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EVALUATION OF THE EFFICIENCY OF THE EXTRACTION OF THE CAROTENOID NORBIXIN FROM ANNAUT SEEDS (Bixa Orellana L.) USING ALKALINE SOLUTION BY TWO EXTRACTION ROUTES

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ABSTRACT

Annatto-based dyes have been one of the most widely used in the world and also have wide applications in the textile, paint, cosmetic, pharmaceutical, and food industries. Among them, the carotenoid norbixin has antioxidant, antimicrobial, and antitumor properties. Thus, the objective of this work is to extract norbixin from annatto seeds through alkaline extraction considering two extraction routes, since the development of an efficient, environmentally safe, and economically viable extraction method has become indispensable. The characterizations performed were UV/vis, XRD, FTIR, TGA, and DSC. UV/vis spectrophotometry revealed that extraction route 2 (NBX_R2) extracted the highest concentration of total soluble solids, however, with lower crystallinity due to the presence of starch as a byproduct identified by the diffractogram (XRD). The FTIR spectrophotometry

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revealed the respective absorption bands and the DSC curve showed greater energy absorption to degrade the greater quantity of residue present in the extracted product and identified in the respective thermogram.

Keywords: Annatto. Norbixin. Alkaline extraction. Concentration. Crystallinity.

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INTRODUCTION

Annatto (Bixa orellana L.) is a species native to the Amazon Rainforest and the Atlantic Forest known for the natural dye extracted from its seeds (PINHEIRO et al., 2020). Due to the size of these biomes, Brazil has established itself as the largest producer of annatto-based seeds and dyes (COLOSIMO et al., 2022), being one of the most widely used dyes in the world and applied in the textile, paint, cosmetic, pharmaceutical and food industries.

Annatto extract has some characteristics that are considered intrinsic, such as abundance, and stability, in addition to the possibility of obtaining water-soluble or fat-soluble extracts from the same source depending on the extraction method (CUNHA et al., 2009). Therefore, the development of an efficient, environmentally safe, and economically viable extraction method has become indispensable (COLOSIMO et al., 2022). Due to the awareness created about ecological issues regarding the use and control of chemicals, alternatives for controlling pathogens have emerged, such as the use of plant extracts, which have been indicated. It is a current trend in food to replace artificial dyes with natural dyes due to growing concerns about body health (LIU et al., 2022). It is worth mentioning that the composition of plant extracts from medicinal plants has fungi-toxic properties and is, therefore, less harmful to humans and the environment, in addition to being cost-effective and available to producers (VENTUROSO et al., 2011).

In addition, consumers have been increasingly seeking products of natural origin, because there is already a health concern, therefore, technological development of processing must keep up with market evolution (HUSA et al., 2018). Regarding extraction methods with organic solvents, they are arduous and require restricted solvents in many countries, in this case, direct alkali extraction is established as an alternative extraction process. The alkaline solution hydrolyzes or saponifies (–COOCH3) bixin and generates dicarboxylic acid salt or norbixin salt (WITONO et al., 2022)

The carotenoids bixin and norbixin (Figure 1) are the main pigments extracted from the pericarp of annatto seeds (Bixa Orellana L). Bixin indicated in Figure 1(a) represents 80% of the carotenoid pigments found in annatto and is chemically characterized as a 25-carbon isoprene chain containing a carboxylic acid and a methyl ester at the ends (KUSMITA et al., 2022). On the other hand, norbixin indicated in Figure 1(b) is found in smaller quantities (20%) and, because of this, is generally obtained from the saponification

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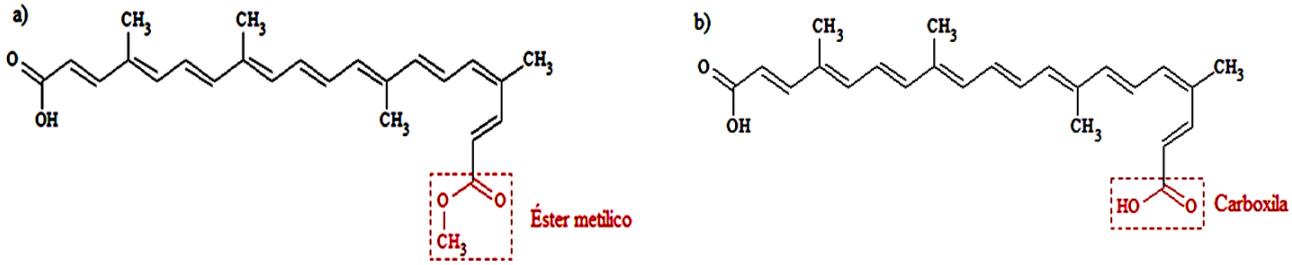
of bixin, in the form of its demethylated derivative, that is, its chain has only 24 carbons (SOUSA *et al.,* 2020).

Figure 1: Structural formula of Bixin (a) and Norbixin (b).



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In this process, the intensity of the bixin saponification reaction depends largely on the concentration and type of base used and, to a lesser extent, on the time and temperature of the process. In general, the more concentrated the base, the more complete the saponification reaction will be. On the other hand, when higher temperatures are used, an increase in the pigment extraction yield is observed, but with little increase in the bixin hydrolysis reaction to norbixin (STRINGHETA; SILVA, 2008).

Norbixin is a naturally occurring, water-soluble apocarotenoid (dicarboxylic acid) that has antioxidant, antimicrobial, and antitumor properties, which can influence and improve tissue healing processes (MUTHUKUMAR et al., 2014). Furthermore, in the context of traditional medicine, some studies demonstrate broad pharmacological activities such as nephroprotective and lipid-lowering effects (KUSMITA et al., 2022).

Since the extraction of carotenoids using an alkaline solution requires a less arduous and safer solvent than extraction using organic solvents, this study aims to extract the carotenoid norbixin using aqueous alkaline extraction considering two extraction routes.

MATERIALS AND METHODS

MATERIALS

Bixa orellana L. seeds were purchased from a local store in the city of Teresina - PI. Potassium hydroxide (85% purity, Vetec, Brazil) was used in the saponification of bixin into norbixin salt. Hydrochloric acid (36% purity, Dinâmica, Brazil) was used to neutralize the extract and convert the norbixin salt into norbixin.

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METHODS

Extraction of norbixin by route 1 (nbx_r1)

The extraction process followed the methodology proposed by Sousa et al., (2020) with minor changes. Initially, 200 grams of seeds were washed, cleaned, and dried in an oven at 40 °C for 24 hours. Then, the seeds were added to the 4% KOH solution (w/w) and heated at 70 °C for 1 hour. After the mixture cooled, the seeds were separated and 12 mol x L-1 HCl was added to the supernatant until a precipitate with a color change was formed. Finally, the precipitate was washed until pH equal to 4 and then dried in an oven at 70 °C for 24 hours.

Extraction of norbixin by route 2 (nbx_r2)

The extraction followed the methodology proposed by Witono et al., (2022) with minor adaptations. 200 grams of seeds were separated, washed with distilled water, and placed in an oven at 50 °C for 12 hours to dry. Then, the seeds were added to a KOH solution at 0.5 mol x L-1 followed by heating at 70 °C for 5 hours with constant stirring. At this stage, the norbixin salt (potassium norbixin) is formed; finally, a hydrochloric acid solution at 3 mol x L-1 was added for neutralization and conversion of the norbixin salt into norbixin.

CHARACTERIZATION

Yield of extracted powder

To determine the yield (Re), after drying the solvent in the oven, the procedure carried out by Oliveira et al. (2021) was used, in which the final mass of the solid extract (me) was weighed and using the value of the mass of annatto seeds used (mss), the total yield of the extract (Re), in percentage, was determined through the Equation 1:

$$Re = \frac{m_e}{m_{ss}} \text{ x } 100 \qquad (1)$$

Analysis by ultraviolet/visible spectrophotometry (UV/vis)

Molecular absorption spectra were performed to determine the presence of characteristic peaks of norbixin together with calibration curves. The characterization was performed on a SHIMADZU double-beam spectrometer, model UV-2401 PC. Solutions (Figure 2) of NBX_R1 and NBX_R2 with defined concentrations were prepared using 0.5%

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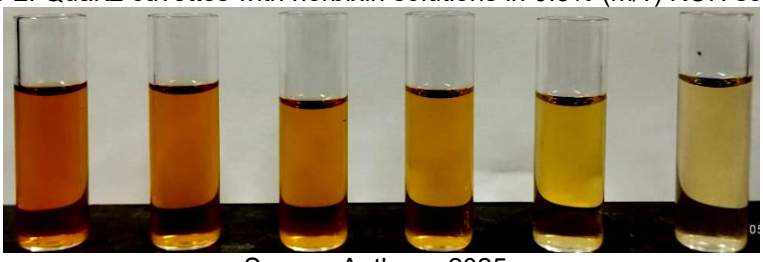
(m/v) KOH solution as solvent. The prepared diluted solutions were placed in quartz cuvettes and subjected to spectral analysis in a scanning range between 200 a 600 nm.

Figure 2: Quartz cuvettes with norbixin solutions in 0.5% (m/v) KOH solution



Source: Authors, 2025.

X-ray diffraction (XRD)

The norbixin powders were analyzed on a Panalytical Empyrean Series 2 X-ray diffractometer, equipped with a cobalt tube with λ = 1.78 nm and a secondary monochromator for Co, at a voltage of 40 kV and a current of 45 mA. The samples were analyzed in the range of diffraction angles 2θ ranging from 5 to 90° and a speed of 2 °C/min–1.

Fourier transform infrared (FTIR)

The analyses were performed on a Fourier Transform Infrared Spectrophotometer Ir Affinity-1 from SHIMADZU with wavenumber recording in the range of 4000 to 400 cm-1. The spectra were obtained at a resolution of 16 cm-1. The samples were prepared in the form of tablets with potassium bromide (KBr) and the bands observed in the spectrum were characterized according to their functional groups.

Thermogravimetric Analysis (TGA)

The thermogravimetric analysis (TGA) was performed on Shimadzu equipment, model TGA-51. The analysis conditions were: test temperature range of 25 °C to 1000 °C, heating rate of 10 °C. min-1, nitrogen atmosphere at 50 mL.min-1 and platinum sample holder.

Differential Scanning Calorimetry (DSC)

The DSC measurement was performed on Shimadzu equipment – Model DSC 60 Plus under the following conditions: test temperature range of 25 °C to 600 °C, heating rate of 10 °C.min-1, nitrogen atmosphere at 50 mL.min-1 and aluminum sample holder.

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RESULTS AND DISCUSSION

YIELD OF EXTRACTED POWDERS

From the 200g of annatto seeds used, 18.36g and 19.27g were obtained, representing a yield of 9.2% and 9.6% (m/m) for NBX_R1 and NBX_R2, respectively. Extractions performed by Pimentel (1995) in KOH solution obtained a yield of 8.55% by mass. However, the extractions performed by Silva; Bizerra; and Fernandes, (2018) obtained a yield of 4.53% with ethanolic extract. The differences observed may be related to the concentration of the KOH solution used and the contact time of the seeds with the extracting solution. In addition, Maniglia; and Tapia-Blácido (2016) reported that alkaline methods aid in the release, providing a higher extraction yield.

ULTRAVIOLET/VISIBLE SPECTROPHOTOMETRY (UV-VIS)

The optical analysis of the solutions of the NBX_R1 and NBX_R2 samples, Figures 3a and 3b, revealed two peaks of maximum absorbance at 453 nm and 482 nm, which according to Witono et al., (2022); Rahmalia et al., (2018) and Silva et al., (2018) these peaks are characteristic of the carotenoid norbixin contained in the annatto extract. According to Rahmalia; Crespo; Usman, (2021) these peaks are related to the fact that norbixin presents an electronic transition mediated by excited electrons $\pi \rightarrow \pi^*$ in the conjugated double bond chain of the molecule, this transition occurs from the lowest vibrational level in the ground state to the lowest vibrational level in the excited state (HOMO → LUMO).

From the calibration curves shown in Figures 3c and 3d, the molar absorptivity coefficients (θ) of the NBX_R1 and NBX_R2 samples with a maximum absorption peak at 453 nm were determined, finding a θ value equal to 1.5 x 104 L.mol-1.cm-1 and 2.3 x 103 L.mol-1.cm-1, respectively. In this case, considering that the aliquots were prepared in 0.5% (m/v) KOH solution. According to Calegaro et al., (2018), carotenoids present light absorption ($\varepsilon \sim 10\ 5$ L.mol −1.cm −1) in the range of 380 to 550 nm of the visible spectrum. However, Carvalho et al., (2020) determined the molar absorptivity coefficient (θ) of the pigment norbixin at $1.4 \times 10\ 4$ L.mol −1.cm −1, a value that guarantees the pigment's great photon capture capabilities.

From the values of the molar absorptivity coefficients θ, the concentrations for samples of NBX_R1 and NBX_R2 were determined, obtaining 1 x 10-2 g.L-1 and 9.8 x 10-2 g.L-1, respectively. The concentration values obtained revealed a yield of 95% and 98%,

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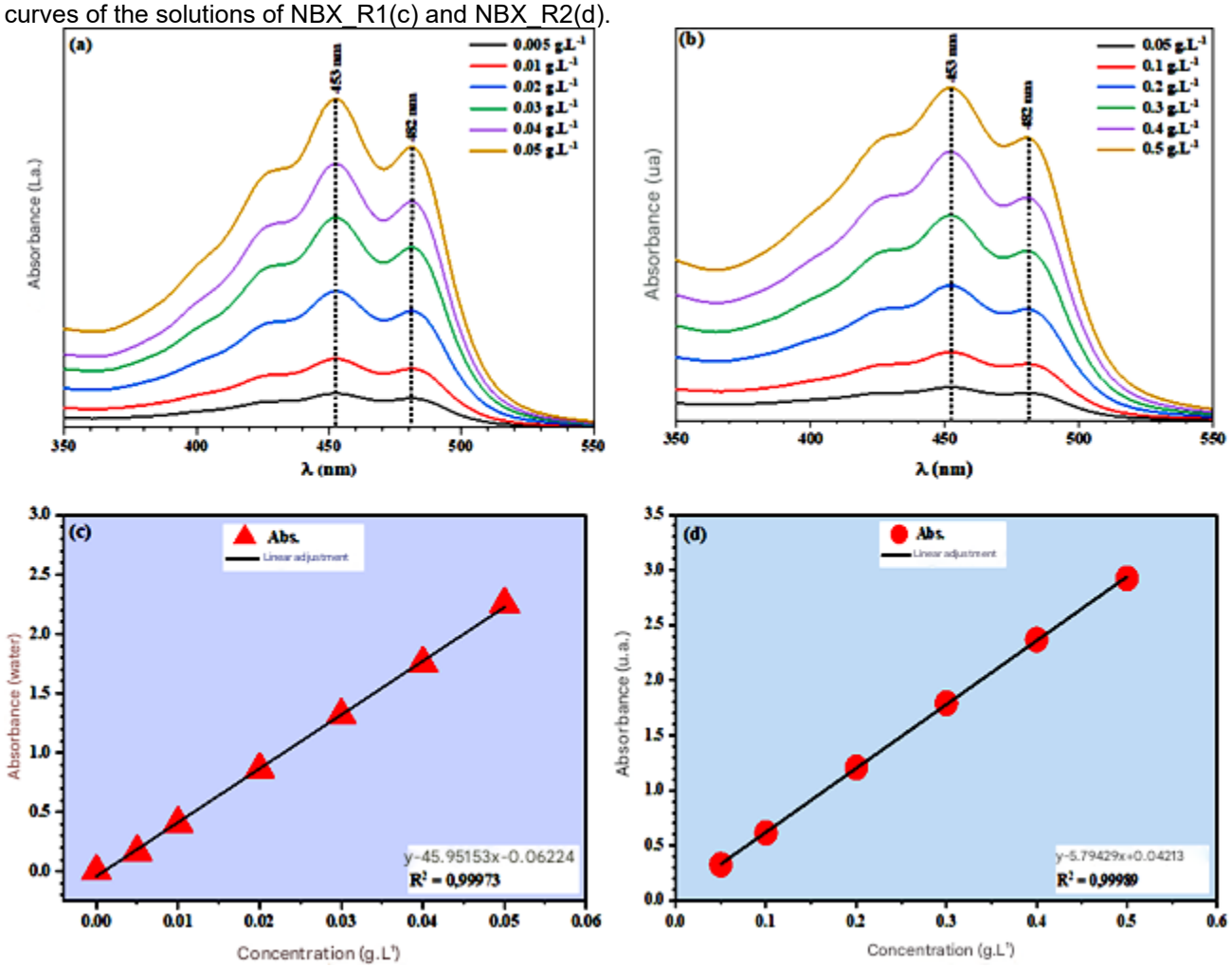
by mass, of total soluble solids. According to studies carried out by Filipini et al., (2022), the efficiency of the extraction of pigments from annatto seeds when the extracting solvent is the KOH solution, obtained a yield of approximately 98%, by mass.

Figure 3: Absorption spectra of the diluted solutions of NBX_R1(a) and NBX_R2 (b) and the calibration curves of the solutions of NBX_R1(c) and NBX_R2(d).



Source: Authors, 2025.

X-RAY DIFFRACTION (XRD)

Figure 4 shows the diffractograms of the NBX_R1 and NBX_R2 samples, where it was possible to identify in the NBX_R1 sample the presence of narrow peaks at 28° and 40°, with a maximum at 28° and narrow peaks of low intensity at 45.3°; 50.2°; 58.6°; 66.4° and 73.6°. In the NBX_R2 sample, the same narrow peaks were observed with lower intensity, which means the existence of a more pronounced amorphous region, however, both samples presented relative crystallinity. According to studies carried out by Matos (2017), norbixin has curves typical of semicrystalline material presenting peaks related to the crystalline regions. Furthermore, the peaks present in the region with 2θ values from 5°

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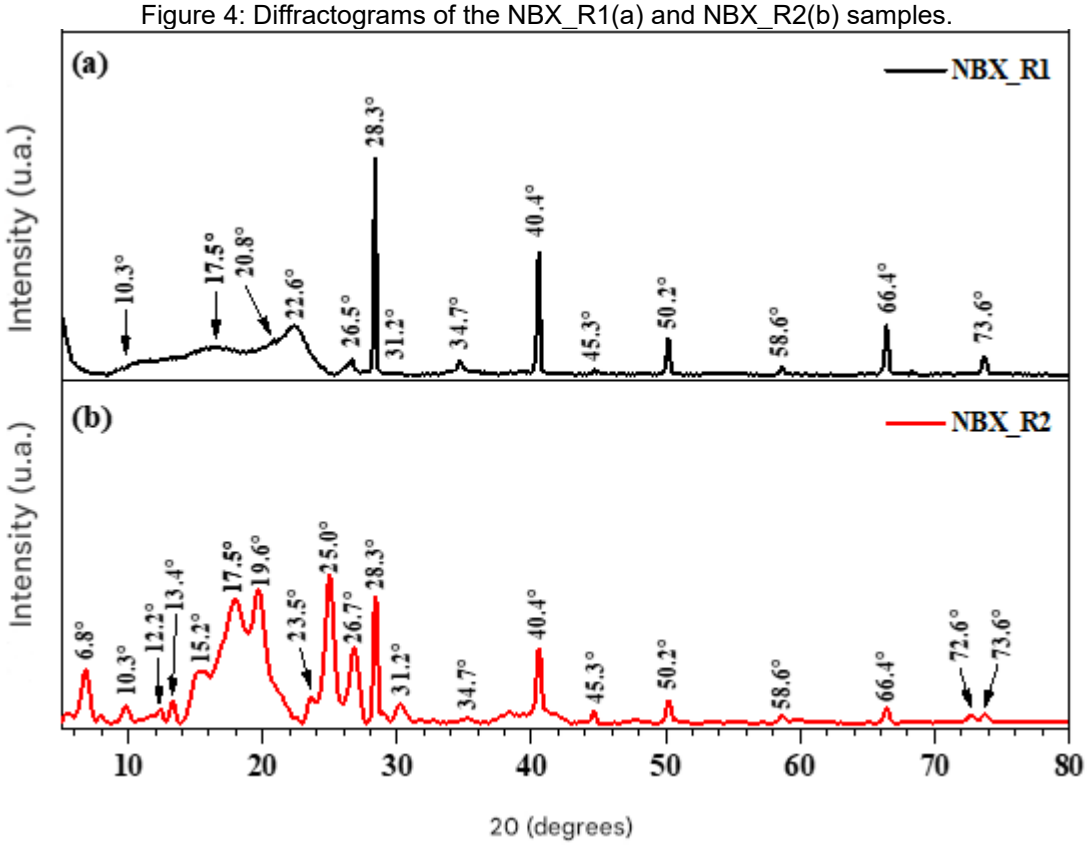
to 26° indicate the presence of a mixture of type A and type B starch, which according to Maniglia; Tapia-Blácido (2018) the peaks at 2θ equal to 5° and 12° are characteristic of type A starch, while the 2θ peaks equal to 15°, 17°, 19° and 23° according to Zabot et al., (2019) are characteristic of type B starch. B.

Figure 4: Diffractograms of the NBX_R1(a) and NBX_R2(b) samples.



Source: Authors, 2025.

From the information in Table 1, the relative crystallinity content of the samples was determined, indicating a crystallinity of 52% for NBX_R1 and 29% for NBX_R2. This difference may be associated with the fact that in extraction route 2, the seeds were placed in a lower-concentration extractive solution for a longer contact time, extracting not only norbixin but also some organic residues. According to Silveira and Tapia-Blácido (2018), the use of alkaline methods isolates some residues, including starch, resulting in a material with lower crystallinity.

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Table 1: Crystallinity content of the norbixin samples NBX_R1 and NBX_R2.

Samples	Total area of crystalline peaks	The total area of all peaks (crystalline + amorphous)	Crystallinity (%)
NBX_R1	13822.567	26771.50182	52.0
NBX_R2	17198.408	59465.42518	29.0

Source: Authors, 2025.

FOURIER TRANSFORM INFRARED SPECTROSCOPY (FTIR)

In Figure 5(a), it can be observed that norbixin NBX_R1 has an absorption band at 3427 cm−1, which is attributed to the (OH) bond stretching of the terminal dimeric carboxylic acid group (—COOH). According to Silva Junior et al. (2021) and Sousa et al. (2020), this band occurs between 3747 and 3180 cm−1, with a maximum absorption at 3394 cm−1. Regarding the band located at 2930 cm−1, Gutierrez and Alvarez (2017) reported that this corresponds to the stretching of the CH2 group bond, with a shoulder at 2860 cm−1 attributed to the asymmetric C–H stretching vibration (DOS SANTOS et al., 2021). There is a band at 1649 cm−1 attributed to the C=O stretching vibration of the carboxyl group, characteristic of norbixin (SILVA JÚNIOR et al., 2021; SOUSA et al., 2020). The bands located in the region between 1000 and 880 cm−1 are attributed to C=C bonds (SOUSA et al., 2020). The band at 1550 cm−1 represents the terminal carbonyls found in the structure of norbixin (SOUSA et al., 2020). The band at 1421 cm−1 is related to C–H bending vibrations of the sp3 type (SOUSA et al., 2020; GUTIERREZ; ALVAREZ, 2017). A band at 1030 cm−1 was also observed, which was attributed to the asymmetric bending of C—H. According to Dos Santos et al. (2021), this band is common in carotenoid compounds, especially in bixin and norbixin. Additionally, two bands at 860 and 763 cm−1 were observed, which according to Silva Júnior et al. (2021) correspond to out-of-plane curves, indicating a natural trans-precipitation, important for the synthesis of stereoselective reactions when obtaining norbixin.

When comparing the absorption bands of NBX_R1 with those of NBX_R2 in Figure 5(b), it is evident that there was an increase in the quantity and intensity of the absorption bands in the NBX_R2 sample. In this case, the bands corresponding to (-OH) shifted from 3427 cm−1 to 3404 cm−1, 1550 cm−1 to 1563 cm−1, 1060 cm−1 to 1030 cm−1, and 1157 cm−1 to 1178 cm−1. However, the band at 1157 cm−1 in NBX_R1 and the band at 1178 cm−1 in NBX_R2 are attributed to the stretching of the C—O bond, which Cruz et al. (2022) reported as characteristic of starch, particularly the band around 1160 cm−1. In this case, the increase in the number of bands and the observed shifts are related to the fact that the seeds used for the extraction of NBX_R2 were in contact with the KOH extractive

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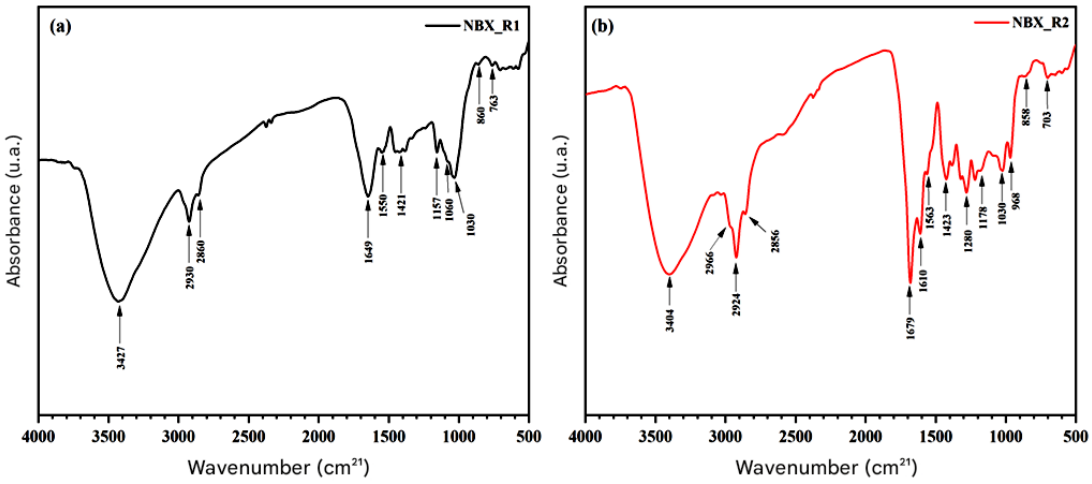
solution for a longer time. According to Witono et al. (2022), this longer time causes the extraction not only of norbixin but also other byproducts present in the seeds.

Figure 5: IR absorbance spectra of NBX_R1(a) and NBX_R2(b)).



Source: Authors, 2025.

THERMOGRAVIMETRIC ANALYSIS (TGA) AND THERMOGRAVIMETRIC DERIVATIVE (DRTG)

Figure 6 shows the thermal behavior of the norbixin powders NBX_R1 and NBX_R2, where it is possible to identify that both samples presented similar thermal stability with 4 degradation stages. In summary, the data contained in Table 2 reveal that in the first event, the samples lost approximately 4% and 6% in mass between 30 °C and 145 °C, respectively. These losses are related to the loss of water adsorbed on the surface, which according to Barrozo; Santos; Cunha (2013); and Sousa et al., (2020) is due to the hygroscopic character of the carotenoid norbixin. The second event presents a maximum degradation rate at temperatures of 260 °C and 296 °C, respectively. Studies carried out by Oliveira et al., (2021) revealed a maximum degradation rate at a temperature of 230 °C. This difference may be related to the parameters used in the extraction of the carotenoid, such as the type of extracting solvent, its concentration, time, and extraction temperature. The thermograms also revealed a loss of approximately 50% in mass, which occurs up to 400 °C in the samples, which according to Oliveira et al., (2022) may be related to the decomposition of starch that occurs between 330 °C and 380 °C.

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Figure 6: DSC curves of norbixin samples NBX_R1 and NBX_R2

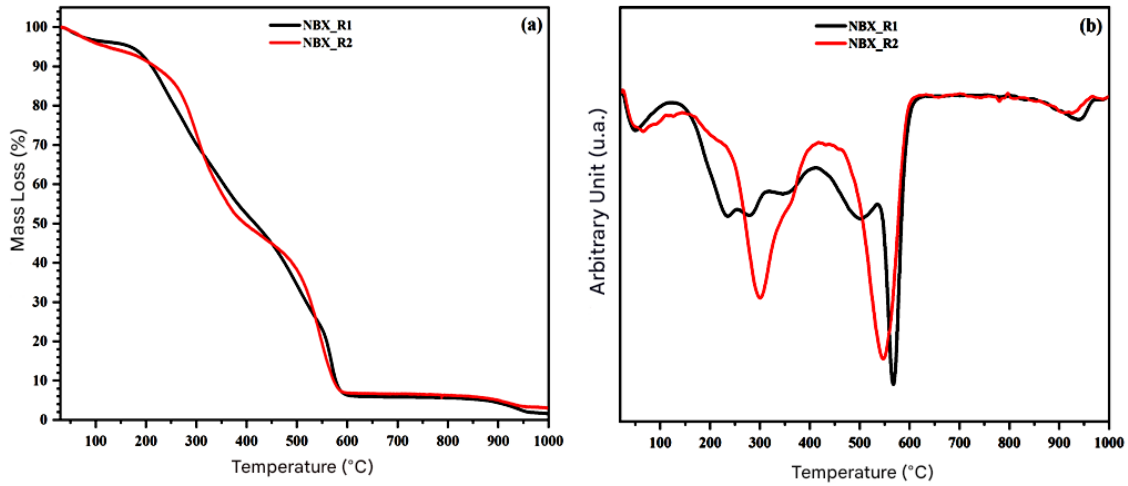

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Table 2: TGA results of the NBX_R1 and NBX_R2 samples.

Samples	Thermal Event	ΔT (°C)	Δm (%)	Residue (%)
NBX_R1	1st	30 to 115	3.6	~ 3.0
	2nd	130 to 410	45.3	
	3rd	415 to 630	44.4	
	4th	840 to 970	3.5	
NBX_R2	1st	30 to 145	5.7	~ 6.0
	2nd	150 to 420	46.3	
	3rd	440 to 630	39.2	
	4th	820 to 970	2.8	

Legend: ΔT = temperature variation, Δm = mass variation.
Source: Authors, 2025.

DIFFERENTIAL SCANNING CALORIMETRY (DSC)

Figure 6 shows that the DSC curves of norbixin NBX_R1 and NBX_R2 revealed two exothermic peaks (peaks 1 and 2) and one endothermic peak (peak 3) in both samples. Table 3 shows that peaks 1 and 2 have temperature variations with energy changes that are close. According to Perotti et al. (2020), from 170°C to 300°C, norbixin undergoes decomposition, releasing primarily H2O and CO2 fragments. However, peak 3 of the NBX_R2 sample shows a higher absorbed energy compared to the same peak in the NBX_R1 sample. This difference, according to Maniglia; and Tapia-Blácido (2016), may be related to the fact that alkaline extraction produces starch granules, which cause an increase in energy. Moreover, studies by Sousa et al. (2020) showed that the presence of an endothermic peak at 416°C is associated with the melting or boiling process of one of the degradation components of norbixin.

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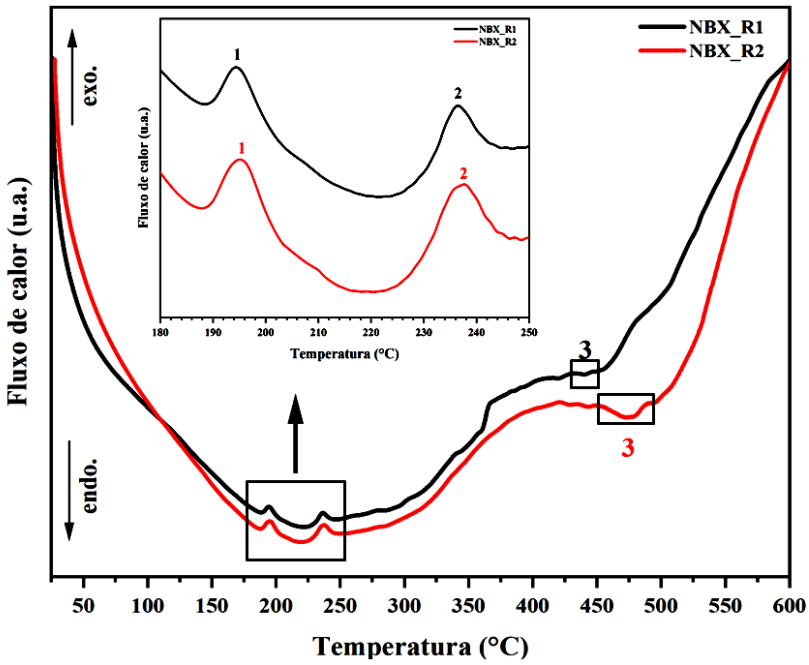
Figure 6: DSC curves of norbixin samples NBX_R1 and NBX_R2



Source Authors

Table 3: Thermal behavior of DSCs of norbixin (NBX_R1) and norbixin (NBX_R2).

Samples	Peaks	Tonset (°C)	Tendset (°C)	Tpico (°C)	ΔH (J/g)
NBX_R1	1	189.63	200.59	194.44	3.25
	2	229.97	241.66	236.54	4.20
	3	434.37	446.53	439.52	0.72
NBX_R2	1	189.38	201.60	195.17	3.29
	2	229.63	243.07	237.67	4.34
	3	459.60	484.68	473.66	11.87

Legend: Tonset (extrapolated initial temperature), Tendset (extrapolated final temperature), ΔH (energy change)
Source: Authors, 2025.

CONCLUSION

The results showed that the extraction of norbixin using an alkaline extractive solution can be a great business opportunity, in addition to being economically viable. The analysis of the extraction time and extractive solution concentration parameters revealed that a more concentrated base promotes a higher conversion of bixin to norbixin during the saponification process. A longer extraction time, despite providing a higher conversion of bixin to norbixin, also causes the extraction and degradation of other organic compounds.

Therefore, extraction route 1 (NBX_R1) was more efficient than extraction route 2 (NBX_R2), even though the yield of the extract in both routes did not show a significant difference. However, UV/Vis spectrophotometry revealed a higher concentration of norbixin in the NBX_R2 sample. Upon analyzing the diffractogram, UV-Vis spectroscopy, and the DSC curve of the NBX_R2 sample, the presence of starch as an extracted byproduct was

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identified, which caused a decrease in crystallinity. Additionally, the thermogram revealed a greater amount of residues being degraded.

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