Journal of the Professional Association for Cactus Development

Pigmenting potential of betacyanins and betaxanthins separated from fruits of *Stenocereus pruinosus* with aqueous biphasic systems

Leticia García-Cruz[1], Diana Guerra-Ramírez[2], Salvador Valle-Guadarrama* [1,3]

[1]Programa de Posgrado en Ciencia y Tecnología Agroalimentaria, Universidad Autónoma Chapingo, Mexico-Texcoco km 38.5, Chapingo, 56230, Texcoco de Mora, Mexico.
[2]Laboratorio de Productos Naturales. Departamento de Preparatoria Agrícola, Universidad Autónoma Chapingo, Mexico-Texcoco km 38.5, Chapingo, 56230, Texcoco de Mora, Mexico.
[3]Departamento de Ingeniería Agroindustrial, Universidad Autónoma Chapingo, Mexico-Texcoco km 38.5, Chapingo, 56230, Texcoco de Mora, Mexico.

*Corresponding Author: svalleg.chapingo@gmail.com

Abstract. The fruit of *Stenocereus pruinosus* is a source of betalains that can be used to provide tonalities ranging from red-violet to yellow, but the partitioning into betacyanins and betaxanthins must be addressed, in addition to the reduction of sugars. The objective was to evaluate the use of extracts of betalains from *Stenocereus pruinosus* fruits with different tones and low sugar content in the pigmentation of food matrices. Aqueous biphasic systems (ABS) based on 1-propanol and sodium citrate were applied in multiple stages to obtain fractions rich in betaxanthins and betacyanins. Subsequently, an ABS based on polyethylene glycol 4000 and sodium citrate was used to reduce the sugar content. The extracts were used to pigment milk and to evaluate the color stability during a storage of 39 d at 4 °C. Four fractions, E_{yellow}, E_{orange}, E_{pink}, and E_{red}, were obtained with betacyanins concentration of 9.23, 22.37, 76.54, and 61.0 mg L^{-1} and betaxanthins concentration of 49.3, 77.5, 35.2, and 53.32 mg L^{-1}, respectively. The hue angle of the pigmented milk with E_{yellow}, E_{orange}, E_{pink}, and E_{red} was 81.9, 60.6, 9.6, and 13.0°, respectively, at the beginning. The color remained without apparent change during 24 d, which allowed extracts of betacyanins and betaxanthins to be qualified as adequate to provide reddish and yellow hue in milk beverages. Although the multistage operation was required, four fractions with different tones were obtained from only one sample, which constitutes an innovative feature. In conclusion, it is feasible the use of betalains obtained with ABS from fruits of *S. pruinosus* as pigmenting agents of foods.

Keywords: *Stenocereus spp., aqueous two-phase extraction, betalain partitioning, pigmentation.*

Citation: García-Cruz, L., Guerra-Ramírez, D., Valle-Guadarrama, S. 2023. Pigmentation potential of betacyanins and betaxanthins separated from fruits *of Stenocereus pruinosus* with aqueous biphasic systems. *Journal of the Professional Association for Cactus Development. 25*: 136-152. https://doi.org/10.56890/jpacd.v25i.522

Associate Editor: Pablo Preciado-Rangel

Technical Editor: Tomas Rivas-Garcia.

Received date: 03 March 2023

Accepted date: 25 July 2023

Published date: 08 August 2023

Introduction

For many years, synthetic colorants have been used in food products for reasons of low cost, stability, and pigmenting potential without altering the flavor of the product. However, these products can affect health (Malabadi *et al.,* 2022), so there is interest in replacing them with natural pigments. Substances as Red 40 and Yellow 5 are artificial colors widely used in the food industry. However, there are reports that relate these compounds with the presence of tumors in mice, with hyperactivity in children, and with carcinogenic chemicals (Okafor *et al.,* 2016).

From different vegetable sources (Kruszewski *et al.,* 2023; Wang *et al.,* 2023), it is possible to obtain betalains, which are compunds that include betacyanins, that give red-violet coloration and are formed by the union of betalamic acid with cyclo-3,4-dihydroxyphenylalanine (cyclo-Dopa), and betaxanthins, which exhibit yellow tones and are formed by the condensation of betalamic acid with an amino acid or an amine (Azeredo, 2009). Betalains have a promising use as natural pigments (Vargas-Campos *et al.,* 2018) in the food industry, since they are stable in the range of pH from 3 to 7, so they can be used both in acidic and neutral foods (Stintzing and Carle, 2007) and can provide a wide range of colors by controlling the proportion of betacyanins and betaxanthins (Gandía-Herrero *et al.,* 2013). Thus, betacyanins and betaxanthins can replace colorants such as Red 40 and Yellow 5 in food matrices to provide similar shades. In fact, betalain extracts have been used to provide color in dairy foods such as milk (Gengatharan *et al.,* 2016; Güneşer, 2016), yogurt (Dias *et al.,* 2020; Guneser, 2021), and ice cream (Gengatharan *et al.,* 2016). However, mainly extracts rich in betacyanins have been evaluated and the use of extracts with betaxanthins as the main component has been little studied.

Beetroot has been the main source of betalains and it is the only one approved in the European Union for using as a colorant in foods, where it is classified by the Codex Alimentarius as E-162, but exhibits an earthy flavor caused mainly by the presence of geosmin (Moreno *et al.,* 2008). On the other hand, the Cactaceae family can also be a source of natural pigments (Reyes-Ulloa *et al.,* 2020), specifically betalains (García-Cruz *et al.,* 2022; Quiroz-González *et al.,* 2018; Rodríguez-Félix *et al.,* 2019). In this regard, the fruit of *Stenocereus pruinosus* (Cactaceae) exhibits high concentrations of betacyanins and betaxanthins in the pulp (García-Cruz *et al.,* 2017; Rodríguez-Sánchez *et al.,* 2017; Vargas-Campos *et al.,* 2018), with a balanced content of both groups, betacyanins and betaxanthins (García-Cruz *et al.,* 2017). However, the separation of betalains from cactus fruits faces the problem of a high concentration of sugars, which can limit their use, due to possible sensory alteration in the target material and the possible rejection by people with diabetes.

The separation of sugars can be done with hot water, acidic procedures, with the use of enzymes, and microbial treatments, among others (Zheng *et al.,* 2023), but these alternatives do not attend the separation of sugars from betalains, which requires a more specific technique. The separation of betalains from sugars can be done through flash chromatography, based on normal or reverse phase separation with high flow rates and low pressure (Fernando *et al.,* 2022), and with the aid of a reversed phase C18 resin adsorption (Thi Tran *et al.,* 2019), however the procedure may be expensive due to the requirement of highly specialized reagents and equipment. Among chromatography alternatives, the operation in open column is a simple, easy, and inexpensive technique, but it has the disadvantage of being a time-consuming process and requires too much solvent for an adequate elusion (Akash and Rehman, 2020). Besides, the preparative chromatography can obtain a high purity specific compound and with the highest yield, but it requires highly expensive equipment (Robards and Ryan, 2022).

Alternatively, Chandrasekhar *et al.* (2015), Vargas-Campos *et al.* (2018), and García-Cruz *et al.* (2021) showed that a procedure based on aqueous biphasic systems (ABS) is a viable option to separate betalains from sugars. An ABS can be formed with the mixture of two polymers, a salt and a polymer (Raja *et al.,* 2011), or a salt in mixture with an organic solvent (García-Cruz *et al.,* 2021; Leong *et al.,* 2019), where a phenomenon of salting-out is developed above certain concentrations, allowing other compounds to be separated between such phases (Gomes *et al.,* 2017). A separation based on ABS has been typified as a simple and low-cost technique, where compounds can even be concentrated through a non-thermal procedure (Rodríguez-Herrera *et al.,* 2023). Besides, Chandrasekhar *et al.* (2015) and García-Cruz *et al.* (2021) showed that the use of aqueous biphasic systems allows fractioning betalains into betacyanins and betaxanthins to obtain fractions of different colors by using a multistage procedure. In this context, the objective of the work was to evaluate the use of betalains extracted with aqueous biphasic systems from *S. pruinosus* fruits with different shades and low sugar content in the pigmentation of food matrices.

Material and Methods

Plant material

Fruits of the red-fleshed variant of *Stenocereus pruinosus* collected in commercial orchards of Santa Clara Huitziltepec, Puebla, Mexico (18º 35' 46'' N, 97º 55' 48'' W; 1940 m above sea level) were used. The physiological condition of fruits corresponded to commercial maturity, which occurred when spines were easily released and skin became bright (García-Cruz *et al.,* 2016).

Obtention of extracts

The procedure described by García-Cruz *et al.* (2021) to obtain extracts of different shades with a multistage operation was applied with aqueous biphasic systems formed by 1-propanol and trisodium citrate (Na_3Cit; $Na_3C_6H_5O_7$) in concentrations of 60.54 and 6.57%, respectively (Figure 1). A 50-g sample of pulp of *S. pruinosus* fruits was used and incorporated into a 320 g mixture of 1-propanol/Na_3Cit. The mixture was stirred with a magnetic element for 5 min, left to stand for 1 h in the dark, and centrifuged at 11,000×g for 5 min. Two liquid phases were formed: a top one, with a majority composition of propanol and a bottom phase, with a majority composition of Na_3Cit. The bottom phase was separated and the top phase of a system prepared *de novo* with water as solvent, without plant material, was added to it, and the procedure was repeated. The bottom phase was recovered once more and the procedure was repeated twice to obtain an extract rich in betacyanins (E_{pink}; Ep) (Figure 1).

The top phases of the first and second separations were mixed with the lower phase of a system prepared *de novo* with water as solvent and without plant material, to obtain a betaxanthin extract (E_{yellow}; Ey). The top phases of these systems were pooled because their betaxanthin content was similar. Another extract was prepared, also with 50 g of sample, and it was subjected to a single separation, from which the bottom phase was obtained, which had a mixture of betacyanins and betaxanthins (E_{red}; Er), and likewise the top phase, with greater betaxanthin content (E_{orange}; Eo). Fractions Ey and Eo were dried in a rotary evaporator and the residue was recovered in 50 mL of distilled water. The Ep and Er fractions were subjected, separately, to another ABS based on polyethylene glycol 4000 (PEG 4000) and trisodium citrate, at concentrations of 21.78 and 17.63%, respectively, in order to remove sugars, in accordance with Jiménez-Velázquez *et al.* (2020). In this system, the betalains were located in the top phase, while sugars remained in the bottom one, thus the top phases were recovered and used in pigmentation assays.

https://www.jpacd.org Electronic ISSN: 1938-6648

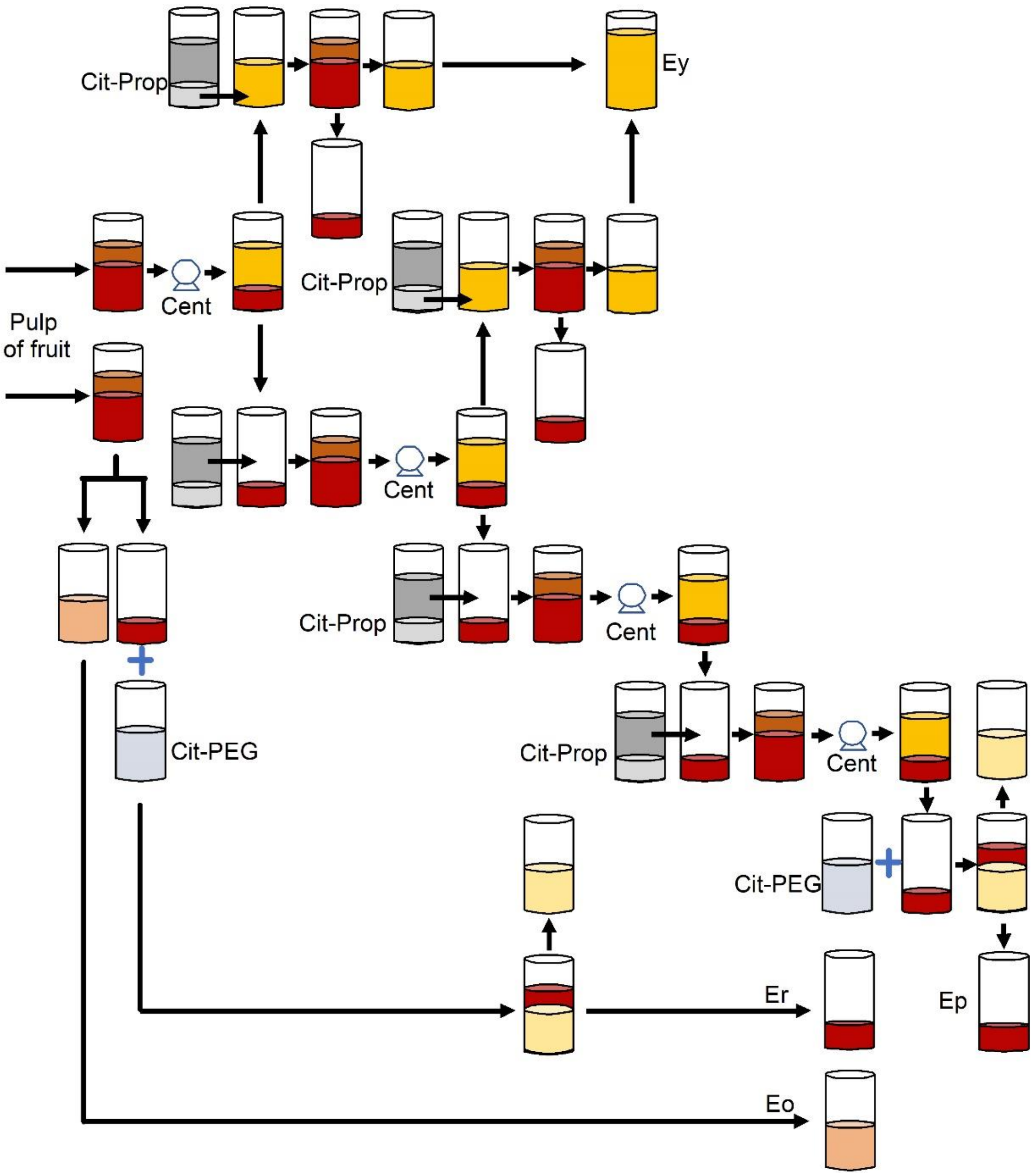

Figure 1. Procedure based on aqueous biphasic systems to obtain extracts E_{yellow} (Ey), E_{orange} (Eo), E_{pink} (Ep), and E_{red} (Er). Cit-Prop: system based on sodium citrate and 1-propanol. Cit-PEG: system based on sodium citrate and PEG 4000. Cent: centrifugation treatment.

Characterization of extracts

Each fraction was subjected to a spectrophotometric analysis with an UV-vis equipment (DR 500 UV-vis HACH, USA) to obtain absorption spectra. The extracts were subjected to evaluation of the content of betacyanin (c_{Bc}, mg L^{-1}) and betaxanthin (c_{Bx}, mg L^{-1}), with a spectrophotometric procedure (Stintzing *et al.,* 2005) through Equation (1), where A_1 is absorbance at 538 nm for betacyanins and 488 nm for betaxanthins, Fd (dimensionless) is a dilution factor at the time of reading in the UV-vis spectrophotometer (DR 500 UV-vis HACH, USA), M is molecular weight (550 g mol^{-1} for betanin and 308 g mol^{-1} for indicaxanthin), ε is molar extinction coefficient (60,000 L mol^{-1} cm^{-1} for betanin and 48,000 L mol^{-1} cm^{-1} for indicaxanthin) and L is the cell length (1 cm).

$$c_{Bc} \ or \ c_{Bx} = \frac{100 \ A_1 \ Fd \ M}{\varepsilon \ L} \tag{1}$$

The total sugar content in extracts was determined with the phenol-sulfuric acid method as described by Nowotny (1979), with some modifications. One hundred microliters of sample were placed in test tubes. Subsequently, 500 µL of phenol at 5% (w/v) and 2500 µL of concentrated sulfuric acid were added. The mixture was left to react for 15 min and absorbance (A_2) was read at a wavelength of 490 nm in a spectrophotometer (DR 500 UV-vis HACH, USA). A standard curve of glucose (c_{Glu}, mg mL^{-1}) was developed, with a determination coefficient R^2 equal to 0.995, which had the form of Equation (2).

$$c_{Glu} = 0.0022\, A_2 + 0.0026 \quad (2)$$

Pigmentation assays

Twelve batches of 20 mL of whole cow's milk (Lala®, Mexico) were used. Extracts Ey, Eo, Ep, and Er were added at different concentrations (c_{ex}, mg mL^{-1}) to vary the hue angle (H^*, degrees). Data were fitted to models with the form of Equation (3), where H_0^* (degrees), k_1 (degrees), and k_2 (mL g^{-1}) were regression constants.

$$H^* = H_0^* + k_1\, [1 - exp(-k_2\, c_{ex})] \quad (3)$$

The addition of extracts caused reduction of the hue angle. Based on Equation (4) (Valle-Guadarrama *et al.,* 2002), the minimum value of hue angle (H_{min}^*, degrees) that could be reached after an infinite addition of extract was determined. Likewise, the required concentration of extract to cause pigmentation of 95% ($c_{0.95}$, mg mL^{-1}), relative to H_{min}^*, was determined with Equation (5).

$$H_{min}^* = H_0^* + k_1 \quad (4)$$

$$c_{0.95} = \left(\frac{1}{k_2}\right) ln(20) \quad (5)$$

Four groups of batches of 20 mL of whole cow's milk (Lala®, Mexico) were prepared and incorporated with the extracts Ey, Eo, Ep, and Er, respectively, in the concentration defined as $c_{0.95}$. Units were stored at 4.0 (±1.0) °C for 39 d. Every three days, three units from each group were retired from the storage room and they were evaluated in terms of hue angle (H^*, degrees (Sant'Anna *et al.,* 2013), pH, and titratable acidity. The hue angle was evaluated with a Hunter Lab colorimeter (Mini Scan XE Plus 45/0-L, USA). The pH was determined using a portable potentiometer (Hanna Instruments, model HI 8420, Padua, Italy). Titratable acidity was evaluated according to the standard NOM-155-SCFI-2012 (STPS, 2012), through titration with NaOH 0.1 N, and phenolphthalein as indicator. Results were expressed as lactic acid content (g L^{-1}).

Data analysis

The phase of obtaining fractions with different composition of betacyanins and betaxanthins was conducted as a completely randomized design, where the type of extract (Ey, Eo, Ep, and Er) constituted the variation factor. The phase of evaluation of the pigmentation stability was conducted as a factorial arrangement, where the type of extract and the storage time constituted the variation factors. In both cases, an analysis of variance was applied, complemented with comparison routines of treatment means, applied with the Tukey test, with a significance level (α) equal to 0.05. All analyses were performed in triplicate.

Results and discussion

Pigmenting extracts

The aqueous biphasic systems applied in a multi-stage strategy based on systems with 60.54% 1-propanol and 6.57% trisodium citrate allowed obtaining four fractions with different hue: E_{red} (Er), E_{pink} (Ep), E_{orange} (Eo), and E_{yellow} (Ey) (Figure 1), which was consistent with that described by García-Cruz *et al.* (2021). The difference in colors was due to different proportions of betacyanins (Bc) and betaxanthins (Bx). The Ep fraction of the propanol-citrate system had a composition of 67.79 (±2.11) and 32.59 (±1.62) mg L^{-1} of Bc and Bx, respectively. An absorption spectrum with a single peak was obtained for this fraction, with the maximum at 540 nm (Figure 2A); however, the appearance was wide and a small shoulder was observed, so there was also a significant absorption reading at 488 nm. Meanwhile, the Er fraction presented concentrations of 101.38 (± 2.85) and 123.14 (± 1.85) mg L^{-1}, in Bc and Bx, respectively. These two fractions were reprocessed with a system based on sodium citrate and PEG 4000 (Figure 1) to reduce the sugar content (Jiménez-Velázquez *et al.,* 2020), which changed the concentration of Bc and Bx and also the proportion between these compounds (Table 1). At the end, the betaxanthin content was 5.35 and 3.46 times higher than the betacyanin one in Ey and Eo, respectively, while a reverse behavior was found in Ep, and Er, since in them betacyanins were found 2.17 and 1.14 times higher than betaxanthins, respectively, with the concentrations described in Table 1. The absorption spectrum of Ey showed only one peak (Figure 2C) and the color of the extract was indeed yellow, indicating the main presence of betaxanthins. The orange and pink fractions had also absorption spectra with a single peak, although they had the combined presence of betacyanins and betaxanthins. Meanwhile, the Er fraction showed a red hue and the presence of two peaks in the absorption spectrum (Figure 2B), derived from a greater presence of betacyanins and a lower presence of betaxanthins. Gandía-Herrero *et al.* (2013) obtained a similar behavior when they mixed extract of *Lampranthus* flowers (pink color) with extract of betaxanthins synthesized in the laboratory from beetroot and, depending on the proportion of betacyanins and betaxanthins, a different coloration was obtained, which varied from yellow (pure betaxanthins), through different shades of orange and red, to pink-purple (pure betacyanins). Similarly to the present work, Chandrasekhar *et al.* (2015) allowed obtaining two fractions from beetroot, one containing only betacyanins and other containing only betaxanthins, through ABS formed with polyethylene glycol and ammonium sulphate. Besides, Rodríguez-Herrera *et al.* (2023) intended the fractionation of betacyanins and betaxanthins from bougainvillea flowers but, due to betacyanins predominated in such material, the fractions had mainly red tonality. However, in the present work, four shades were obtained from a single plant source, which constitutes an innovative feature.

Table 1. Concentration of betacyanins (Bc) and betaxanthins (Bx) in extracts obtained with aqueous biphasic systems with 60.54% 1-propanol and 6.57% trisodium citrate applied in multistage.

Extract	Bc (mg L^{-1})	Bx (mg L^{-1})	Proportion Bc/Bx
E_{yellow}	9.23 (±0.83) d	49.34 (±1.11) c	1.00/5.35
E_{orange}	22.37 (±0.21) c	77.51 (±0.21) a	1.00/3.46
E_{pink}	76.54 (±1.19) a	35.29 (±1.12) d	2.17/1.00
E_{red}	61.00 (±1.79) b	53.32 (±1.25) b	1.14/1.00

Values in parentheses express standard deviation. Means with the same letter indicate non-significant difference (Tukey, 0.05) within a column.

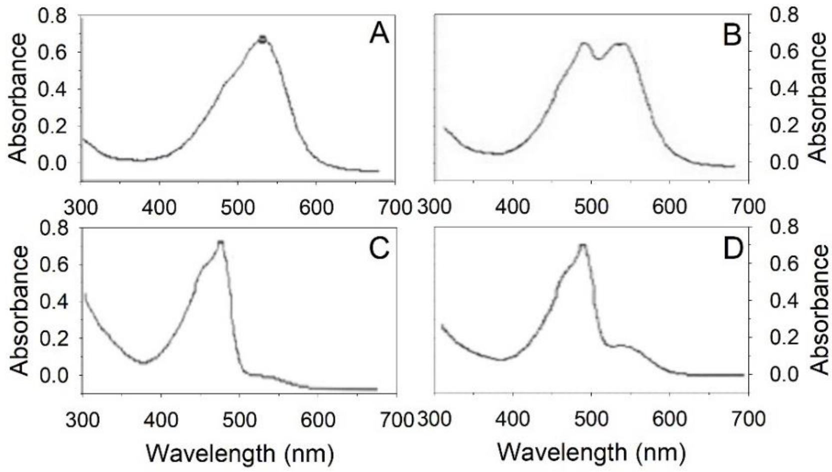

Figure 2. Absorption spectra of extracts E_{pink} (A), E_{red} (B), E_{yellow} (C), and E_{orange} (D).

Concentration of total sugars

The E_{yellow} and E_{orange} extracts had total sugar concentrations of 6.45 (±0.25) and 25.45 (±1.57) mg L^{-1}, respectively. The difference was due to E_{orange} came from a single extraction stage (Figure 1), while E_{yellow} came from a multi-stage extraction, which favored the gradual reduction of these compounds. It has been reported that a multi-stage ABS procedure can reduce the sugar content through a selective partition of compounds (Chandrasekhar *et al.,* 2015; García-Cruz *et al.,* 2021). Meanwhile, the E_{pink} and E_{red} fractions had sugar concentrations of 30.99 (±0.42) and 69.31 (±0.77) mg mL^{-1} after processing them with the propanol-citrate system, but the content was reduced to 8.52 (±0.66) and 20.91 (±0.66) mg mL^{-1}, respectively, with the reprocessing with ABS based on PEG 4000 and sodium citrate. In this regard, it has been observed that when the ABS technique is used to remove sugars with a system formed by a polymer and a salt, such compounds go towards the bottom saline phase (Jampani and Raghavarao, 2015; Sandate-Flores *et al.,* 2020; Vargas-Campos *et al.,* 2018). In this work, a similar situation was observed, since the bottom saline phase of the PEG 4000-citrate system presented the highest concentration of sugars, while the top polymeric phase had a reduction of 70% of them relative to the original extract.

Pigmentation curves

The gradual addition of extracts in samples of milk caused reduction of the hue angle with logarithmic behavior (Figure 3). Data fitted well to models with the form of Equation (3), with determination coefficients (R^2) that varied between 0.963 and 0.995 and the regression constants shown in Table 2. With this basis and the use of Equation (4), it was determined that the minimum hue angle (H^*_{min}) that could be achieved after a large addition of E_{yellow}, E_{orange}, E_{pink}, and E_{red} extract was 78.44°, 59.85°, -13.80° (346.20°), and 5.00°, respectively. In the context of the CIELab color sphere, the values of 0 and 90° indicate red and yellow hue angles, respectively (Sant'Anna *et al.,* 2013), while intermediate values correspond to orange hue, which was consistent with the results of the present work.

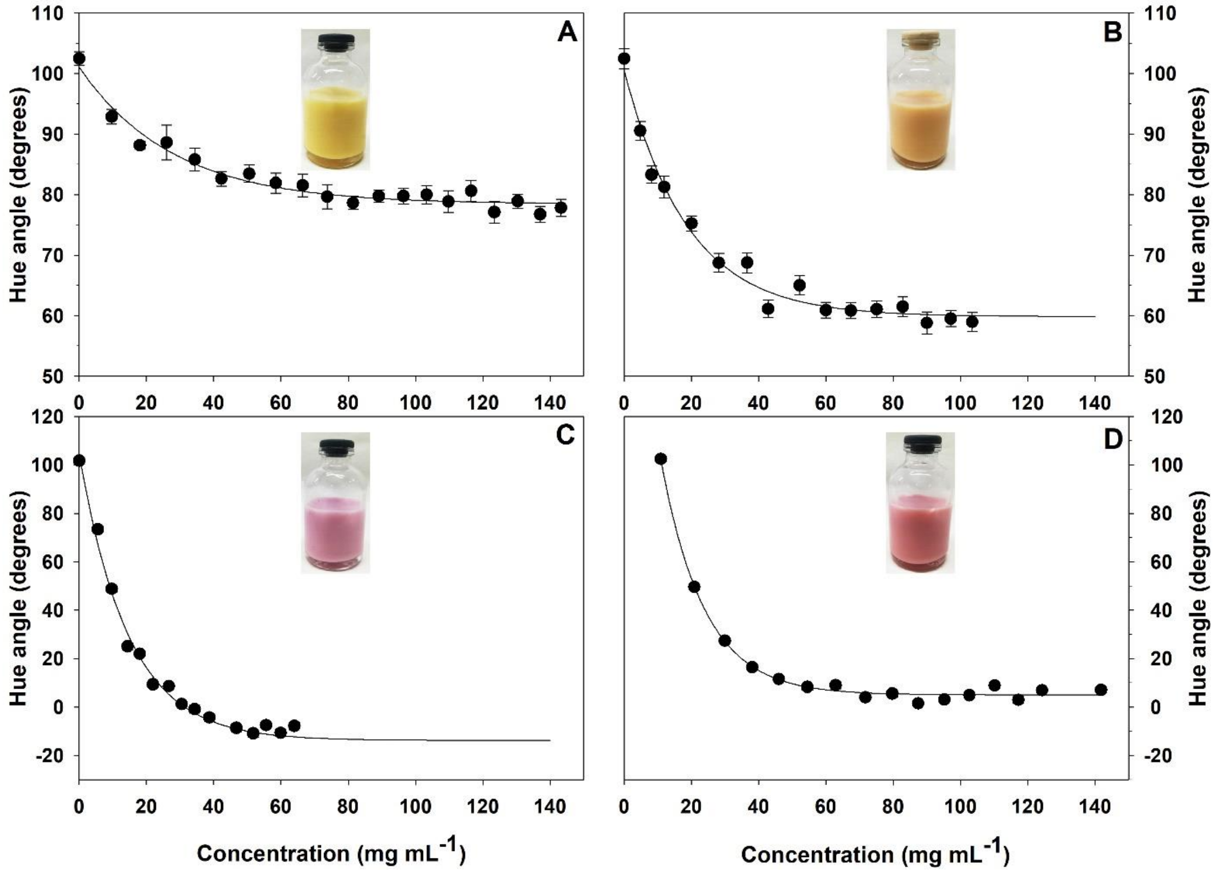

Figure 3. Pigmentation curves of milk incorporated with extracts E_{yellow} (A), E_{orange} (B), E_{pink} (C), and E_{red} (D). Points are experimental data. Solid lines correspond to regression adjustment.

Table 2. Pigmentation parameters with extracts obtained from *S. pruinosus* fruits through aqueous biphasic systems applied in multiple stages.

Parameter	Extract			
	E_{yellow}	E_{orange}	E_{pink}	E_{red}
H_0^* (degrees)	101.0714	100.6050	104.8972	233.1062
k_1 (degrees)	-22.6319	-40.7553	-118.6956	-228.1047
k_2 mL g^{-1}	0.0412	0.0447	0.0743	0.0779
R^2	0.9629	0.9826	0.9813	0.9946
H_{min} (degrees)	78.4395	59.8497	-13.7984	5.0015
$c_{0.95}$ (mg mL^{-1})	72.7119	67.0186	40.1035	38.4561

By analogy with similar kinetic phenomena (Muñoz-González *et al.,* 2022; Valle-Guadarrama *et al.,* 2018), the constant k_2 corresponded to a pigmentation coefficient and expressed the ability of the added extract to modify the hue angle. In this sense, the highest pigmentation capacity was found in E_{pink} and E_{red} extracts, while the lowest in E_{yellow} and E_{orange} extracts. On the other hand, due to the logarithmic behavior, it was considered adequate to have pigmentation of 95% in relation to the limit value and, based on Equation (5), it was determined that this condition could be achieved with concentrations of 72.71, 67.02, 40.10, and 38.46 mg mL^{-1} of extracts ($c_{0.95}$) in the medium to be pigmented with E_{yelow}, E_{orange}, E_{pink}, and E_{red}, respectively. In this regard, the best result was the feasibility of pigmenting food matrices with pink and red tones, due to the lower quantities of extract required in relation to the feasibility of obtaining yellow and orange tones. In addition, the required concentrations were similar to the value of 50 mg mL^{-1} determined by

Gengatharan *et al.* (2016) to use betacyanin extract from pitahaya (*Hylocereus polyrhizus*). Likewise, for the same purpose of pigmentation of dairy products, Güneşer (2016) used 0.3% w/v of a commercial beetroot extract.

Pigmentation stability

Pigmentation stability monitoring routines were conducted based on hue angle variation. The pigmentation of 20 mL units of whole cow's milk was based on the incorporation of 1.45, 1.34, 0.80, and 0.77 g of the extracts E_{yellow}, E_{orange}, E_{pink}, and E_{red}, respectively, which acquired hue angle of 82.05° (±1.06), 60.57° (±1.61), 9.65° (±1.37), and 12.88° (±1.80), and the appearance shown in Figure 4. In addition, commercial pigmented milks with similar colors to those obtained with the extracts were used as references. In this regard, milks commercially available with denomination of "strawberry flavor" (Figure 4c) and "vanilla flavor" (Figure 4f) had hue angles of 14.59° (±2.72) and 84.16° (±0.77), respectively, indicating that the E_{yellow} extract caused pigmentation similar to that shown by "vanilla flavor" type products, while the E_{pink} and E_{red} extracts caused pigmentation similar to that shown by "strawberry flavor" type commercial products. However, the potential to substitute artificial colorants in yellow foods is lesser than in the case of red systems, due to the higher quantities of extract required in the former than in the latter. On the other hand, the hue angle in materials pigmented with E_{yellow} and E_{orange} remained without significant change during the storage time (Figure 5A). However, the cases that used E_{pink} and E_{red} showed an upward trend from day 27, going from red to orange tones, which could be due to betacyanin degradation. In this regard, the loss of the carboxyl group in the C17 position of these compounds causes a color change from red to orange, in addition to dehydrogenation, which causes a yellow coloration (Herbach *et al.,* 2004).

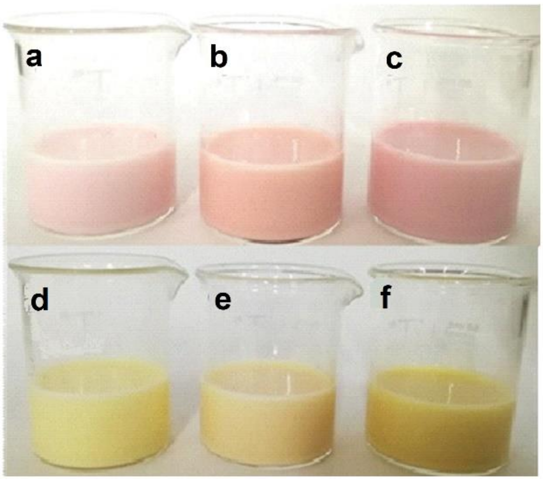

Figure 4. Appearance of pigmented milk with extracts E_{pink} (a), E_{red} (b) E_{yellow} (d), E_{orange} (e), commercial milk "strawberry flavor" (c), and commercial milk "vanilla flavor" (f).

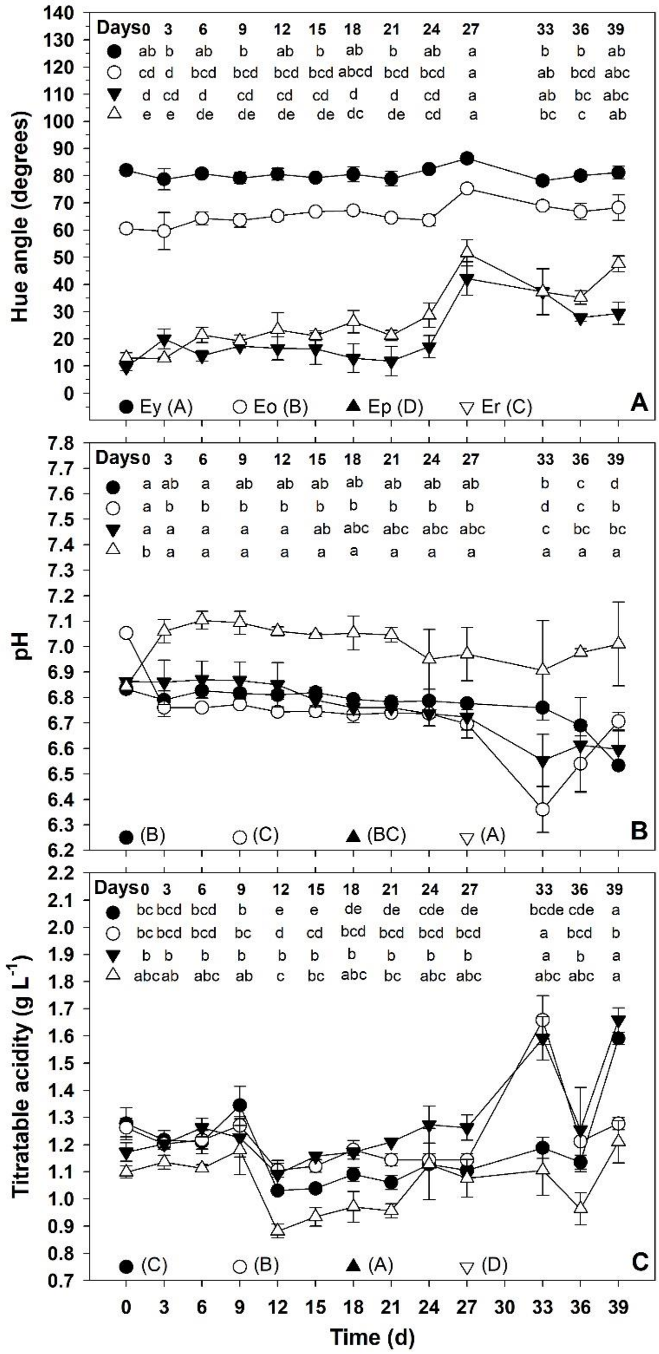

Figure 5. Variation of hue angle, pH, and acidity in batches of pigmented milk with betalain extracts and stored at 4 °C. Different capital letters in parentheses of legends indicate significant difference between treatments (Tukey, 0.05). Different lowercase letters indicate a significant difference between days of evaluation inside each treatment (Tukey, 0.05). Error bars indicate standard deviation. Ey: E_{yellow}; Eo: E_{orange}; Ep: E_{pink}; Er: E_{red}.

In all treatments, the hue angle values were without significant change until day 24 (Figure 5A), with average values of 79.9° (±1.3), 63.9° (±2.5), 14.7° (± 3.2), and 19.8° (±5.3), in systems with E_{yellow}, E_{orange}, E_{pink}, and E_{red}, respectively, whose differences relative the initial conditions were not significant. On the other hand, the period with stable color in the present work was similar to that found in yogurt samples stored for 21 d at 4 °C and pigmented with betalain extract from beetroot (Dias *et al.,* 2020). Other authors have found greater stability in yogurt, but this could be due to the use of higher concentrations of the pigmenting agent (Guneser, 2021). Likewise, betacyanins have been used to provide pink-red color in ice cream, with minimal color changes during 21 d at -18 °C (Gengatharan *et al.,* 2021).

pH and titratable acidity

The pH of the milk was 6.79 (±0.01) before pigmentation. After the addition of extracts, the average pH values were 6.8 (±0.02), 6.7 (±0.1), 6.8 (±0.05), and 7.0 (±0.8) in systems with E_{yellow}, E_{orange}, E_{pink}, and E_{red}, respectively (Figure 5B) and, although the case of E_{red} was statistically different from the rest, it was considered without practical relevance. Throughout storage, the units remained without significant change in pH until day 27, which indicated that there was no alteration in the quality attributes of the product. In addition, the commercial products typified as "vanilla flavor" and "strawberry flavor" had pH of 6.67 (±0.08), which confirmed that the extracts of the present work can reproduce the conditions of materials available in the market. On the other hand, betalains are stable at pH between 3 and 7 (Stintzing *et al.,* 2003), so they can be used to provide color in both neutral and slightly acid foods. Betaxanthins are more stable at pH 6 (Stintzing and Carle, 2007), while betacyanins are at pH 4 (Herbach *et al.,* 2006), which could have caused that the hue angle of milks colored with E_{yellow} and E_{orange} remained without significant changes during the 39 d of storage, compared to the pigmented milks with E_{pink} and E_{red}, where there were notable changes from day 27.

In relation to titratable acidity, the NOM-155-SCFI-2012 (STPS, 2012) establishes that whole milk must have values between 1.3 and 1.7 g L^{-1}. In this regard, values of 1.31 (±0.034) g L^{-1} were obtained before pigmentation, while average values of 1.27 (±0.02), 1.26 (±0.07), 1.18 (±0.04), and 1.10 (±0.04) g L^{-1}, were obtained with E_{yellow}, E_{orange}, E_{pink}, and E_{red}, respectively, after the addition of extracts (Figure 5C). Similarly to pH, acidity remained constant until day 27, but from day 33 such variable showed an irregular behavior in batches with E_{orange} and E_{pink}.

Potential uses and limitations

The food industry requires the use of additives such as colorants, because they improve the attractiveness of the products (Albuquerque *et al.,* 2021) but, due to the evidence that artificial colors can affect health (Malabadi *et al.,* 2022), it is convenient to replace them with natural pigments. Cejudo-Bastante *et al.* (2019) demonstrated the value of betalains obtained from *Stenocereus* fruits as pigmenting agents and Soto-Castro *et al.* (2019) demonstrated that, through encapsulation, this potential can be improved. In this sense, the present work has shown that it is feasible to pigment foods with betalains obtained from *Stenocereus pruinosus* fruits using aqueous biphasic systems. However, a practical implementation requires the setting of parameters to scale up the results from the laboratory level to the industrial one. In addition, it is necessary to improve the production of fruits of this species to ensure the quantities required to meet the demand of the food industry.

On the other hand, the pigmentation routines were developed with milk samples already exposed to the environment. However, the food industry works with processes where the products have low exposure to light, low exposure to natural air, and to other deterioration factors, so it is feasible to expect that the pigmentation can be maintained for longer times than those shown in the present work.

Conclusions

The aqueous two-phase extraction of betalains from *Stenocereus pruinosus* fruits with systems based on trisodium citrate and 1-propanol and trisodium citrate and polyethylene glycol 4000 allowed obtaining fractions with hue that varied between red and yellow. The extracts rich in betacyanins (E_{pink} and E_{red}) provided a similar color to that of a commercial strawberry-flavored milk, while the E_{yellow} extract provided similar color to that of a commercial vanilla-flavored milk. The stability of the hue angle was maintained for 24 d of storage at 4 °C, so the extracts obtained from fruits of *Stenocereus pruinosus* represent a viable option to give color and replace synthetic dyes in the pigmentation of fluid milk.

ETHICS STATEMENT

Not applicable.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF SUPPORTING DATA

All data generated or analyzed during this study are included in this article.

COMPETING INTERESTS

The authors declare that they have no competing interests.

FUNDING

Not applicable.

AUTHOR CONTRIBUTIONS

Conceptualization, L.G.C. and S.V.G.; methodology, L.G.C., D.G.R. and S.V.G.; formal analysis, L.G.C., D.G.R. and S.V.G.; investigation, L.G.C., D.G.R. and S.V.G.; resources, S.V.G.; data curation, L.G.C.; writing—original draft preparation, L.G.C., D.G.R. and S.V.G.; writing—review and editing, L.G.C., D.G.R. and S.V.G.; project administration, S.V.G.; funding acquisition, S.V.G.

ACKNOWLEDGMENTS

Authors wish to acknowledge the financial support received from Universidad Autónoma Chapingo, and from the scholarship program of Consejo Nacional de Humanidades, Ciencias y Tecnologías (CONAHCyT) of Mexico.

References

Akash, M.S.H. and Rehman, K. 2020. Column chromatography. In *Essentials of Pharmaceutical Analysis* (pp. 167–174). Singapore: Springer Nature Singapore. https://doi.org/10.1007/978-981-15-1547-7_13

Albuquerque, B.R., Oliveira, M.B.P.P., Barros, L. and Ferreira, I.C.F.R. 2021. Could fruits be a reliable source of food colorants? Pros and cons of these natural additives. *Critical Reviews in Food Science and Nutrition.* 61(5): 805–835. https://doi.org/10.1080/10408398.2020.1746904

Azeredo, H.M.C. 2009. Betalains: Properties, sources, applications, and stability - A review. *International Journal of Food Science and Technology.* 44*:* 2365–2376. https://doi.org/10.1111/j.1365-2621.2007.01668.x

Cejudo-Bastante, M.J., Hurtado, N., Muñoz-Burguillos, P. and Heredia, F.J. 2019. *Stenocereus griseus* (Haw) pitaya as source of natural colourant: technological stability of colour and individual betalains. *International Journal of Food Science & Technology.* 54(11): 3024–3031. https://doi.org/10.1111/ijfs.14215

Chandrasekhar, J., Sonika, G., Madhusudhan, M.C. and Raghavarao, K.S.M.S. 2015. Differential partitioning of betacyanins and betaxanthins employing aqueous two phase extraction. *Journal of Food Engineering.* 144: 156–163. https://doi.org/10.1016/j.jfoodeng.2014.07.018

Dias, S., Castanheira, E.M.S., Fortes, A.G., Pereira, D.M. and Gonçalves, M.S.T. 2020. Coloring soy-based yogurt alternative. *Foods.* 9(771): 1–13.

Fernando, G.S.N., Sergeeva, N.N., Frutos, M.J., Marshall, L.J. and Boesch, C. 2022. Novel approach for purification of major betalains using flash chromatography and comparison of radical scavenging and antioxidant activities. *Food Chemistry.* 385*:* 132632. https://doi.org/10.1016/j.foodchem.2022.132632

Gandía-Herrero, F., Cabanes, J., Escribano, J., García-Carmona, F. and Jiménez-Atiénzar, M. 2013. Encapsulation of the most potent antioxidant betalains in edible matrixes as powders of different colors. *Journal of Agricultural and Food Chemistry.* 61(18): 4294–4302. https://doi.org/10.1021/jf400337g

García-Cruz, L., Dueñas, M., Santos-Buelgas, C., Valle-Guadarrama, S. and Salinas-Moreno, Y. 2017. Betalains and phenolic compounds profiling and antioxidant capacity of pitaya (*Stenocereus* spp.) fruit from two species (S. Pruinosus and S. stellatus). *Food Chemistry.* 234: 111–118. https://doi.org/10.1016/j.foodchem.2017.04.174

García-Cruz, L., Valle-Guadarrama, S., Guerra-Ramírez, D., Martínez-Damián, M. T. and Zuleta-Prada, H. 2022. Cultivation, quality attributes, postharvest behavior, bioactive compounds, and uses of *Stenocereus*: A review. *Scientia Horticulturae.* 304*:* 111336. https://doi.org/10.1016/j.scienta.2022.111336

García-Cruz, L., Valle-Guadarrama, S., Salinas-Moreno, Y. and Luna-Morales, C. del C. 2016. Postharvest quality, soluble phenols, betalains content, and antioxidant activity of *Stenocereus pruinosus* and *Stenocereus stellatus* fruit. *Postharvest Biology and Technology.* 111*:* 69–76. https://doi.org/10.1016/j.postharvbio.2015.07.004

García-Cruz, L., Valle-Guadarrama, S., Soto-Hernández, R.M., Guerra-Ramírez, D., Zuleta-Prada, H., Martínez-Damián, M.T. and Ramírez-Valencia, Y.D. 2021. Separation of pitaya (*Stenocereus pruinosus*) betaxanthins, betacyanins, and soluble phenols through multistage aqueous two-phase systems. *Food and Bioprocess Technology.* 14(10): 1791–1804. https://doi.org/10.1007/s11947-021-02676-1

Gengatharan, A., Dykes, G.A. and Choo, W.S. 2016. Stability of betacyanin from red pitahaya (*Hylocereus polyrhizus*) and its potential application as a natural colourant in milk. *International Journal of Food Science and Technology.* 51(2): 427–434. https://doi.org/10.1111/ijfs.12999

Gengatharan, A., Dykes, G. and Choo, W.S. 2021. Betacyanins from *Hylocereus polyrhizus*: pectinase-assisted extraction and application as a natural food colourant in ice cream. *Journal of Food Science and Technology.* 58(4): 1401-1410. https://doi.org/10.1007/s13197-020-04651-8

Gomes, J., Ferreira, S., Reinert, O., Gandol, R., Ayra, L., Santos, V., Martins, V.C., da Costa, I.F.R., Santos, S.V. and Ferreira, B.R.C. 2017. Fluid Phase Equilibria Evaluation of salting-out effect in the liquid e liquid equilibrium of aqueous two-phase systems composed of 2-propanol and Na 2 SO 4 / MgSO 4 at different temperatures. *Fluid Phase Equilibria.* 450: 184–193. https://doi.org/10.1016/j.fluid.2017.08.001

Guneser, O. 2021. Kinetic modelling of betalain stability and color changes in yogurt during storage. *Polish Journal of Food and Nutrition Sciences.* 71(2): 135–145. https://doi.org/10.31883/pjfns/134393

Güneşer, O. 2016. Pigment and color stability of beetroot betalains in cow milk during thermal treatment. *Food Chemistry.* 196: 220–227. https://doi.org/10.1016/j.foodchem.2015.09.033

Herbach, K.M., Rohe, M., Stintzing, F.C. and Carle, R. 2006. Structural and chromatic stability of purple pitaya (*Hylocereus polyrhizus* [Weber] Britton & Rose) betacyanins as affected by the juice matrix and selected additives. *Food Research International.* 39: 667–677. https://doi.org/10.1016/j.foodres.2006.01.004

Herbach, K.M., Stintzing, F.C. and Carle, R. 2004. Impact of thermal treatment on color and pigment pattern of red beet (*Beta vulgaris* L.) preparations. *Journal of Food Science.* 69(6): 491–499. https://doi.org/10.1111/j.1365-2621.2004.tb10994.x

Jampani, C. and Raghavarao, K.S.M.S. 2015. Differential partitioning for purification of anthocyanins from *Brassica oleracea* L. *Separation and Purification Technology.* 151: 57–65. https://doi.org/10.1016/j.seppur.2015.07.030

Jiménez-Velázquez, P., Valle-Guadarrama, S., Alia-Tejacal, I., Salinas-Moreno, Y., García-Cruz, L., Pérez-López, A. and Guerra-Ramírez, D. 2020. Separation of bioactive compounds from epicarp of 'Hass' avocado fruit through aqueous two-phase systems. *Food and Bioproducts Processing.* 123: 238–250. https://doi.org/10.1016/j.fbp.2020.07.004

Kruszewski, B., Domian, E. and Nowacka, M. 2023. Influence of high-pressure homogenization on the physicochemical properties and betalain pigments of red beetroot (*Beta vulgaris* L.) juice. *Molecules.* 28(5): 2018. https://doi.org/10.3390/molecules28052018

Leong, H.Y., Ooi, C.W., Law, C.L., Julkifle, A.L., Katsuda, T. and Show, P.L. 2019. Integration process for betacyanins extraction from peel and flesh of *Hylocereus polyrhizus* using liquid biphasic electric flotation system and antioxidant activity evaluation. *Separation and Purification Technology.* 209: 193–201. https://doi.org/10.1016/j.seppur.2018.07.040

Malabadi, R.B., Kolkar, K.P. and Chalannavar, R.K. 2022. Plant natural pigment colorants-health benefits: toxicity of synthetic or artificial food colorants. *International Journal of Innovation Scientific Research and Review.* 4(10): 3418–3429.

Moreno, D.A., García-Viguera, C., Gil, J.I. and Gil-Izquierdo, A. 2008. Betalains in the era of global agri-food science, technology and nutritional health. *Phytochemistry Reviews.* 7: 261–280. https://doi.org/10.1007/s11101-007-9084-y

Muñoz-González, B.I., Sandoval-Castilla, O., Meléndez-Gómez, N., Hahn-Schlam, F. and Valle-Guadarrama, S. 2022. Release kinetics modeling and fungal control potential of encapsulated thyme (*Thymus vulgaris*) essential oil. *Revista Bio Ciencias.* 9(e1168): 1–20. https://doi.org/10.15741/revbio.09.e1168

Nowotny, A. 1979. Carbohydrate determination by phenol-sulfuric acid. In: *Basic Exercises in Immunochemistry* (pp. 171–173). Berlin: Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-642-67356-6_52

Okafor, S.N., Obonga, W., Ezeokonkwo, M.A., Nurudeen, J., Orovwigho, U. and Ahiabuike, A. 2016. Assessment of the health implications of synthetic and natural food colourants – A critical review. *Pharmaceutical and Biosciences Journal.* 4(4): 1–11. https://doi.org/10.20510/ukjpb/4/i4/110639

Quiroz-González, B., García-Mateos, R., Corrales-García, J.J.E. and Colinas-León, M.T. 2018. Pitaya (*Stenocereus* spp.): an under-utilized fruit. *Journal of the Professional Association for Cactus Development.* 20: 82–100.

Raja, S., Murty, V.R., Thivaharan, V., Rajasekar, V. and Ramesh, V. 2011. Aqueous two phase systems for the recovery of biomolecules – a review. *Science and Technology.* 1(1): 7–16. https://doi.org/10.5923/j.scit.20110101.02

Reyes-Ulloa, D., Camarena-Gómez, D.M. and Salgado-Beltrán, L. 2020. Exploring the attitudes of consumers towards a sustainable cactus-based paint (*Opuntia* spp). *Journal of the Professional Association for Cactus Development.* 22: 29–48.

Robards, K. and Ryan, D. 2022. Preparative chromatography. In: *Principles and practice of modern chromatographic methods* (Second edi., pp. 495–513). Elsevier Ltd. https://doi.org/10.1016/C2019-0-03803-4

Rodríguez-Félix, A., Fortiz-Hernández, J. and Tortoledo-Ortiz, O. 2019. Physico-chemical characteristics , and bioactive compounds of red fruits of sweet pitaya (*Stenocereus thurberi*). *Journal of the Professional Association for Cactus Development.* 21: 87–100.

Rodríguez-Herrera, V.V., García-Cruz, L. and Valle-Guadarrama, S. 2023. Aqueous two-phase extraction: A non-thermal technique to separate and concentrate betalains from *Bougainvillea glabra* Choisy bracts. *Industrial Crops and Products.* 193: 116245. https://doi.org/10.1016/j.indcrop.2023.116245

Rodríguez-Sánchez, J.A., Cruz y Victoria, M.T. and Barragán-Huerta, B.E. 2017. Betaxanthins and antioxidant capacity in *Stenocereus pruinosus*: Stability and use in food. *Food Research International.* 91: 63–71. https://doi.org/10.1016/j.foodres.2016.11.023

Sandate-Flores, L., Rodríguez-Rodríguez, J., Velázquez, G., Mayolo-Deloisa, K., Rito-Palomares, M., Torres, J.A. and Parra-Saldívar, R. 2020. Low-sugar content betaxanthins extracts from yellow pitaya (*Stenocereus pruinosus*). *Food and Bioproducts Processing.* 121: 178–185. https://doi.org/10.1016/j.fbp.2020.02.006

Sant'Anna, V., Gurak, P.D., Ferreira, M.L.D. and Tessaro, I.C. 2013. Tracking bioactive compounds with colour changes in foods – A review. *Dyes and Pigments*, *98*(3), 601–608. https://doi.org/10.1016/j.dyepig.2013.04.011

Soto-Castro, D., Chávez, G.M., León-Martínez Frank, M., Araceli, S.-G.P., Irais, A.-L. and Franco, A.A. 2019. Spray drying microencapsulation of betalain rich extracts from *Escontria chiotilla* and *Stenocereus queretaroensis* fruits using cactus mucilage. *Food Chemistry.* 272: 715–722. https://doi.org/10.1016/j.foodchem.2018.08.069

Stintzing, F.C. and Carle, R. 2007. Betalains - emerging prospects for food scientists. *Trends in Food Science and Technology.* 18(10): 514–525. https://doi.org/10.1016/j.tifs.2007.04.012

Stintzing, F.C., Herbach, K.M., Mosshammer, M.R., Carle, R., Yi, W., Sellappan, S., Akoh, C.C., Bunch, R. and Felker, P. 2005. Color, betalain pattern, and antioxidant properties of cactus pear (*Opuntia* spp.) clones. *Journal of Agricultural and Food Chemistry. 53*(2): 442–451. https://doi.org/10.1021/jf048751y

Stintzing, F.C., Schieber, A. and Carle, R. 2003. Evaluation of colour properties and chemical quality parameters of cactus juices. *European Food Research and Technology.* 216: 303–311. https://doi.org/10.1007/s00217-002-0657-0

STPS. 2012. *Norma oficial mexicana NOM-155-SCFI-2012, leche-denominaciones, especificaciones fisicoquímicas, información comercial y métodos de prueba*. México: Diario Oficial. Secretaría del Trabajo y Previsión Social.

Thi Tran, T.M., Nguyen Thanh, B., Moussa-Ayoub, T.E., Rohn, S. and Jerz, G. 2019. Profiling of polar metabolites in fruits of *Opuntia stricta* var. Dillenii by ion-pair high-performance countercurrent chromatography and off-line electrospray mass-spectrometry injection. *Journal of Chromatography A.* 1601: 274–287. https://doi.org/10.1016/j.chroma.2019.06.009

Valle-Guadarrama, S., Saucedo-Veloz, C., Peña-Valdivia, C.B., Corrales-García, J.J.E., Chávez-Franco, S.H. and Espinosa-Solares, T. 2002. Skin Permeance and Internal Gas Composition in `Hass' Avocado (*Persea americana* Mill.) Fruits. *Food Science and Technology International.* 8(6): 365–373. https://doi.org/10.1106/108201302031477

Valle-Guadarrama, S., Cruz-Pérez, D., López-Cruz, I.L. and Hahn-Schlam, F. 2018. Thermal convection coefficient in cooling process of lime fruit: Study through the Galerkin finite element method. *Journal of Food Process Engineering.* 41(2): e12653. https://doi.org/10.1111/jfpe.12653

Vargas-Campos, L., Valle-Guadarrama, S., Martínez-Bustos, F., Salinas-Moreno, Y., Lobato-Calleros, C. and Calvo-López, A.D. 2018. Encapsulation and pigmenting potential of betalains of pitaya (*Stenocereus pruinosus*) fruit. *Journal of Food Science and Technology.* 55(7): 2436–2445. https://doi.org/10.1007/s13197-018-3161-7

Wang, H., Xu, D., Wang, S., Wang, A., Lei, L., Jiang, F., Yang, B., Yuan, L., Chen, R., Zhang, Y. and Fan, W. 2023. Chromosome-scale Amaranthus tricolor genome provides insights into the evolution of the genus *Amaranthus* and the mechanism of betalain biosynthesis. *DNA Research.* 30(1). https://doi.org/10.1093/dnares/dsac050

Zheng, Y., Zhang, P. and Fu, L. 2023. Advances on polysaccharides from cactus: analysis and review based on bibliometrics. *Journal of the Professional Association for Cactus Development.* 25: 1–22. https://doi.org/10.5689