds, especially betalains, which are responsible for the strong and attractive colour of the fruit, making it a promising source of natural colorant. This is attractive to the food industry, because the search for natural pigments to replace synthetic ones has intensified (Gengatharan et al., 2016).

Pitaya has gained interest because of the pulp's eye-catching appearance and the potential health benefits of the high levels of betalains in red or purple pitaya peel (Amaya, 2019). Although pitaya peel is rich in antioxidant compounds and a good source of natural pigments, pigment stability after extraction may be affected by extrinsic factors such as light, oxygen, metal ions, pH levels, and high temperatures. Reactions such as isomerisation, glycolysis, and hydrolysis may occur during the extraction process, causing rapid degradation of extracted pigments (Kushwaha et al., 2018; Stintzing & Carle, 2007).

Pitaya pulp is increasingly being either consumed, *in natura* or processed in jams, juices, ice creams, and sweets (Braga et al., 2020). Pitaya peel represents 18–24% of the whole fruit and is often discarded during processing (Chuck-Hernánde et al., 2016). Therefore, the use of this by-product as the raw material for natural dye production through the extraction of pigments could be a waste treatment alternative for the food industry (Ho & Latif, 2016). This study aimed to evaluate the best way to extract pigments from pitaya peel with different solvents and to assess the pigment stability.

2 Materials and methods

2.1 Samples

Mature red-peeled pitaya fruits (*Hylocereus costaricensis*) were purchased from Ceasa in Goiânia, State of Goiás (the central supply centre of the State of Goiás), Brazil, and forwarded to the Food Chemistry and Biochemistry Laboratory of the Faculty of Pharmacy of the Federal University of Goiás. Fruits with an absence of defects were selected, washed in running water,

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and sanitised with 100 ppm sodium hypochlorite for 15 min. The peel and pulp were then separated. The whole peels and the inner layer of the peels were cut into small pieces and dried in an air-circulated oven at 55 °C until constant weights were obtained a forced air velocity of 1.0 m s^{-1} for 72 h. Once dry, they were ground in a mill (Quimis, Q298 A21) to obtain the flour, which was packed in low-density polyethylene plastic bags, vacuum sealed, covered with aluminium foil to exclude light, and stored in a freezer at -18 °C until analysis.

2.2 Experimental conditions

Experiments to determine the best extraction solution were conducted with four extraction solutions (50% methanol and 50 water v/v; 95% ethanol and 5% water v/v; 80% acetone and 20% water v/v; and 100% water) at six pH values (4.0, 5.0, 6.0, 7.0, 8.0, and 9.0); spectrophotometric readings were taken at three wavelengths (485, 535, and 700 nm). For the stability study, three temperatures were tested (about of 23 °C for ambient, 4 °C for chilled, and -8 °C for freezing), two storage conditions (presence and absence of light), and two wavelengths (485 and 535 nm) were assessed over time (0, 15, 30, 45, and 60 days).

2.3 Extraction of pigments

The extraction of natural dyes from red pitaya peel flour was carried out by following the method of Naderi et al. (2012), modified by Fathordoobady et al. (2016), with a 6×4×3 experimental design (six pH values, four extraction solutions, and three wavelengths). Flour (2.5g) was mixed in each extracting solution (50 mL) at six pH values, adjusted with 0.1 mol 1 hydrochloric acid or sodium hydroxide buffer solutions, if necessary, stirred with a magnetic stirrer for 20 minutes, and then filtered. The solutions containing the extracted pigmented material were taken to the UV-Vis spectrophotometer, and the absorbance at different wavelengths was measured. The readings were performed at different wavelengths. Analyses were performed with three repetitions and in quintuplicate.

2.4 Study of extracted pigment stability

Pitaya peel flour (2.5 g) was mixed with water (50 mL) under mechanical stirring for 20 min until complete solubilisation occurred, as described by Naderi et al. (2012) and modified by Fathordoobady et al. (2016). Subsequently, solutions were filtered in the absence and presence of light, at room temperature (30 °C).

Aliquots of raw extract were transferred into 10-mL flasks and analysed for stability, by following the methodology of Cejudo-Bastante et al. (2016), with modifications. Samples stored in the absence of light were wrapped in aluminium foil. Other samples were subjected to direct light in chambers containing LED (Light Emitting Diode) lamps throughout the entire storage period.

2.5 Fourier Transform Infrared (FTIR) spectroscopy

The FTIR spectra of the extracts were recorded with an Agilent ATR FTIR-Cary 630 instrument at room temperature (30 °C), at wavelengths between 4000 and 400 cm-1, with 32 scans performed. We emphasize that for this analysis only the extracts that present the best results will be analysed. The position and intensity of the absorption bands in the FTIR spectra were used to analyse the functional groups, according to the literature; the change in intensity was used to assess pigment degradation.

2.6 Statistical analysis

To evaluate the best extraction solution, a 6×4×3 design was used, with three repetitions in quintuplicate; the response variable was the absorbance. For data processing, analysis of variance (ANOVA) was used to assess the significance of the variables and Tukey's test was applied at 95% confidence level ($p < 0.05$) to evaluate differences between the means, by using *Statistica 7.0* software; results are presented as mean ± standard deviation. In the stability study, ANOVA was used to check for significant differences between treatments (time, temperature, storage conditions, and wavelength), and Tukey's test was applied at 95% confidence level ($p < 0.05$) and followed by regression analysis to separate the influences of the variables on the pigment. The suitability of the model was determined by the lack of fit test and R^2 (the coefficient of determination). For statistical analysis, we used a complete 5×3×2×2 design by means of *Statistica 7.0* software.

3 Results and discussion

3.1 Extraction efficiency

The various extraction conditions for red pitaya peel flour pigments described in the methodology were evaluated by ANOVA. Table 1 shows a significant influence of the extraction solvent and wavelength, both in isolated form and interactively, with consideration that the extraction solvent is closely related to the result. The pH values are not significant for the extraction process; this can be explained by the presence of betalain-rich compounds, which may not be affected by the medium's pH level (Cejudo-Bastante et al., 2019).

Table 1. Result of variance analysis by the F test to evaluate the best pigment extraction solution for pitaya peel flour at different pH values and detection wavelengths.

Causes of variation	Mean square of variables	
	GL	ABS
Solvent	3	13.686398*
pH	5	0.480471
Wavelength	2	12.063116*
Solvent×pH	15	0.329651
Solvent×wavelength	6	1.491123*
pH×wavelength	10	0.037703
Solvent×pH×wavelength	30	0.013095
Error	144	0.297548
Adjusted total	215	
CV (%)	46.51	
General mean	1.172	

*Significant at 5% level by the F test. GL: Degree of freedom; ABS: Absorbance.

Furthermore, there is no significant difference between the ethanol and acetone solvents (Table 2); however, the 50% methanolic extract has the highest absorbance in the pigment extract from red pitaya peel flour. This result can be justified by the methanol dipole moment value, which is close to that of water, and both solvents are more polar than ethanol and acetone. (Martins et al., 2013).

In addition, Fathordoobady et al. (2016) state that polar organic solvents like acetone and ethanol have low extraction solubility, especially for pigments with hydrophilic structures and high polarity such as those in pitaya, which contains a high proportion of betalains. These pigments have high solubility in water because of the hydroxy groups in betacyanins, which cause charge polarisation and hydrogen bonding and are responsible for the hydrophilic properties (Faridah, 2017; Fathordoobady et al., 2016). The findings are corroborated by Al-Alwani et al. (2015), who reported that the solvent affects the absorption spectrum of dyes.

As for the wavelength, the best results appeared in the 485 nm spectrum, followed by the 535 nm one, for all tested solvents (Table 2). This may be because the betaxanthins (yellow) absorb visible light at 470–480 nm and the betacyanins (red) at 530–540 nm (Román et al., 2014).

The presence of an aromatic ring in the cyclo-dopa radical changes the maximum absorption of visible radiation from 480 nm (yellow, betaxanthins) to near 540 nm (red, betacyanins) (Azeredo, 2009; Cai et al., 2005; Strack et al., 2003). The groups that make up the pigment vary according to the different sources from which the pigments are captured and determine the tone and stability (Khan, 2016).

The results of this study corroborate those described by Azeredo (2009), in which the betacyanin and betaxanthin pitaya peel flour pigments absorb at around 540 and 480 nm, respectively; it is also ratified that betacyanins have maximum wavelengths ranging from 534 to 555 nm (Darmawi, 2013).

As this study aimed to obtain the largest amount of extracted pigment and the most direct way to quantify this pigment was spectrophotometry, we considered 485 and 535 nm to be the most effective wavelengths for the readings. For the solvent, methanol is the most common one in use for extraction, because of its polarity, but it is also considered more toxic and dangerous to handle than other alcohols (Faridah et al., 2015), so water is a viable alternative solvent for extraction without environmental harm. In this context, the methanolic solution was disregarded, and only water was considered as the extraction solvent to proceed with the stability experiments. Fathordoobady et al. (2016) reported in their studies that the extraction yield is potentiated with water as the solvent because of the highly hydrophilic nature of pigments such as betacyanins.

Table 2. Average tests to evaluate the solubility of the pigment of red pitaya peel flour in different extraction solutions at different wavelengths.

Wavelength (nm)	Extract 1	Extract 2	Extract 3	Extract 4
485	0.846 ± 0^{aC}	1.039 ± 0^{aC}	1.853 ± 0^{aB}	2.326 ± 0^{aA}
535	0.715 ± 0^{aB}	0.770 ± 0^{aB}	1.670 ± 0^{aA}	1.970 ± 0^{aA}
700	0.616 ± 0^{aB}	0.548 ± 0^{bB}	0.705 ± 0^{bA}	1.009±A

Data are the mean of five repetitions ± standard deviation. Extract 1: ethanol/water (95:5, v/v); extract 2: acetone/water (80:20, v/v); extract 3: water (100, v/v); extract 4: methanol/ water (50:50, v/v). Lower case letters in columns compare the wavelengths and upper-case letters in rows compare the extracts. The same letters do not differ from each other by Tukey's test ($P < 0.05$).

3.2 Pigment stability

In this study, temperature, storage conditions, times and wavelengths, showed a significant influence ($p < 0.05$) in all variables in an isolated manner and combination, demonstrating that the extracted pigments are thermally unstable. However, the storage conditions versus wavelength interaction and the quadruple interaction between all variables showed no significant differences. The results for each response variable are shown in Table 3.

The storage temperature significantly influenced ($p < 0.05$) the pigment concentration between 0 and 60 days (Figure 1A). This can be justified by the fact that, among the studied variables, temperature is the most relevant factor and may cause degradation in pigments (Reshmi et al., 2012).

Pigment degradation is faster at ambient temperature than in refrigerated conditions (Figure 1A). A similar phenomenon was observed by Azeredo (2009): the degradation rate of betacyanins was faster at higher temperatures than at lower ones. Temperature, light, and acidity can increase isomerisation, decarboxylation, or cleavage of betanin (Azeredo, 2009; Czyżowska et al., 2006; Fernández-López; Almela, 2001).

The pigments in pitaya peel flour were most stable at freezing temperatures among those studied (Figure 1A). Miguel et al. (2018) highlighted that, in the food industry, betalain pigments are only used in frozen foods, low-temperature dairy products, and foods with a short shelf-life, specifically to avoid their degradation.

Table 3. ANOVA for the aqueous extract obtained from red pitaya peel flour by the F test to check pigment stability.

Causes of variation	Mean square of variables	
	GL	ABS
Temperature (T)	2	10.9183*
Storage conditions (Ca)	1	1.3925*
Time (t/days)	4	4.3049*
Wavelength (Co)	1	4.9072*
T×Ca	2	0.7154*
T×t	8	0.8363*
T×Co	2	0.3834*
Ca×t	4	1.4704*
Ca×Co	1	0.0054
t×Co	4	0.0896*
T×Ca×t×Co	1.172	0.0121
Error	262	0.0088
Adjusted Total	299	
CV (%)	4.26	
General mean	2.21	

*Significant at 5% level by the F test.

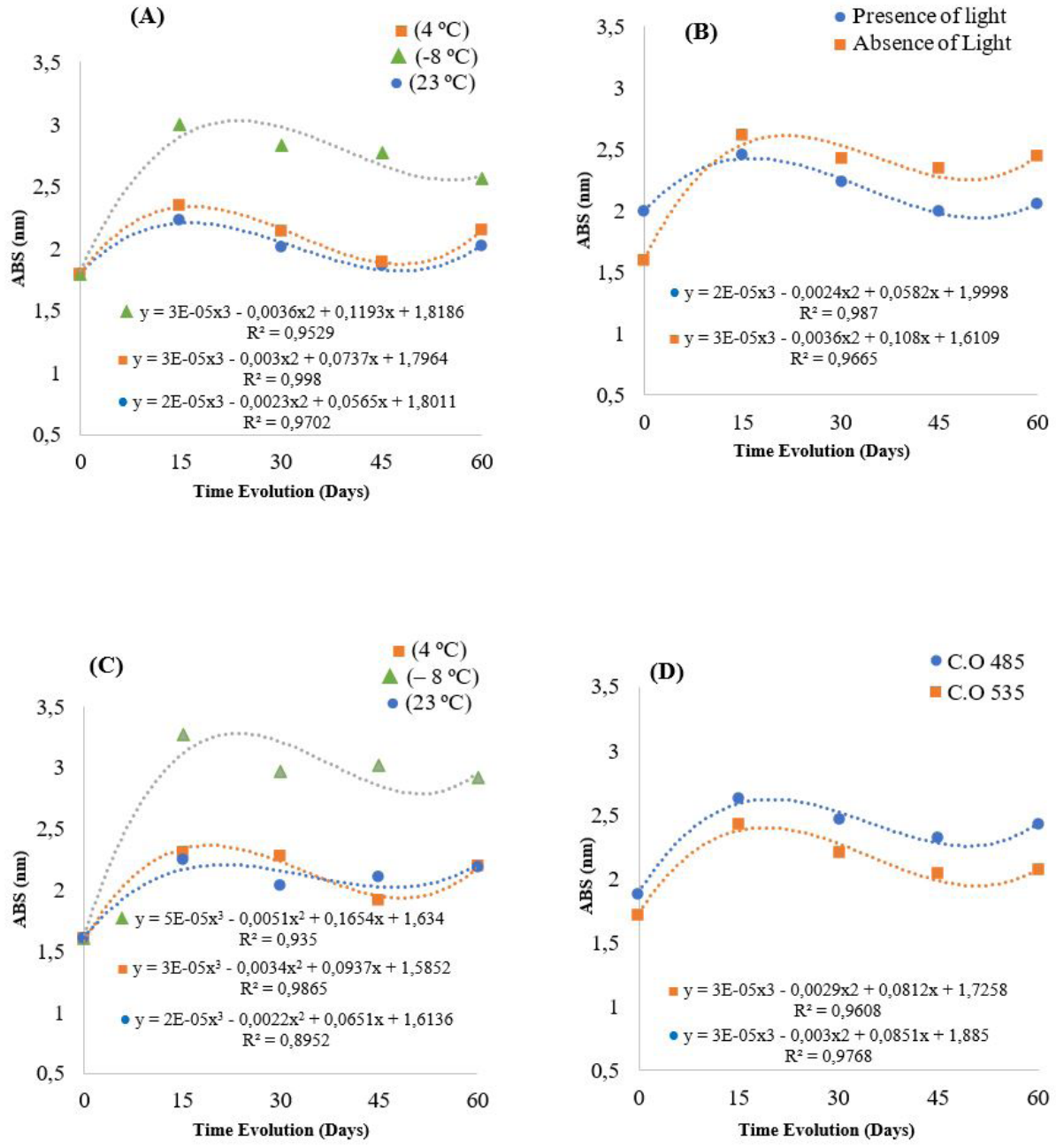

Figure 1. Effect of temperature in relation to storage time (A); effect of storage conditions in relation to time (B); interaction between temperature and storage conditions (C); and effect of wavelength in relation to storage time (D). *Denotes significant differences ($P < 0.05$) between all variations according to Tukey's test.

The results show that pigments from pitaya peel flour extract are thermally unstable during storage (Figure 1A). This is because betalains, the pigment in pitaya peel flour, are degraded by deglycosylation, decarboxylation, dehydrogenation, isomerisation (Sawicki & Wiczkowski, 2018), and hydrolysis (Vergara et al., 2014). Betalains can be regenerated by the use of acids after thermal processing, according to Herbach et al. (2006); the incorporation of isoascorbic acid in betacyanin solutions before or after heating (at 100 °C) helped to regenerate betacyanins.

Noticeably, at freezing temperatures, the absorbance values increase considerably between 0 and 15 days and the degradation of the pigment starts after 30 days of storage (Figure 1A). This degradation may occur because of organic acids that interact with the pigments at freezing temperatures, producing polymeric degradation products and decreasing the stability (Maeda et al., 2007). Organic acids can inhibit the oxygen-sequestrating activity of ascorbic acid, thus degrading the pigment (Levy et al., 2019).

Pitaya peel flour are a promising source of natural phenols. The pigment stability during freezing may be a result of the presence of these compounds, which can act as co-pigments (Kim et al., 2011). Molecular complexation of pigments with other phenolic compounds is the main mechanism of colour stabilisation (Cavalcanti et al., 2011).

Pitaya peel flour extracts stored under refrigeration demonstrate a high absorbance value in the first 15 days, which decreases in the interval from 15 to 45 days and increases at 60 days (Figure 1A). The initial increase can be justified by the fact that betalains, one of the pigments in pitaya peel flour, have greater stability at lower temperatures, with 4 °C being the temperature that allows betalamic compounds to remain stable. Corroborating the results described by Leong et al. (2018), the pigment retention of pitaya peel flour extract stored at 4 °C in the dark was 42.77 ± 1.14% after 10 days.

Endogenous enzymatic activities can also contribute to discoloration during processing and storage (Jagannath et al., 2015). Degradation after 15 days during refrigerated storage (Figure 1A) may be caused by endogenous enzymes, mainly glycosyltransferases and acyltransferases, involved in betacyanin biosynthesis, which contributes to pigment discoloration (Slimen et al., 2017).

Another possibility for degradation is hydrolysis as a result of a betacyanin-bleaching enzyme, as reported by Gengatharan et al. (2016): they observed hydrolysis in the red pitaya pigment forming betalamic acid and cyclo-dopa-5-*O*-glycoside, which induced loss of pigment during refrigeration.

However, betacyanins can also degrade and regenerate continually during storage, because the reaction is reversible, thus maintaining pigment concentrations (Gandía-Herrero et al., 2013; Silva et al., 2019). The process of pigment regeneration (Figure 1A) following refrigerated storage is based on hydrolytic products of betaine. The process comprises condensation of the amine group of cyclo-dopa-5-*O*-glucoside with the betalamic acid aldehyde group (Stintzing & Carle, 2007). Regeneration accelerates when each component is in solution and may occur because betacyanins can regenerate, as the basic building blocks, such as the cyclo-dopa ring and betalamic acid, are present to reform betacyanin chromophores (Herbach et al., 2006).

Considering the degradation behavior of the pigment, it is necessary to choose the storage conditions of the extract based on temperature and consequently on time, as the pigment content after the storage period differs significantly depending on the temperature at which it is stored. The stability can also be affected by other external factors, such as exposure to light (Solovchenko et al., 2019). We observed a significant influence ($p < 0.05$) on the stability of pitaya peel flour pigments in the presence and absence of light (Figure 1B).

The stability of betalains, the pigments in pitaya peel flour, is affected by light; the colour degradation is caused by UV wave absorption (Manchali et al., 2013; Janiszewska, 2014). Exposure to light significantly affects the stability of betalains and other natural pigments. Absorption of UV or visible light activates the π-electrons of betalain chromophores into a more energetic state (π*), increasing the reactivity or decreasing the molecule's activation energy (Wong & Siow, 2015).

Samples were exposed to light with LED lamps, and the absorbance spectra were recorded at regular intervals; the results of the pigment stability are shown in Figure 1B. The study showed that the pigments are unstable in the presence of light over the complete storage time.

The observed behaviour (Figure 1B) is consistent with that observed by Wu et al. (2020), who assessed the degradation of pitaya fruit and noticed a significant delay with light treatment. The decay rate reduced from 86.22% in the control to 15.23% in the light-exposed fruit, suggesting that light treatment could effectively delay pitaya fruit deterioration, although deterioration was not completely stopped (Herbach et al., 2007; Wu et al., 2020).

It was observed (Figure 1B) that there was a lower pigment concentration in the treatment with no light at time zero, than in the light treatment at the same time. This was explained by Manchali et al. (2013) and Khan & Giridhar (2014) who determined that not all light sources cause betalain degradation and that light of different wavelengths can cause an additive colour effect on betalains. Shin et al. (2003) also suggest that blue light in combination with infrared light induces greater accumulation of betacyanins.

Zhao et al. (2010) reported that the betacyanin content initially increased and reached its highest level under a certain amount of light and then decreased, in general, with an increase in the intensity of white, red, or blue light.

Our results suggest that the storage conditions of 45 days, freezing temperatures, and a dark environment (Figure 1C) may retain a greater number of pigments, especially betacyanins, because they are relatively stable at low temperatures with no light. There was a significant influence ($p < 0.05$) of the absorption wavelength on stability (Figure 1D). The absorbances of the spectrum with corresponding defined peaks at wavelengths of 485 and 535 nm were best in this study.

As a result, it was found that the pigment in aqueous solutions was more stable with a wavelength of 485 nm (Figure 1D). This corroborates the report of Fathordoobady et al. (2016) that betalain pigments are characterised by a maximum absorbance (λ_{max}) of approximately 535 nm for purple-red betacyanins (betanin: λ_{max} = 535 nm; betanidine: λ_{max} = 542 nm) and close to 480 nm for yellow betaxanthins (λ_{max} = 482 nm for indicaxanthin, the common betaxanthin found in red beetroot); for betalamic acid, λ_{max} = 424 nm.

There are first order kinetics in relation to the absorbance value of the aqueous pitaya bark flour (Figure 1D). In this sense, the highest point in relation to the absorbance value occurs after 15 days of storage and declines between 15 and 45 days, with an increase after 60 days; the 485 nm wavelength always gave higher absorbance results.

3.3 Extract analysis by FTIR spectroscopy

The FTIR spectrum in Figure 2 shows two distinct peaks at 3263.870 and 1636.807. These absorption bands are characteristic

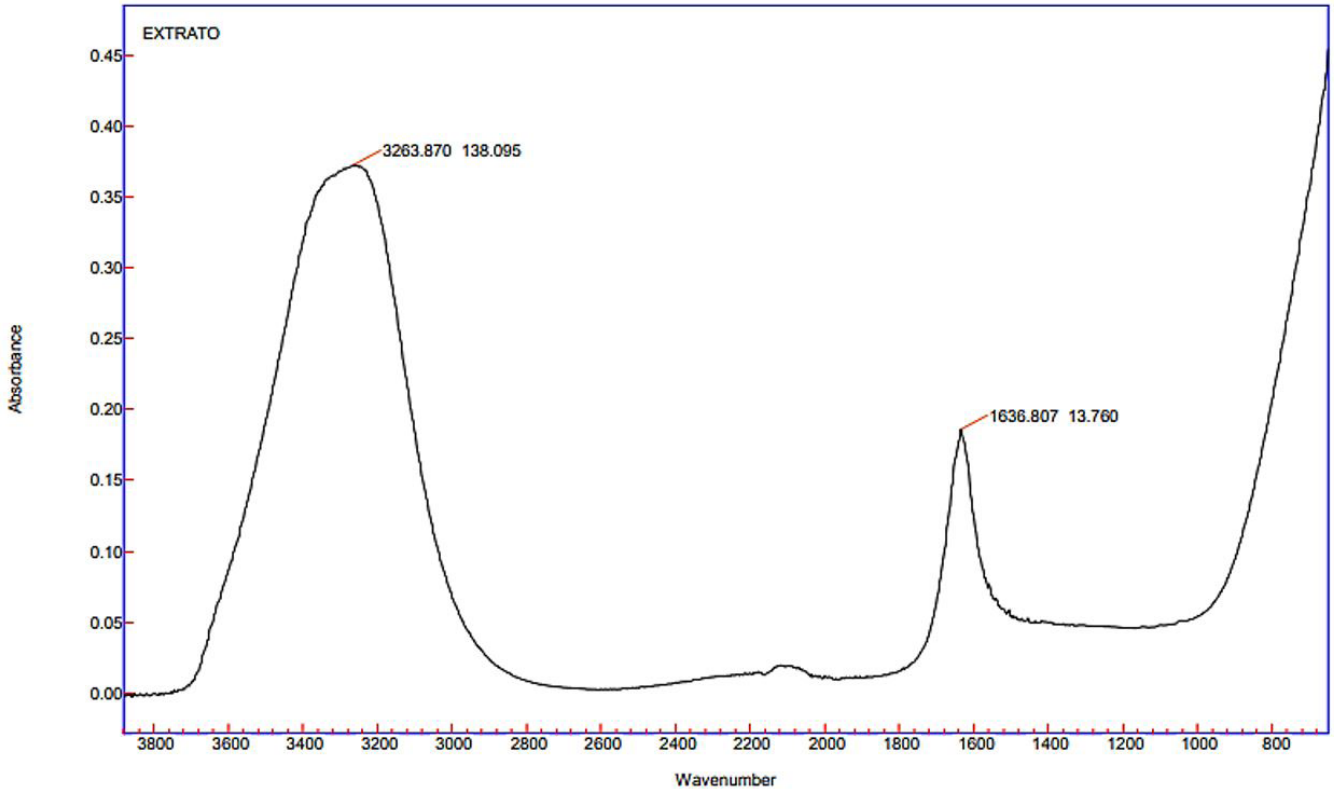

Figure 2. FTIR spectrum of the aqueous extract of red pitaya peel flour from 4000 to 600 cm^{-}.

of betalain functional groups. The spectrum indicates the presence of hydroxy and amine groups as strong, broad, stretching-mode bands, appearing between 3200 cm^{-1} and 3600 cm^{-1}, in agreement with Purushothamreddy et al. (2020).

The broad and strong band at 3263.870 cm^{-1} suggests O–H bonding in stretching vibration mode, and the band at 1636.807 cm^{-1} confirms the presence of carbonyl groups in the stretching mode associated with amide bonding.

Kumar et al. (2017) observed a band at 3359 cm^{-1}, which was attributed to the stretching vibration of O–H bonding, and a band located at 1624 cm^{-1} was attributed to the stretching vibration of C=N bonding (Molina et al., 2014). The main stretching vibration characteristics and the band at 1636.807 cm^{-1} are associated with characteristic carbonyl compounds of the betanin pigment molecule present in pitaya peel flour. The presence of amine, carbonyl, and other functional groups confirmed that the purple-red pigment extracted from the pitaya peel flour belongs to the betalain family.

4 Conclusions

Red pitaya is a possible source of food pigments and has great potential for pharmaceuticals and industrial products. The results indicated here indicate that polar solvents, such as methanol and water, were efficient for the extraction of pigments. The pH value had no significant influence on the process of extracting pigments from the red pitaya bark flour. A determining factor for stability was temperature, which had a significant influence; the colour exhibited greater durability at low temperatures. Furthermore, at low temperatures, pigment regeneration can occur. Samples stored in the absence of light showed the least loss of colour in the aqueous extract. These results enable the use of red pitaya bark flour as a natural source of pigments, rich in bioactive compounds, whose characteristics may vary according to storage conditions, temperature and time. Although more in-depth knowledge of the composition and profile of pigments is needed, this study is a step forward in the awareness and use of dyes of natural origin.

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