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Cosmos bipinnatus Cav., an alternative to synthetic dyes? Study of its stability against degrading factors

Cosmos bipinnatus Cav., ¿una alternativa a los colorantes sintéticos? Estudio de su estabilidad frente a factores degradativos

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ABSTRACT

The pigments of *Cosmos bipinnatus* were characterized for their potential application as natural dyes. In the extraction process, three solvents were evaluated, with the combination of water and ethanol yielding the best results. The stability of the pigments was then assessed under various conditions of pH, temperature, and light radiation over a 41 day storage period. Analysis of the degradation kinetics of anthocyanins revealed a first-order reaction with a half-life ranging from 0.7 to 63 days. At the end of storage, polymeric color formation increased significantly due to the pH effect (1.6 - 96.2 %), while anthocyanin concentration decreased due to temperature effects (1.1 - 0 mg cyn-3-glu g^{-1}). Antioxidant activity was also affected by storage conditions, decreasing from 251 mg ET g^{-1} to 69 mg ET g^{-1}. In terms of color preservation, pH 4 samples retained their color longer, unlike those at pH 10 and 7. Based on these results, it is recommended to use the extracted pigments at pH 4 to maximize their stability and potential applications.

KEY WORDS: Cosmos bipinnatus, bioactive pigments, stability, degradative factors.

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RESUMEN

Los pigmentos de *Cosmos bipinnatus* se caracterizaron por su potencial aplicación como colorantes naturales. En el proceso de extracción, se evaluaron tres solventes, siendo la combinación de agua y etanol la que presentó los mejores resultados. Posteriormente, se evaluó la estabilidad de los pigmentos bajo diversas condiciones de pH, temperatura y radiación lumínica durante un período de almacenamiento de 41 días. El análisis de la cinética de degradación de las antocianinas reveló una reacción de primer orden con una vida media de entre 0.7 y 63 días. Al final del almacenamiento, la formación de color polimérico aumentó significativamente debido al efecto del pH (1.6 – 96.2 %), mientras que la concentración de antocianinas disminuyó debido a los efectos de la temperatura (1.1 - 0 mg cyn-3-glu g^{-1}). La actividad antioxidante también se vio afectada por las condiciones de almacenamiento, disminuyendo de 251 mg ET g^{-1} a 69 mg ET g^{-1}. En términos de conservación del color, las muestras de pH 4 conservaron su color durante más tiempo, a diferencia de las de pH 10 y 7. Con base en estos resultados, se recomienda utilizar los pigmentos extraídos a pH 4 para maximizar su estabilidad y posibles aplicaciones.

PALABRAS CLAVE: *Cosmos bipinnatus*, pigmentos bioactivos, estabilidad, factores degradativos.

Introduction

The use of synthetic dyes in food has been widely questioned in developed countries due to concerns over their potential health impacts. Research has linked the indiscriminate consumption of these pigments to various adverse health effects, including attention problems, hyperactivity, irritability, sleep disorders, and aggressiveness in infants (Ngamwonglumlert *et al*., 2017; Amchova *et al*., 2015; de Oliveira *et al*., 2024). Furthermore, studies have associated synthetic dye consumption with the development of degenerative diseases, including cancer, in adults (Salinas-Moreno *et al*., 2005; Orozco-Villafuerte *et al*., 2018; Mota *et al*., 2023). As a result, the food industry has shifted towards using plant-based pigments as a natural alternative to artificial colorants, promoting healthier food options (Montibeller *et al*., 2018). Meanwhile, growing health awareness among consumers has driven demand for natural colorants, making them an increasingly popular choice (Ngamwonglumlert *et al*., 2017). In this context, anthocyanin pigments, which are primarily responsible for the red, pink, blue, and purple colors of fruits, vegetables, and flowers (Hurtado & Perez, 2014), offer significant potential. These phenolic secondary metabolites of plants belong to the flavonoid family and can be leveraged as natural food colorants. Their incorporation into foods not only imparts color but also provides antioxidant benefits due to their

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structural characteristics (Saint-Cricq *et al*., 1999; Noda *et al*., 2002). As a result, anthocyanins are recognized as bioactive pigments (Salinas-Moreno *et al*., 2005) with documented health benefits (Tsada *et al*., 1999; Morimitsu *et al*., 2002; Lazze *et al*., 2003). In the European Union, anthocyanins are classified as food additives with the code E163 (Martins *et al*., 2016). At the industrial level, companies like Givaudan Sense Colour® offer natural pigments derived from anthocyanins sourced from plants, such as berries, grapes, and other fruits.

Despite the growing demand for anthocyanin pigments, current sources are limited and insufficient to meet industrial needs. Therefore, exploring alternative plant sources for obtaining these pigments is essential. It is crucial to note that the intensity and stability of anthocyanin pigments depend on various factors, including pigment structure and concentration, pH, temperature, light intensity, and the presence of copigments, metal ions, enzymes, oxygen, ascorbic acid, carbohydrates, and sulfur dioxide (Laleh *et al*., 2006; Ngamwonglumlert *et al*., 2017). Kinetic studies are useful for determining parameters during storage, predicting changes in product composition and color, and determining their characteristics at the end of shelf life (Krapfenbauer *et al*., 2006). Furthermore, the plant source of the pigments plays a significant role in their stability. Consequently, identifying suitable plant sources and optimizing extraction and storage conditions are critical to ensuring the stability and quality of anthocyanin pigments.

On the other hand, *Cosmos bipinnatus*, a member of the Asteraceae family, is an annual plant commonly known as garden cosmos due to its vibrant pink, red, violet, or white flowers. In traditional medicine, the flowers and leaves of *C. bipinnatus* have been extensively used to treat various ailments, including headaches, intermittent fever, jaundice, pain, and stomach disorders. Research has demonstrated that *C. bipinnatus* possesses anti-inflammatory properties, as evidenced by tests on murine macrophages, antioxidant activity in *in vitro* models, and antigenotoxic effects, as assessed by the comet assay (Bijani *et al*., 2021). Notably, the flowers of *C. bipinnatus* contain a significant amount of anthocyanins, a natural source that has been understudied for its potential to yield biologically active pigments. Recent studies have revealed that *C. bipinnatus* extracts are rich in anthocyanin pigments (Fernandes *et al*., 2019). Therefore, the present study aimed to extract bioactive pigments from *C. bipinnatus* flowers and characterize their physicochemical properties.

Material and Methods

Ethanol (98 %) was sourced from J.T. Baker S.A. de C.V. (Mexico City, Mexico). The following reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA): 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)-diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), Folin-Ciocalteau's reagent, gallic acid (98 %), quercetin, anhydrous sodium carbonate (99 %), aluminum trichloride, potassium chloride, sodium acetate, ascorbic acid, and potassium metabisulfite.

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Specimen collection

Specimens of *Cosmos bipinnatus* Cav., including flowers, stems, and leaves, as well as fresh flowers, were collected in September and October in Toluca, Mexico. The specimens were identified by the Medicinal Herbarium of the Mexican Institute of Social Security at the National Medical Center, and assigned the registry number 17004. For further analysis, intact, clean, and visually undamaged petals were selected from the flowers. These petals were then dried using freeze-drying (FreeZone 2.5, Labconco, MO, USA) and subsequently ground into a fine powder using a food processor.

Pigment extraction

Pigment extraction was performed using a modified version of the methodology reported by Medina-Torres *et al*. (2017). Briefly, 2 g of dried and powdered flowers were suspended in 120 mL of each solvent: water (aqueous), water:ethanol (80:20, v/v) (hydroethanolic), and water:methanol (80:20, v/v) (hydromethanolic), for 1 hour. The extraction was then assisted by ultrasonication (Ultrasonic cleaner, SK2210HP, Shanghai, China) at a constant frequency of 53 kHz for 40 minutes at 40 °C, followed by shaking at 40 °C for 60 minutes. After the extraction period, the samples were vacuum-filtered and concentrated under reduced pressure (RV10, IKA, USA). Finally, the extracts were lyophilized and stored at 4 °C until further analysis.

Quantification of total phenols

The total phenol content (TPC) in the extracts was determined using the Folin-Ciocalteu method described by Skerget *et al*. (2005) with minor modifications. Briefly, 200 µL of the sample or standard was mixed with 100 µL of Folin's reagent, and the mixture was homogenized and incubated in the dark for 8 minutes. Subsequently, 200 µL of sodium carbonate solution (20 g/100 mL) and 1.5 mL of distilled water were added, and the resulting sample was homogenized and incubated in the dark for 60 minutes. The absorbance was measured at 765 nm using a spectrophotometer (Genesys 10S UV-VIS, Thermo Scientific, China). A standard curve was generated using gallic acid, and the results were expressed as milligrams of gallic acid equivalents per gram of dry extract.

Quantification of flavonoids.

The flavonoid content in the extracts was analyzed using a modified version of the spectrophotometric method described by Pękal & Pyrzynska (2014). A 1000 µL aliquot of the sample was mixed with 500 µL of 2 % aluminum chloride ($AlCl_3$) and 500 µL of distilled water. The samples were shaken, incubated in the dark for 10 minutes, and then read at an absorbance of 425 nm using a spectrophotometer (Genesys 10S UV-VIS). A standard curve was generated using quercetin, and the results were expressed as milligrams of quercetin equivalents per gram of dry extract.

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Quantification of monomeric anthocyanins

The differential pH method described by Giusti & Wrolstad (2001) was employed to determine the monomeric anthocyanin content. Two aliquots of the extract were prepared, one of which was diluted with a pH 1.0 buffer (0.025 M KCl) and the other with a pH 4.5 buffer (0.4 M CH_3CO_2Na). Both mixtures were then scanned from 400 to 700 nm using a spectrophotometer (Genesys 10S UV-VIS). The pigment content was calculated as cyanidin 3-glucoside, using an extinction coefficient of 26,900 L cm^{-1} mol^{-1} and a molecular weight of 449.2 g mol^{-1}.

Determination of antioxidant activity

Antioxidant activity was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical method described by Brand-Williams *et al*. (1995). A 100 µL sample was mixed with 3.9 mL of a 0.1 mM DPPH solution in methanol. The reaction mixture was incubated in the dark for 30 minutes. The absorbance was then measured at 517 nm using a spectrophotometer (Genesys 10S UV-VIS). A standard curve was generated using a 2 mM Trolox solution. The results were expressed as milligrams of Trolox equivalents per gram of dry extract.

Antioxidant activity was also evaluated using the ABTS radical (2,2'-azino-bis(3-ethylbenzothiazolin-6-sulfonic acid)) method described by Pellegrini *et al*. (1999) with minor modifications. Initially, 5 mL of 7 mM ABTS and 88 µL of 140 mM potassium persulfate were mixed and allowed to stand for 12 hours in the dark. The resulting solution was then diluted with methanol to achieve an absorbance of 0.74 at 734 nm. For the reaction, 1 mL of the sample was mixed with 3 mL of the ABTS solution. After a 10-minute incubation in the dark, the absorbance of the samples was measured at 734 nm using a spectrophotometer (Genesys 10S UV-VIS). A standard curve was generated using a 2 mM Trolox solution. The results were expressed as milligrams of Trolox equivalents per gram of dry extract.

Determination of the stability of the bioactive pigment

Aqueous solutions (2 mg/mL) of the hydroethanolic extract were prepared. The pH of each solution was adjusted to 4, 7, or 10 using saturated solutions of citric acid ($C_6H_8O_7$) and sodium carbonate ($NaHCO_3$). The hydroethanolic solutions were then exposed to three different temperature conditions (4, 25, and 65 °C) and two light conditions (light or dark), resulting in a total of 18 treatments. The stability of the resulting extracts was evaluated by monitoring anthocyanin degradation kinetics (Section 2.8.1), monomeric anthocyanin content (Section 2.6), antioxidant activity by DPPH (Section 2.7), polymeric color, and color (Sections 2.8.2 and 2.8.3) at a frequency of twice a week over a period of 41 days.

Degradation kinetics of anthocyanins

The degradation kinetics of anthocyanins can be expressed through kinetic parameters such as the first-order reaction rate constant (k) and the half-life ($t_{1/2}$), i.e., the time required for the

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degradation of 50 % of anthocyanins, which were calculated by the following equations (Yang *et al*., 2008; Hou *et al*., 2013; Martynenko & Chen, 2016):

$$ln\frac{[C_t]}{[C_o]} = -kt \quad \text{(Eq. 1)}$$

$$t_{1/2} = -\frac{\ln 0.5}{k} \quad \text{(Eq. 2)}$$

Where Co is the initial monomeric anthocyanin content and Ct is the monomeric anthocyanin content after t days of storage. $t_{1/2}$ is the half-life time, k is the rate constant of the first order kinetics ($days^{-1}$).

Color density and polymeric color percentage

The color density (CD) was determined according to the method described by Giusti and Wrolstad (2001). A 20 % potassium metabisulfite solution was prepared prior to analysis. Each sample (2.8 mL) was then treated with either 0.2 mL of the $K_2S_2O_5$ solution for bleaching or 0.2 mL of distilled water as a control. After a 15-minute incubation in the dark, the samples were scanned from 420 to 700 nm against a distilled water blank using a spectrophotometer (Genesys 10S UV-VIS). The color density (CD) was calculated using the control sample and Equation 3, where A represents the absorbance of the blank or sample, and DF is the dilution factor of the extract.

$$CD = \left[(A_{420} - A_{700}) + (A_{\lambda\,\mathrm{vis-max}} - A_{700)}\right] \times DF \quad \text{(Eq. 3)}$$

The polymeric color (PC) was determined using the bleached sample and according to equation (4), where A corresponds to the absorbance of the blank or bleached sample, and DF is the dilution factor of the extract.

$$PC = \left[(A_{420} - A_{700}) + (A_{\lambda\,\mathrm{vis-max}} - A_{700)}\right] \times DF \quad \text{(Eq. 4)}$$

The percentage of polymeric color (% PC) was calculated according to the following relationship:

$$\%PC = \left(\frac{PC}{CD}\right) \times 100 \quad \text{(Eq. 5)}$$

Color determination

The color analysis was performed using a colorimeter (Chroma Meter CR-400, Konica Minolta Sensing Inc., Japan). The CIE L*, a*, b* color space parameters were measured, where

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L* represents lightness (ranging from 0 for black to 100 for white), a* indicates red-green color opposition (with positive values indicating red and negative values indicating green), and b* represents yellow-blue color opposition (with positive values indicating yellow and negative values indicating blue). Color measurements were taken on 20 mL of extract, placed in 4 cm diameter white plastic capsules. The total color difference between the initial pigment and its color at various time points was calculated using the following equation:

$$\Delta E = [(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2]^{1/2} \quad \text{(Eq. 6)}$$

The color difference (ΔE) was calculated using the following equation, where ΔL, Δa, and Δb represent the differences in L*, a*, and b* values between day 1 and subsequent exposure times up to day 41. The color difference (ΔE) is a quantitative measure determined by a mathematical relationship involving the three CIE parameters L*, a*, and b*, allowing for objective assessment of color variations between samples over time. According to Wang *et al*. (2014), the color differences were classified as follows: $\Delta E < 0.2$ (non-perceptible difference), $0.2 \leq \Delta E < 0.5$ (very small difference), $0.5 \leq \Delta E < 2$ (small difference), $2 \leq \Delta E < 3$ (highly perceptible difference), $3 \leq \Delta E < 6$ (perceptible difference), $6 \leq \Delta E < 12$ (strong difference), and $\Delta E \geq 12$ (different colors).

Statistical analysis

All experiments were conducted in triplicate, and the results are presented as the mean ± standard deviation (SD). The data were analyzed using analysis of variance (ANOVA), and significant differences between means were determined by Tukey's test ($p \leq 0.05$). Statistical analysis was performed using Statgraphics Centurion 16 software.

Results and Discussion

Partial characterization of the extracts of *C. bipinnatus* flower petals

Among the three solvents evaluated, the aqueous extract exhibited the highest content of phenolic compounds (112.7 mg GAE g^{-1}), followed by the hydroethanolic and hydromethanolic extracts (72.2 ± 2.2 and 77 ± 1.1 mg GAE g^{-1}, respectively), with no significant differences between the latter two (Table 1). A study by Gutierrez *et al*. (2008) reported a phenolic content of 65 mg GAE g^{-1} in hydromethanolic extracts of 14 flowers, including *C. bipinnatus*, which ranked third. In contrast, Chensom *et al*. (2019) reported a lower phenolic content (13 mg GAE g^{-1}) in hydroethanolic extracts of Cosmos yellow. The higher phenolic content determined in this study can be attributed to the combination of the solvent and the ultrasound-assisted extraction method employed, as well as the higher sample:solvent ratio, which enhanced extraction efficiency. The determination of total phenols in *C. bipinnatus* petals is relevant for its potential use as a natural pigment in food formulations, given its associated health benefits (Butts-Wilmsmeyer *et al*., 2018; Pires *et al*., 2019). For instance, the high phenolic content in *Cosmos caudatus*, *Pluchea indica*, and *Lawsonia inermis* has been linked to antioxidant activity and pancreatic lipase inhibitory activity, both of which contribute to combating obesity (Abdul *et al*., 2017).

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Table 1. Effect of the solvent on the partial phytochemical characterization of the bioactive pigments extracted from *Cosmos bipinnatus* flowers.

Phytochemical	Aqueous extract	Hydroethanolic extract	Hydromethanolic extract
Total phenols (mg AGE g^{-1})	112.7 ± 0.5^{b}	72.2 ± 2.2^{a}	77 ± 1.1^{a}
Flavonoids (mg QE g^{-1})	13.1 ± 0.7^{a}	24.7 ± 5^{b}	23.7 ± 3.9^{b}
Anthocyanins (mg Cyn-3-glu g^{-1})	2.3 ± 0.4^{a}	3.6 ± 0.3^{b}	4.9 ± 0.5^{c}
Antioxidant activity-DPPH (mg TE g^{-1})	727.1 ± 19^{b}	321 ± 47^{a}	305.7 ± 13^{a}
Antioxidant activity-ABTS (mg TE g^{-1})	695.4 ± 58^{b}	317.9 ± 19^{a}	379.5 ± 58^{a}

Data represents the mean ± standard deviation. Values in the same row with different letters show significant differences ($p \leq 0.05$).

On the other hand, the flavonoid content in the extracts exhibited a high degree of variation (13.1-24.7 mg EQ g^{-1}), with significantly higher values observed in the hydroalcoholic extracts (Table 1). The extraction efficiency is related to the solubility of flavonoids in the solvent, as well as the intensity of interactions with the plant matrix (Chaves *et al*., 2020). Sulaiman *et al*. (2011) reported that *C. caudatus* exhibits a higher flavonoid content in solvents such as acetone and 70 % methanol compared to water. Similarly, other studies have investigated the solvent effect on flavonoid extraction in edible flowers, observing lower extraction yields with water (Dian-Nashiela *et al*., 2015; Petrova *et al*., 2016; Fernandes *et al*., 2019). Flavonoids are easily oxidizable substances that exhibit antioxidant effects, including the inhibition of lipoperoxidation, antimutagenic effects, and enzyme inhibition, primarily due to their ability to reduce free radicals and chelate metals (Lopez, 2002). Consequently, conserving flavonoids is desirable, as they have been shown to possess antioxidant, antibacterial, antiviral, anti-inflammatory, diuretic, antispasmodic, and gastric antiulcer bioactivities (López, 2002; Chaves *et al*., 2020). Studies by Amamiya and Iwashina (2015) and Bijani *et al*. (2021) identified the presence of three flavonoids, apigenin 7-O-glucuronide, chrysoeriol 7-O-glucuronide, and luteolin 7-O-glucuronide, in the petals of *C. bipinnatus*.

Significant differences in anthocyanin content were observed among the extracts. The hydromethanolic extract exhibited the highest content (4.9 mg Cyn-3-glu g^{-1}), followed by the hydroethanolic and aqueous extracts (Table 1). According to Santos-Buelga *et al*. (2012), polar solvents such as methanol, acetone, and their aqueous mixtures are commonly used to extract polyphenols, including anthocyanins. However, there is a growing trend towards using more environmentally friendly solvents, such as water, ethanol, or a combination of both, to produce “green” products that meet the requirements of the food and pharmaceutical industries (Pires *et al*., 2019). Furthermore, anthocyanins have been reported to possess vitamin-like properties, beneficial effects on the capillary and venous vascular system, and antioxidant, anti-inflammatory, and antiplatelet activities, with very low toxicity (López, 2002)

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The antioxidant activity of *C. bipinnatus* flower petals extracts is presented in Table 1. The values obtained using both radicals varied depending on the solvent type. The aqueous extract exhibited the highest antioxidant activity in both tests (727.1 and 695.4 mg ET g^{-1} for DPPH and ABTS, respectively), which was nearly twice that of the hydroalcoholic extracts. The ability to inhibit DPPH and ABTS free radicals is influenced by the content of phenolic compounds and flavonoids, as well as the degree of polymerization, concentration, and interaction of their various chemical structures with colorimetric assays (Sulaiman *et al*., 2011). Notably, *C. caudatus* has been found to contain flavonol glucoside, kaempferol, and flavone C-glucoside, which are considered anti-aging agents for the skin due to their ability to inhibit tyrosinase, collagenase, and elastase (Phong *et al*., 2022).

Stability of anthocyanins from flower petals of *C. bipinnatus*

To evaluate the stability of the pigment against degradative factors, the hydroethanolic extract was selected for its high anthocyanin content and its wide pigmentation capacity; the hydromethanolic was not considered because of its known toxic effects for humans; however, it was considered for the characterization to have it as a reference because of its known affinity for anthocyanins (Santos-Buelga *et al*. 2012). Anthocyanins are the compounds responsible for the color in *C. bipinnatus* flower petals, and in turn, color hue depends on pH, temperature, light, the presence of metal ions, copigments, enzymes, oxygen, and sugars (Ngamwonglumlert *et al*., 2017). To select extracts with suitable colors for further application in food and/or pharmaceutical matrices, a pH sweep (1-14) was performed. As shown in Figure 1, at acidic pH values the characteristic pink color of the flower petals is preserved, at neutral pH the lilac color is generated, and at alkaline pH, the pigment can pass through brown, green, and yellow colors. According to these color shades of the hydroalcoholic extract derived from the pH change, the extracts at pH 4, 7, and 10 were selected.

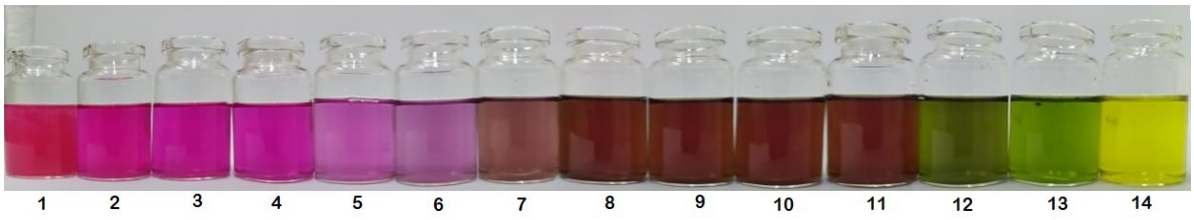

Figure 1. The color range is exhibited by the bioactive pigments of *Cosmos bipinnatus* at different pH

Source: Own elaboration

Delgado-Vargas *et al*. (2003) and Ngamwonglumlert *et al*. (2017) reported color changes in anthocyanin solutions, which are attributed to the equilibrium between different chemical

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species. In aqueous solutions, anthocyanins exist in a dynamic equilibrium between the flavilium cation, carbinol pseudobase, quinoidal base, and chalcone forms. At pH 1, the flavilium cation, which exhibits a red color, is the dominant species. However, as the pH increases to 4 or 5, the concentration of the colorless carbinol pseudobase increases at the expense of the flavilium cation. At pH 6, the hydroxyl groups in the anthocyanin structure become more pronounced, and the quinoidal base concentration increases, resulting in an unstable blue or violet color. As the pH continues to rise to 8, the quinoidal base is transformed into chalcone, which exhibits a yellow color.

As illustrated in Figure 2a, the monomeric anthocyanin content decreased in all treatments after 41 days of storage. The most extensive anthocyanin degradation (74-100 %) occurred in extracts exposed to 65 °C at pH 10, whereas the lowest loss (64 % retention) was observed in extracts stored at 4 °C at pH 4 (Table 2), regardless of light conditions. This behavior can be attributed to the cumulative effects of time, temperature, pH, and light exposure, which collectively promote anthocyanin degradation. Statistical analysis of the main effects revealed that time was the primary contributor to anthocyanin degradation, followed by temperature, pH, and finally light conditions. The stability of anthocyanins in foods is known to be influenced by various physical and chemical factors, including enzymatic activity, pH, sugar content, and the presence of copigments, metals, ions, hydrogen peroxide, and other compounds (Martynenko & Chen, 2016). Temperature has been identified as a crucial factor influencing the degradation of anthocyanins. Ngamwonglumlert *et al*. (2017) reported that temperature induces logarithmic destruction of the pigment over time, with greater degradation occurring at 100 °C. This thermal degradation involves the formation of a colorless carbinol pseudobase and the subsequent opening of the pyrilium ring to form chalcone, ultimately leading to the formation of a brown degradation product. Similarly, Sinela *et al*. (2017) observed a decrease in anthocyanin concentration in *Hibiscus sabdariffa* extracts after 60 days, indicating degradation of the original pigment. Specifically, delphinidin 3-O-sambubioside and cyanidin 3-O-sambubioside showed degradation rates of 11 % and 17 % at 4 °C, and 99 % and 98 % at 37 °C, respectively. The degradation of anthocyanins due to temperature and pH has also been reported in extracts of various flowers, including rose and Chrysanthemum (Xu *et al*., 2021). Patras *et al*. (2010) emphasized the critical role of storage temperature in anthocyanin loss, observing slower degradation at 20 °C compared to 37 °C. Furthermore, Brownmiller *et al*. (2008) found that more than 50 % of anthocyanins in blueberry puree (cv. Bluecrop) were lost after 6 months of storage, accompanied by increased polymeric color values, indicating extensive polymerization of anthocyanins during storage.

The loss of anthocyanins is also influenced by the pH effect. According to Tan *et al*. (2014) and Hou *et al*. (2013), at pH values below 4, anthocyanins are primarily present in the form of the flavylium cation, which enhances their stability. In contrast, an increase in pH can lead to a reduction in pigment stability. Cevallos-Casals & Cisneros-Zevallos (2004) reported that at pH 0.9-4.0, red sweet potato and purple carrot pigments exhibited high stability during storage at 20 °C for 134 days. However, at pH values above 4, the color shifted from purple-blue to brown-yellow. In a separate study, Gamage & Choo (2023) observed that dyes prepared from black goji (*Lycium ruthenicum*) and purple sweet potato powder at pH 3, 4, 5, and 6 retained up to 70 % of anthocyanins at pH 3, indicating their suitability as a coloring agent in acidic foods stored

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in cold rooms. Hu *et al*. (2014) determined the optimal conditions for extracting anthocyanins from methanolic extracts of black goji berries (*Lycium ruthenicum Murray*) and investigated their stability at various pH levels (1, 3, 5, 7, 9, 11, 13). The results showed that at lower pH values (1 and 3), up to 90 % of anthocyanins were retained after 12 days of observation. In contrast, in alkaline solutions (pH 11 and 13), the retention was approximately 20 %. These findings indicate that the stability of anthocyanins also depends on the plant source.

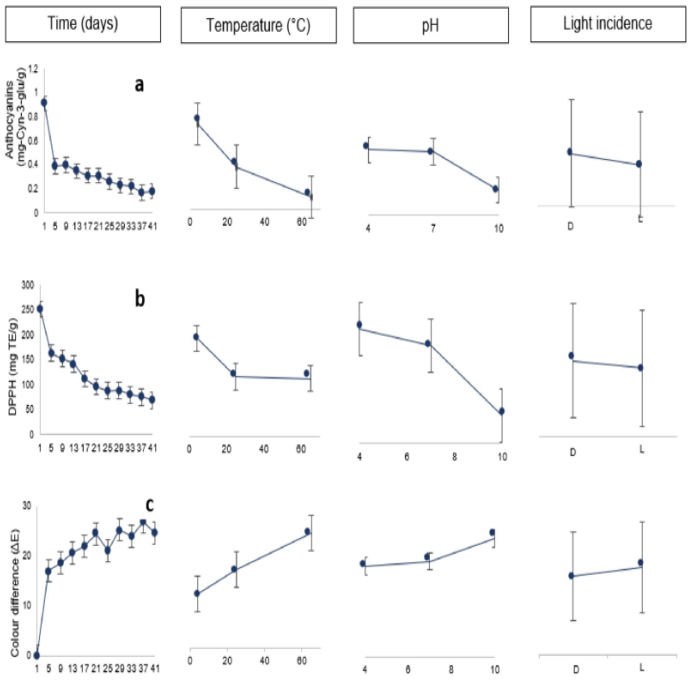

Figure 2. Effect of time, temperature, pH, and light incidence on bioactive pigments of *Cosmos bipinnatus* flower petals

during 41 days of storage on (a) Monomeric anthocyanins (mg cyn-3-glu g-1), (b) Antioxidant activity by DPPH (mg TE g-1), and (c) Colour difference (ΔE).

Source: Own elaboration

Anthocyanin degradation kinetics

The degradation kinetics of anthocyanins in *C. bipinnatus* extracts were plotted as a function of the logarithm of concentration versus time, and linear regression analysis was used to determine the degradation rate constant (k) and half-life time (t1/2) (Figure 3). The anthocyanin degradation over time was fitted to a first-order equation, with variations in the linear regression

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coefficient (0.7739 < R2 < 0.9871) (Table 2). Among the evaluated factors, temperature had the most significant impact on anthocyanin degradation (Figure 3A), exhibiting a direct proportional relationship between temperature increase and anthocyanin degradation. Notably, the temperature of 65 °C caused the most extensive degradation from day 1. In contrast, when samples were exposed to different pH values (Figure 3B), an increase in alkalinity (pH 10) promoted anthocyanin degradation within a few days of storage.

The kinetic parameters of anthocyanin degradation are presented in Table 2. The results indicate that increasing temperature leads to an increase in the rate constant (k) and a decrease in half-life values ($t_{1/2}$), similar trends were observed with increasing pH and exposure to light. In general, the values of k and $t_{1/2}$ demonstrate that anthocyanin stability decreases with increasing temperature and pH, primarily. This behavior was observed most pronouncedly in samples at 65 °C and pH 10. In contrast, the highest $t_{1/2}$ value (63 days) was determined at 4 °C, pH 4, and in darkness, indicating optimal anthocyanin protection (64.4 %) under these conditions. Analysis of treatments at different temperatures revealed that $t_{1/2}$ values at 4 °C ranged from 10.4 to 63 days, at 25 °C from 6.9 to 35.3 days, and at 65 °C from 0.7 to 7.8 days, indicating that anthocyanins are less stable at elevated temperaturas.

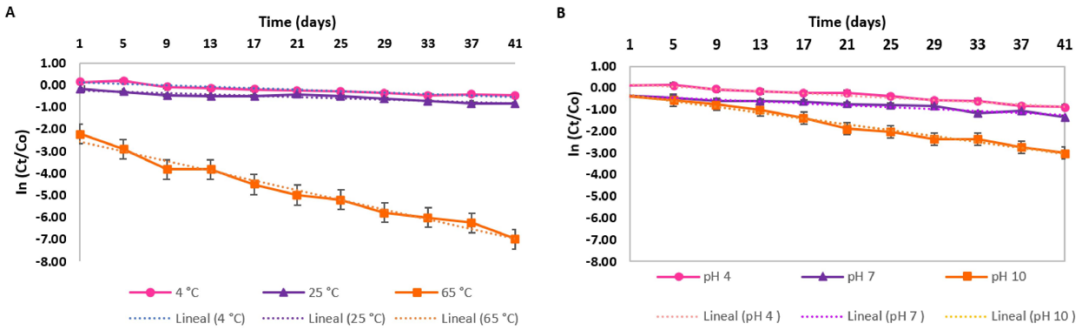

Figure 3. Anthocyanin degradation kinetics of *Cosmos bipinnatus* flower petals extracts.

A) Effect of temperature (4, 25 and 65 °C). **B)** Effect of pH (4, 7 and 10). The dashed lines represent the behavior predicted by the first-order kinetic model.

Source: Own elaboration

Exposure to heat treatment can trigger a multitude of mechanisms, including glycosylation, nucleophilic attack by water, cleavage, and polymerization, which can lead to the degradation of anthocyanins. As a result, both the intensity of color, determined by monomeric anthocyanins, and their quantity decrease with time and temperature, while the amount of brown pigments resulting from browning or polymerization increases (Jiang *et al*., 2019; Enaru *et al*., 2021). This thermal degradation phenomenon has been observed in blueberry purees subjected to

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hydrothermodynamic (HTD) processing at temperatures ranging from 70 to 105 °C. The results show a significant decrease in half-life ($t_{1/2}$) from 346.6 to 38.7 minutes, indicating that anthocyanins are more susceptible to thermal degradation at temperatures above 95 °C. This increased sensitivity may be attributed to several factors, including the formation of melanoidin pigments, as well as condensation, copigmentation, and degradation reactions that result in the formation of chalcone, which further degrades to brown products during thermal treatment (Martynenko & Chen, 2016).

Regarding the influence of pH on anthocyanin stability, the half-life ($t_{1/2}$) values ranged from 2.2 to 63 days at pH 4, 1.3 to 35.3 days at pH 7, and 0.7 to 21.8 days at pH 10. These results suggest that increasing the pH value accelerates anthocyanin degradation, leading to a decrease in color intensity or a change in color, which affects the pigment's application and shelf life. To counteract this phenomenon and enhance anthocyanin stability, intermolecular copigmentation mechanisms, such as associations between anthocyanins and phenols, flavonoids, polysaccharides, amino acids, alkaloids, or organic acids, can prevent the hydration of the flavylium cation and subsequent chalcone formation (Enaru *et al*., 2021).

Table 2. Parameters of anthocyanin degradation kinetics of *Cosmos bipinnatus* flower petals by the effect of temperature, pH, and light incidence.

Temperature	pH	Light incidence	*K* (days $^{-1}$)	$t_{1/2}$ (days)	r^2	Anthocyanin loss (%)
4 °C	4	Dark	0.01 ± 0.001^{f}	63.0 ± 5.4^{a}	0.9069	35.6
		Light	0.02 ± 0.003^{f}	32.4 ± 5.2^{b}	0.9676	57.8
	7	Dark	0.02 ± 0.007^{f}	35.3 ± 2.1^{b}	0.8543	51.8
		Light	0.03 ± 0.011^{f}	32.6 ± 13^{b}	0.9227	74.5
	10	Dark	0.03 ± 0.004^{f}	21.8 ± 2.9^{b,c}	0.9025	74
		Light	0.07 ± 0.019^{f}	10.4 ± 2.7^{c,d}	0.9871	94.5

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Table 2. Parameters of anthocyanin degradation kinetics of *Cosmos bipinnatus* flower petals by the effect of temperature, pH, and light incidence.

Temperature	pH	Light incidence	*K* (days $^{-1}$)	$t_{1/2}$ (days)	r^2	Anthocyanin loss (%)
25 °C	4	Dark	0.02 ± 0.006^{f}	35.3 ± 10.9^{b}	0.8821	56.7
		Light	0.03 ± 0.001^{f}	21.5 ± 1.3^{b,c}	0.7739	100
	7	Dark	0.05 ± 0.007^{f}	13.4 ± 2.2^{c,d}	0.8252	93.6
		Light	0.08 ± 0.039^{f}	9.6 ± 4.7^{c,d}	0.9498	100
	10	Dark	0.05 ± 0.007^{f}	13.1 ± 1.8^{c,d}	0.9023	94.5
		Light	0.1 ± 0.026^{e,f}	6.9 ± 1.8^{d}	0.9519	97.3
65 °C	4	Dark	0.21 ± 0.001^{d,e}	3.2 ± 0.02^{d}	0.9413	100
		Light	0.33 ± 0.05^{d}	2.2 ± 0.3^{d}	0.9825	100
	7	Dark	0.50 ± 0.01^{c}	1.3 ± 0.3^{d}	0.9264	100
		Light	0.89 ± 0.11^{a}	7.8 ± 0.9^{c,d}	0.9000	100
	10	Dark	0.78 ± 0.15^{b}	0.9 ± 0.19^{d}	0.9000	100
		Light	0.93 ± 0.02^{a}	0.7 ± 0.1^{d}	0.8256	89

Results are expressed as mean ± standard deviation. Values followed by different letters are significantly different ($p \leq 0.05$).

Additionally, intramolecular copigmentation mechanisms, involving associations between anthocyanins, can also contribute to stability. It has been observed that the presence of acyl groups and a higher number of methoxyl groups in the B ring enhance anthocyanin stability, whereas an increase in hydroxyl groups or the absence of acyl groups decreases stability (Escribano-Bailón *et al*., 2004). Similarly, Hou *et al*. (2013) found that anthocyanins from black rice (*Oryza sativa* L.) extracts degraded more rapidly with increasing temperature (100 °C) and at pH values above 5. Other studies have also investigated the impact of pH on anthocyanin stability. Cisse *et al*. (2009) found that a pH value greater than 3 significantly reduces the stability of anthocyanins in blood orange juice (*Citrus sinensis*) extracts. Similarly, Tüker and Erdogdu (2006) reported that anthocyanins extracted from black carrot (*Daucus carota* var. L.) are more stable at pH 2, and that

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increasing the pH can lead to reduced pigment stability. A study on red cabbage demonstrated that acylated anthocyanins are more stable than their non-acylated counterparts under conditions of pH 7 and 50 °C, which helps maintain the blue color (Fenger *et al*., 2020). Although anthocyanin stability has been extensively studied, it is clear that their stability is also influenced by the plant source from which they are extracted. Therefore, to consider a potential source for obtaining these pigments, specific studies are necessary depending on the plant source. Notably, there is a lack of research on the stability of *Cosmos bipinnatus* pigments and their potential use as natural colorants in the food industry.

Polymeric color

Polymeric color is a measure that indicates the degree of anthocyanin polymerization, which is also related to their stability. The degradation of anthocyanins is always accompanied by the formation of polymeric color (Martynenko & Chen, 2016). Table 3 shows significant differences (p <0.05) in the percentage of polymeric color at day 0 and its formation after 41 days of storage for the different treatments. The effects of pH and temperature on polymeric color formation are also evident. When samples were exposed to pH 4, regardless of temperature, the polymerization reaction of anthocyanins was favored, resulting in an increase in polymeric color during storage (from 32.19 to 96.19 %). This polymerization is considered a priority under acidic conditions (pH 4), as the flavylium cation is in its most stable form, allowing it to polymerize with other anthocyanins, phenolic compounds (phenolic acids, flavonoids, tannins), or non-phenolic compounds (amino acids, organic acids) present in the environment (Türkyılmaz & Özkan, 2012; Sinela *et al*., 2017). However, increasing the pH to 7 and 10 leads to additional reactions, such as degradation, hydrolysis, glycosylation, and oxidation reactions, which reduce polymeric color formation. Compared to samples at pH 4, alkaline conditions result in less polymeric color formation (at pH 7 from 22.37 to 83.59 % and at pH 10 from 22.44 to 31.95 %, respectively). This difference in behavior may be attributed to the rapid degradation of anthocyanins under alkaline conditions and high temperatures, leading to the opening of the pyrilium ring, formation of chalcones, and brown degradation products, such as coumaric glucosides, and hydrolysis of the sugar fraction of anthocyanins to form anthocyanidins, aldehydes, and benzoic acid derivatives, resulting in the loss of the original pigment (Seeram *et al*., 2001; Patras *et al*., 2010).

Danişman *et al*. (2015), Martynenko & Chen (2016), and Ngamwonglumlert *et al*. (2017) observed that temperatures above 60 °C lead to a decrease in monomeric anthocyanins and an increase in polymeric color formation. This phenomenon involves the formation of a colorless carbinol pseudobase and the subsequent opening of the pyrilium ring to form chalcone, which ultimately transforms into a brown degradation product. In extracts of purple sweet potato and purple carrot stored for 134 days at pH values greater than 4.0, Cevallos-Casal & Cisneros-Zevallos (2004) found that anthocyanin degradation led to color changes from purple to blue to brown and finally to yellow. According to Seeram *et al*. (2001), increasing the pH to 7 or 10, combined with high temperatures, causes rapid degradation of anthocyanins in cherries (*Prunus cerasus* L. cv. Balaton) to benzoic acid derivatives. Additionally, Gössinger *et al*. (2009) reported a significant positive effect of precooling strawberries (*Fragaria ananassa*, cultivar Elsanta) on the color stability of puree nectars, which can be stored for over 12 months. They also found that cold

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storage at 4 °C is an effective way to stabilize the color of strawberry nectars.

Effect of storage on antioxidant activity

As shown in Figure 2b, during storage, the treatments at pH 4 and 7 exhibited a lower loss of antioxidant activity, whereas the extracts at pH 10 showed a drastic loss of activity. The retention of antioxidant capacity during storage can be attributed to the formation of copigments (Tsai *et al*., 2004), which compensate for the loss of monomeric anthocyanins, or to the formation of hydrogen donor products that inhibit the chain reaction of free radicals, thereby contributing to the DPPH-reducing effect. These results are consistent with the trend observed by Tsai & Huang (2004) for acidified ethanolic extracts of *Hibiscus sabdariffa* L. flowers after heat treatment (90 °C) for 147 hours, which showed a 18-43 % reduction in DPPH. Similarly, Chaovanalikit & Wrolstad (2004) found that the antioxidant activity of canned cherries (*Prunus cerasus* L.), measured by oxygen radical absorption capacity (ORAC), increased after 5 months of storage at 22 °C. Hager *et al*. (2008) observed an increase in ORAC values of 32 % and 27 % after 3 and 6 months of storage, respectively, in black raspberries (*Rubus occidentalis* L.) in syrup. In contrast, the treatments at pH 10 showed a lower antioxidant activity value at the end of storage, which is likely related to the degradation of anthocyanins associated with the temperature effect.

Table 3. Influence of degradative factors, temperature, pH, and light incidence on polymeric color in extracts of *Cosmos bipinnatus* flower petals during 41 days of storage.

Temperature	pH	Light incidence	Polymeric color (%)	
			Day 0	Day 41
4 °C	4	Dark	32.19 ± 0.31^{f}	91.69 ± 1.25^{a,b}
		Light	32.19 ± 0.31^{f}	96.19 ± 2.05^{a}
	7	Dark	22.37 ± 2.58^{g}	29.64 ± 0.42^{f}
		Light	22.37 ± 2.58^{g}	28.35 ± 2.09^{f}
	10	Dark	22.44 ± 2.22^{g}	1.65 ± 0.62^{j}
		Light	22.44 ± 2.22^{g}	4.19 ± 0.79^{i,j}
25 °C	4	Dark	32.19 ± 0.31^{f}	86.47 ± 0.05^{b,c}
		Light	32.19 ± 0.31^{f}	95.85 ± 2.01^{a}
	7	Dark	22.37 ± 2.58^{g}	57.6 ± 2.24^{e}
		Light	22.37 ± 2.58^{g}	58.13 ± 3.74^{d,e}
	10	Dark	22.44 ± 2.22^{g}	31.95 ± 2.69^{f}
		Light	22.44 ± 2.22^{g}	15.64 ± 0.19^{h}
65 °C	4	Dark	32.19 ± 0.31^{f}	95.36 ± 0.75^{a}
		Light	32.19 ± 0.31^{f}	95.18 ± 0.49^{a}
	7	Dark	22.37 ± 2.58^{g}	63.97 ± 2.05^{d}
		Light	22.37 ± 2.58^{g}	83.59 ± 1.51^{c}
	10	Dark	22.44 ± 2.22^{g}	12.14 ± 0.02^{h}
		Light	22.44 ± 2.22^{g}	9.85 ± 1.37^{h,i}

Results are expressed as mean ± standard deviation. Values followed by different letters are significantly

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different ($p \leq 0.05$).

Analysis of color stability

The development of natural pigments for potential use in foods necessitates an analysis of color stability prior to application. In this study, the CIE Lab color space analysis and color difference (ΔE) were employed to determine the pigment's behavior over 41 days of storage under various conditions of light exposure, temperature, and pH (Table 4).

Table 4. Effect of degradative factors, temperature, pH, and light incidence on color in extracts of *Cosmos bipinnatus* flower petals during 41 days of storage.

Temperature (°C)	pH	Day 0				Day 41			
		L*	a	b	ΔE	L*	a	b	ΔE
Dark									
4	**4**	25.87 (0.12)	28.25 (1.34)	-4.23 (0.04)	0	30.39 (0.12)	18.81 (0.04)	-3.52 (0.04)	10.53 (1.34)
	7	25.25 (0.11)	13.56 (0.17)	-0.92 (0.23)	0	36.51 (0.37)	-0.28 (0.04)	9.60 (0.18)	20.71 (0.12)
	10	16.70 (0.14)	10.43 (0.13)	9.01 (0.17)	0	36.15 (1.04)	-1.71 (0.16)	12.03 (0.21)	23.12 (0.94)
25	**4**	25.87 (0.12)	28.25 (1.34)	-4.23 (0.04)	0	35.05 (0.52)	5.93 (0.24)	5.73 (0.07)	30.02 (1.18)
	7	25.25 (0.11)	13.56 (0.17)	-0.92 (0.23)	0	35.63 (0.46)	0.33 (0.05)	15.85 (0.14)	28.67 (1.98)
	10	16.70 (0.14)	10.43 (0.13)	9.01 (0.17)	0	36.52 (0.74)	-0.10 (0.08)	12.10 (0.52)	21.65 (1.12)

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Table 4. Effect of degradative factors, temperature, pH, and light incidence on color in extracts of *Cosmos bipinnatus* flower petals during 41 days of storage.

Temperature (°C)	pH	Day 0				Day 41			
		L*	a	b	ΔE	L*	a	b	ΔE
	4	25.87	28.25	-4.23	0	30.84	2.47	19.74	38.53
		(0.12)	(1.34)	(0.04)		(0.28)	(0.06)	(0.04)	(0.87)
65	**7**	25.25	13.56	-0.92	0	36.41	1.32	22.22	28.67
		(0.11)	(0.17)	(0.23)		(0.31)	(0.14)	(0.39)	(0.59)
	10	16.70	10.43	9.01	0	35.79	2.09	28.99	28.86
		(0.14)	(0.13)	(0.17)		(0.28)	(0.04)	(1.61)	(0.48)
Light									
	4	25.87	28.25	-4.23	0	32.73	8.45	0.39	21.45
		(0.12)	(1.34)	(0.04)		(0.25)	(0.13)	(0.06)	(1.47)
4	**7**	25.25	13.56	-0.92	0	40.31	-1.05	11.17	24.21
		(0.11)	(0.17)	(0.23)		(0.97)	(0.17)	(0.93)	(1.15)
	10	16.70	10.43	9.01	0	33.17	-2.35	23.02	25.11
		(0.14)	(0.13)	(0.17)		(0.23)	(0.15)	(2.15)	(0.17)
	4	25.87	28.25	-4.23	0	42.89	3.72	4.42	31.08
		(0.12)	(1.34)	(0.04)		(0.37)	(0.10)	(0.12)	(1.27)
25	**7**	25.25	13.56	-0.92	0	39.55	0.77	17.31	28.67
		(0.11)	(0.17)	(0.23)		(0.15)	(0.01)	(0.36)	(0.22)
	10	16.70	10.43	9.01	0	27.59	4.32	28.08	22.79
		(0.14)	(0.13)	(0.17)		(0.08)	(0.07)	(0.65)	(0.74)
	4	25.87	28.25	-4.23	0	40.21	0.39	16.62	38.53
		(0.12)	(1.34)	(0.04)		(1.79)	(0.10)	(0.04)	(1.80)
65	**7**	25.25	13.56	-0.92	0	44.77	-0.27	11.81	28.67
		(0.11)	(0.17)	(0.23)		(0.40)	(0.02)	(0.53)	(1.47)
	10	16.70	10.43	9.01	0	38.33	-1.26	21.27	21.65
		(0.14)	(0.13)	(0.17)		(0.28)	(0.03)	(0.16)	(0.04)

For the 18 samples studied, a significant increase in the L* value was observed starting on day 2, which was maintained until the end of storage. This increase in L* was also reported by Montibeller *et al*. (2018) when evaluating the stability of anthocyanins from grape residues applied as a food coloring in kefir and carbonated water. This behavior may be related to the degradation

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of anthocyanins over time, resulting in discoloration of the pigment and an increase in the L* value. In contrast, the a* parameter decreased significantly over time, with the greatest change observed in the pigment with pH 10 exposed to light and darkness, as well as at the three temperatures. According to Hubbermann *et al*. (2006), the decrease in the a* value is associated with the loss of anthocyanins and the resulting loss of red color. This change can be explained by the equilibrium between the four anthocyanin chromophores in aqueous solutions and the subsequent hydration reaction of the flavylium cation, involving the entry of a water molecule, the release of a proton, and the nucleophilic attack of the hydroxyl of the water, which neutralizes the charge and causes the red color to disappear. On the other hand, the b* parameter increased over time, evident in all treatments, showing a change in the blue color (-b*) towards yellow (+b*). This phenomenon is related to the formation of chalcones favored by the increase in temperature and pH, which corresponds to the results observed in the samples subjected to pH 7 and 10 at 25 and 65 °C. Regarding the color difference (ΔE) for all samples at pH 7 and 10, a marked change was observed from day 5 onwards, indicating color instability under these conditions (Figure 2c). Specifically, in treatments at pH 4, significant changes occurred over time: at 4 °C, a strong difference followed by a color change was observed from day 21; at 25 °C, the color difference appeared from day 13; and at 65 °C, the color difference was evident from day 2.

Conclusions

The present study proposes the use of the hydroethanolic extract of *Cosmos bipinnatus* flower petals as a natural pigment with bioactive properties, attributed to its high content of monomeric anthocyanins. Phytochemical characterization using various polar solvents (aqueous, hydroethanolic, and hydromethanolic) confirmed the suitability of the hydroethanolic extract. The extract's stability was evaluated under different conditions, including pH (4, 7, and 10), temperature (4, 25, and 65 °C), and light radiation (light and dark). The results showed that anthocyanin degradation follows first-order reaction kinetics, with a maximum half-life of 63 days. Over time (41 days), the stability of monomeric anthocyanins decreased, while polymeric color formation increased with rising temperature and pH. The most significant color change from pink to yellow, as well as loss of antioxidant activity, occurred in treatments at pH 10 and 65 °C, just a few days after exposure. In contrast, anthocyanins exhibited greater stability and preserved their pigment color under conditions of pH 4, 4 °C, and darkness. These findings suggest that these conditions should be considered when developing future functional foods or using the extract as a functional colorant.

Authors' contribution

Conceptualization of the work, HJC, OVJ; development of the methodology, ALAN, BGL; software management, ALAN; experimental validation, HJC, BGL; analysis of results, ALAN, OVJ;

management of data, ALAN, OVJ; writing and preparation of the manuscript, writing, reviewing and editing, ALAN, HJC, BGL; project manager and funding acquisition, OVJ.

All authors of this manuscript have read and accepted the published version of it.

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Conflict of interest

The authors declare that they have no conflict of interest.

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