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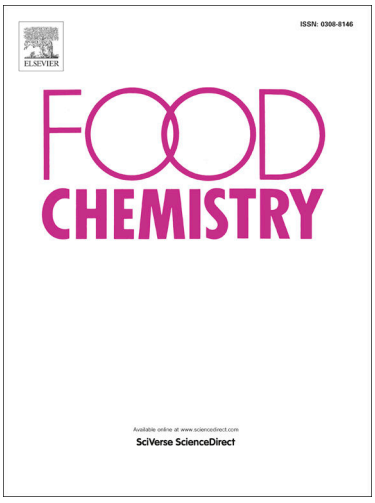

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Physicochemical properties, degradation kinetics, and antioxidant capacity of aqueous anthocyanin-based extracts from purple carrots compared to synthetic and natural food colorants

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ABSTRACT

As a means to evaluate the potential of carrot anthocyanins as food colorants and nutraceutical agents, we investigated the physicochemical stability and antioxidant capacity of purple carrot extracts under different pH (2.5-7.0) and temperature (4-40 °C) conditions, in comparison to a commercial synthetic (E131) and a natural grape-based (GRP) colorant. During incubation, the colorants were weekly-monitored for various color parameters, concentration of anthocyanins and phenolics, and antioxidant capacity. Carrot colorants were more stable than GRP; and their thermal stability was equal (at 4°C) or higher than that of E131 (at 25-40°C). Carrot anthocyanins had lower degradation rate at low pH and temperature, with acylated anthocyanins (AA) being significantly more stable than non-acylated anthocyanins (NAA). Anthocyanins acylated with feruloyl and coumaroyl glycosides were the most stable carrot pigments. The higher stability of carrot colorants is likely due to their richness in AA and –to a lesser extent- copigmentation with other phenolics.

Keywords: natural pigments, anthocyanins, physical-chemical stability, degradation kinetics, pH, temperature.

1. Introduction

Food colorants are among the most interesting additives used in the food industry, because the color is intrinsically-linked to the visual appeal and the quality perception of food products (Kucharska & Grabka, 2010). Among them, synthetic dyes are generally considered to have high stability under different conditions of temperature, pH, light intensity, and oxygen concentration (Kucharska & Grabka, 2010). However, their consumption has been associated with various health problems, including allergic reactions, behavioral and neurological adverse effects, and potential carcinogenesis (Houghton, Appelhagen, & Martin, 2021). As a result, there is increasing interest in the development of chemically-stable food colorants from natural sources that may help replace synthetic dyes.

Anthocyanins are natural flavonoid water-soluble pigments that provide red, violet, and blue hues to different organs and tissues of many plant species (Strack & Wray, 1994; Koes, Quattrocchio, & Mol, 1994), serving various roles in the plant, including attraction of insects and animals for pollination and seed dispersal, and protection against biotic and abiotic stresses (Koes et al., 1994). In addition, these pigments have potent antioxidant and anti-inflammatory properties, and their consumption has proven beneficial effects on human health, with regards to reducing the risk of cardiovascular disease and some cancer types, improving glucose regulation, and aiding in the prevention neurological disorders (Joseph, Shukitt-Hale, & Casadesus, 2005).

The chemical structure of these pigments strongly influences their bioavailability, nutraceutical properties, and chemical stability. In particular, glycosylation and acylation have been shown to increase the chemical stability of anthocyanins, and therefore their potential utilization as food colorants (Giusti & Wrolstad, 2003). According to Mazza, Cacace, & Kay (2004), glycosylation confers increased stability and water solubility, whereas acylation of the sugar residues significantly increases stability. Interestingly, while acylated anthocyanins are

generally considered to have higher stability than their non-acylated counterparts, the latter have shown significantly higher bioavailability in some species (Kurilich, Clevidence, Britz, Simon, & Novotny, 2005).

Some purple or black carrots (*Daucus carota* ssp. *sativus* var. *atrorubens* Alef.) can accumulate large quantities of anthocyanins in their roots, reaching concentrations of ~2000 mg/kg fw, or higher, in some genetic stocks (Bannoud et al., 2018; Kammerer, Carle, & Schieber, 2004); thereby representing an excellent dietary source of these pigments. Cyanidin is the major type of anthocyanidin accumulating in purple carrot roots, with five major cyanidin glycosides –three monoacylated and two non-acyalated anthocyanins- being reported for this vegetable to date (Supplementary Table S1) (reviewed by Cavagnaro & Iorizzo, 2019). Extensive variation has been found in the purple carrot germplasm for root total anthocyanin concentration (reported mean values range from 5 to 2290 mg/kg fw) and composition, with most of the accessions presenting a predominance of acylated anthocyanins (AA) over non-acylated anthocyanins (NAA), with AA accounting for 55-99% of the total anthocyanins content (Bannoud et al., 2018; Kammerer et al., 2004; Montilla, Arzaba, Hillebrand, & Winterhalter, 2011). Such broad variation for root anthocyanin content and AA:NAA ratio suggests that different carrot genetic stocks vary in their suitability as potential sources of food dyes. In addition, significant variation for total phenolics and antioxidant activity was reported in the carrot germplasm (Leja et al., 2013), suggesting that important variation exists also for their root content of antioxidant phytochemicals.

To date, only a few studies have evaluated color stability of carrot anthocyanins, when used as additive for coloring different food matrices (fruit juices and nectars, hard candy, jelly, and sunflower oil), providing the first published data on the utilization of these pigments as food dyes. However, in these studies, the carrot anthocyanin extracts were added to food matrices with different pH values that contained many other constituents that may affect anthocyanins stability, and no comparisons with other natural or synthetic dyes were

performed. Also, they used a single and uncharacterized carrot cultivar (obtained from a local market) as source of anthocyanins, and total anthocyanins content –rather than individual pigments- was analyzed (Kırca, Özkan, & Cemeroğlu, 2007; Kumar et al., 2020).

Comparative analysis of pigment stability from different purple carrot accessions varying in anthocyanin composition, along with other commercially-available natural and synthetic food dyes, under ranges of temperature and pH conditions commonly used for storage of food products, is necessary to evaluate the potential of carrot anthocyanins as colorants for the food industry. Furthermore, comparative analysis of the stability –under these conditions- of individual anthocyanin pigments would reveal important information for carrot breeding programs aiming at developing varieties for the production of chemically-stable food colorants (e.g., by means of increasing the concentration of highly-stable compounds). Also, time-course analysis of other water-soluble compounds that may interact with anthocyanins and influence their stability (e.g., polyphenols), as well as the antioxidant capacity of anthocyanin extracts, may evidence other factors conditioning the stability and functional value of these pigments.

In the present study we investigated color physical-chemical properties, stability of total and individual anthocyanin pigments (by HPLC analysis), phenolics content, and antioxidant activity of aqueous extracts from two purple carrot accessions varying in anthocyanin composition, in comparison (side-by-side) to similar extracts prepared from a commercial synthetic dye and a natural grape-based food colorant, under different temperature and pH conditions used for storage of food products. In addition to characterizing color and total pigment variation in these colorant sources, the results of this study provide novel and relevant information concerning the stability of individual anthocyanin pigments, phenolics content, and antioxidant activity in purple carrots extracts. These data are relevant for carrot breeders and food technologists aiming at developing natural and chemically-stable dyes with added functional value for the food industry.

2. Materials and methods

2.1. Plant materials and commercial colorants

The physical-chemical stability of carrot anthocyanins was evaluated in two purple carrot accessions; the commercial hybrid cultivar 'Purple 68', obtained from Territorial Seed Company (Cottage Grove, Oregon, USA), and the inbred line 'INTA43', developed from the carrot breeding program at INTA, Argentina. These materials were selected for their high concentration of anthocyanins in the roots and their different pigment composition (Bannoud et al., 2018). Additional information regarding the carrot accessions is presented in Supplementary Table S2.

For comparison purposes, a natural food colorant developed from grape anthocyanins (catalog number BFTU-42-EA, TruColor LLC, California, USA) (from here on referred to as 'GRP'); and the synthetic food dye 'E131 Patent Blue' (Laboratorio Fleibor S.R.L., Buenos Aires, Argentina) (from here on referred to as 'E131'), were included in the experiments and evaluated under the same conditions as the carrot anthocyanin extracts. Thus, E131 and GRP were included as representatives of currently-used synthetic and natural food dyes, respectively.

2.2. Preparation of anthocyanin aqueous extracts

The anthocyanins from Purple 68 and INTA43 were extracted as follows: anthocyanin-containing tissues from fresh carrot roots were mixed with an aqueous acid formic solution (1 % v/v) in a 1:1 w/v proportion until a uniform consistency was reached. Then, the mixture was

filtered and centrifuged (11000 rpm, 4°C), and the clear supernatant was separated and stored at -20°C (Espinosa-Acosta et al., 2018).

2.3. Colorant stability experiments

The effect of pH on the stability of color and antioxidant constituents of the aqueous extracts of Purple 68 and INTA43 was analyzed at pH 2.5, 4.5, and 7.0. For this purpose, an aliquot of the carrot extract was diluted in different pH buffers (Supplementary Table S3) (Reyes & Cisneros-Zevallos, 2007). All chemicals and reagents were of analytical grade and were supplied from Sigma Aldrich (Atlanta, USA). The extract/buffer ratio used was such that the maximum absorbance in the visible wavelength range of the solution at pH 2.5 was about 0.7. This ratio was used as criterion to standardize the preparation of the other pH solutions. The final pH of the solutions was measured and adjusted by adding a few drops of HCl (37 % v/v). The aqueous solutions of the natural (GRP) and synthetic (E131) commercial colorants were prepared similarly. Supplementary Figure S1 shows the absorbance spectra of the colorants aqueous solutions at pH 2.5. All colored solutions were then allowed to equilibrate at room temperature in their respective buffer solutions for 1 h, after which baseline measurements for all the variables were taken. The colored solutions were then incubated at 25°C in the dark for 42 days. The first sampling date was three days later, followed by weekly samplings and measurements until the 42nd day of incubation, for a total of eight sampling moments. Overall, the experiment consisted of 24 50-mL caramel-glass jars containing 25 mL of colorant solution per colorant type and pH value, for a total of 288 jars, placed in a thermostat-regulated temperature incubator (CLN 32, POL-EKO, Wodzisław Śląski, Poland), and at each sampling time three jars of each colorant type were taken out of the incubator and analyzed.

An experiment to test the effect of temperature on colorant stability was performed for all the colorant sources, using aqueous solutions at pH 2.5, prepared as described above (Reyes & Cisneros-Zevallos, 2007). The jars containing the colorant solutions were placed in the dark at 4°C (in a refrigerator), 25°C and 40°C (in thermostat-regulated temperature incubators). Thermal degradation was monitored for 56 days. After the initial baseline measurements, taken as described above, the first sampling moment was three days later, followed by weekly samplings and measurements until the 56th day of incubation. Overall, the experiment consisted of 27 colorant-containing jars for each type of colorant and temperature treatment, for a total of 324 jars, and at each sampling time three jars of each colorant type and temperature treatment were taken out of the incubator or refrigerator and analyzed.

2.4. Color measurements

The color intensity (CI) was determined according to the formula ($A_{\lambda max}$ - A_{700}) x DF; where $A_{\lambda max}$ is the absorbance at the wavelength of maximum absorbance, A_{700} is the absorbance at 700 nm, and DF is a dilution factor (Marpaung, Zhang, & Sutanto, 2019). The relative CI (%) was calculated as the percent relative to the initial (baseline) conditions. Spectrophotometric measurements were carried out using a T60 visible spectrophotometer (PG Instruments, Leicestershire, United Kingdom). The changes in visual color were assessed with a Minolta CR-300 colorimeter (Konica Minolta Inc.; Mahwah, NJ) by measuring Hunter L*C*h° values.

2.5. Spectrophotometric determinations of total phenolics, total anthocyanins, and chlorogenic acid concentrations

Total phenolics concentration was determined colorimetrically with Folin's reagent (Leja et al., 2013). For this purpose, 50 µL of the colorant aqueous solution or water (as sample blank) were mixed with 3700 µL of water and 250 µL of the Folin–Ciocalteu reagent. The mixture was stirred and after 3 min, 1 mL of a 20 % Na_2CO_3 solution (w/v) was added. Subsequently, the mixture was incubated at 37 °C in the dark. After 60 min, absorbance was read at 765 nm. Gallic acid was used as the reference compound for calibration.

The pH differential method was used to determinate total anthocyanins concentration of the colorant aqueous solutions (Kang, Ko, & Chung, 2021). The absorbance of the solutions was measured at pH 1.0 and pH 4.5 at two wavelengths, 520 nm and 700 nm, to correct the haze. The absorbance difference was calculated using the following equation:

$$A = (A_{520} - A_{700})_{pH\,=\,1.0} - (A_{520} - A_{700})_{pH\,=\,4.5}$$

The absorbance difference was used to calculate the anthocyanins content (C, mg L^{-1}) as expressed in the following equation:

$$C = \frac{DF\ x\ MW\ x\ 1000\ x\ A}{\varepsilon\ x\ d}$$

Where, DF is the dilution factor, MW is molecular weight of 449.2 g mol^{-1}, A is the absorbance difference, ε is the molar absorptivity (26900), and d is the cuvette width (1 cm).

The content of chlorogenic acid was estimated by measurement of UV/Vis absorbance at 330 nm (Leja et al., 2013). A calibration curve was built using chlorogenic acid as reference compound. No purifications were performed prior to taking chlorogenic acid measurements.

The relative content of total phenolics, anthocyanins and chlorogenic acid (%) was calculated as the percent relative to the initial conditions. Spectrophotometric measurements

were performed with a T60 visible spectrophotometer (PG Instruments, Leicestershire, United Kingdom). All standards and reagents were of analytical grade and were supplied from Sigma Aldrich (Atlanta, USA).

2.6. Anthocyanin HLPC analysis

High-performance liquid chromatography (HPLC) was used to determine the concentration of anthocyanin pigments in the carrot aqueous extracts, according to methods previously described (Kurilich et al., 2005). HPLC analysis was carried out using a UHPLC apparatus (Shimadzu, SIL30-AC, Japan) equipped with a binary pump system (LC-30AD, Nexera, Shimadzu), an autosampler-injector (SIL-30AC, Nexera X2, Shimadzu), a photodiode array UV-VIS detector (SPD-M30A Nexera X2, Shimadzu), and a C18 column (3 µm, 2.1 x 150 mm, UFLC Aqueous, RESTEK). The mobile phase was distilled water acidified with 1% (v/v) formic acid as solvent A and methanol as solvent B. The gradient system was 0/5, 20/55, 21/100, 26/100, 27/5, and 40/5 (min / % solvent B). A commercial standard of cyanidin (Sigma Aldrich, Atlanta, USA) was used for quantitation purposes. Peak assignment was performed by LC-MS/MS analysis of the column eluate, which was carried out with the HPLC system described above coupled to electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) with an ESI-QTOF instrument model G6560A from Agilent (Santa Clara, California, USA). Supplementary Table S1 presents the five major carrot anthocyanin pigments, along with their HPLC retention times and molecular masses, identified and quantified in the present study. The anthocyanin pigments content was expressed as the percent relative to the initial conditions.

2.7. Antioxidant capacity

The antioxidant activity of the colorant solutions was determined using the ABTS assay (Thaipong, Boonprakob, Crosby, Cisneros-Zevallos, & Byrne, 2006). The $ABTS^{\cdot+}$ working solution was prepared by mixing in equal quantities a 7.4 mM $ABTS^{\cdot+}$ solution and a 2.6 mM potassium persulfate solution. Both reagents were from Cayman (Michigan, USA). The mixture was allowed to react for 12 h at room temperature in the dark. The solution was then diluted with methanol to obtain an absorbance of 1.0 at 734 nm. Fresh dilute $ABTS^{\cdot+}$ solution was prepared for each assay. Colorants aqueous solutions (150 μL) were allowed to react with 2850 mL of the $ABTS^{\cdot+}$ solution for 2 h in the dark. Then, absorbance readings were taken at 734 nm using a T60 visible spectrophotometer (PG Instruments, Leicestershire, United Kingdom). The standard curve was made using Trolox (Cayman, USA) as standard.

The antioxidant activity of the colorant solutions was also determined using the DPPH assay according to Thaipong et al. (2006), with some modifications. A DPPH working solution was prepared by dissolving 40 mg of DPPH reactant per 1 L of methanol. Colorants aqueous solutions (50 μL) were allowed to react with 1000 mL of the DPPH solution for 1 h in the dark. Then, absorbance readings were taken at 515 nm using a T60 visible spectrophotometer (PG Instruments, Leicestershire, United Kingdom). The standard curve was made using Trolox (Cayman, USA) as standard.

2.8. Statistical analysis

All the instrumental measurements were performed by triplicates. The data were evaluated using analysis of variance (ANOVA), and the means were compared by Tukey's test, considering significant p values ≤ 0.05. Correlation analyses was made using Pearson's correlation coefficient. In all the cases, the InfoStat software was used.

3. Results

3.1. Effect of the pH on the stability of colorant solutions

3.1.1. Color intensity (CI)

The effect of different pH values on color intensity (expressed as percentage relative to the CI in the samples prior to incubation) of aqueous solutions of Purple 68, INTA43, GRP, and E131, is illustrated in Figure 1A. CI had a similar variation trend for Purple 68, INTA43, and GRP, with CI decreasing at faster rates as pH increased. Despite their general similar trend, color intensity decreased faster in GRP than in the two carrot anthocyanin extracts, particularly at low pH, with extracts of Purple 68 and INTA43 retaining 56% and 53% of their initial color intensities at the end of the experiment (at day 42) at pH 2.5, whereas GRP solutions only retained 16% of their initial color intensity under these same conditions. Conversely to the trend observed for the three natural colorants, the synthetic dye E131 revealed higher degradation rate (as evidenced by decreasing CI values) at the lowest pH value (pH 2.5), retaining only 4% of the initial color at the end of the experiment, whereas for higher pH values (pH 4.5 and 7.0) color retention was always above 75%.

3.1.2. Changes in visual color attributes

The time-course variation of color visual attributes for the four colorant sources is depicted in Supplementary Figure S2. Bright and attractive colors were initially observed in all the colorant solutions. Both of the carrot anthocyanin solutions changed their initial color as pH values increased, from a bright reddish hue (h°~25°) at pH 2.5, to purple (h°~45°) at pH

4.5, to a blueish hue (h° ~350°) at pH 7. At pH 2.5, carrot extracts from INTA43 and Purple 68, maintained nearly unaffected their color visual aspect throughout the 6-week incubation period, whereas at pH 4.5 and 7.0 a noticeable degree of color loss and change of hue was observed for both carrot anthocyanin sources after 21 days of incubation. The GRP colorant suffered extensive color degradation at all the pH values analyzed, evidencing such changes early in the experiment (after 14 days of incubation). In contrast to the three natural colorants, the synthetic dye E131 revealed color degradation at the lowest pH value after three weeks of incubation, changing its hue from blue to red, whereas at higher pH values (pH 4.5 and 7.0) no color degradation was observed through the entire incubation period.

Figure 2 depicts variation for the L*C*h° color parameters for all the colorants incubated at different pH values. In general, the lightness (L*) increased through time, whereas the chroma (C*) parameter decreased. GRP exhibited the highest decrease rate of C* at all pH values. The hue angle (h°) tended to turn towards ~50° for INTA43 and Purple 68 and towards ~8° for GRP (Supplementary Figure S3). In E131, h° did not change significantly at high pH, but it decreased rapidly, form 300° to ~0°, at pH 2.5.

3.1.3. Total phenolics and anthocyanins concentration

The time-course variation for total phenolics content in the colorant solutions prepared from purple carrot roots and grapes, relative to their initial content, revealed decreasing phenolics levels with time, and the rate of decrease was slower at low pH (Figure 2B). Comparisons among the three natural colorants showed a similar variation trend at pH 2.5. However, at higher pH values of 4.5 and 7, the GRP colorant retained a higher proportion of its initial phenolics content than the carrot extracts, with relative phenolics content of 39.3-49.3%, 6.0-23.0%, and 3.0-6.0% found for GRP, Purple 68 and INTA43, respectively, at the end of the incubation period.

Figure 2A depicts the time-course variation for relative total anthocyanins content (%) in the purple carrot and grape colorant solutions. In general, total anthocyanin concentration (determined spectrophotometrically) decreased at higher rates as pH values of the solutions increased, coincidently with result found for total phenolics. At the lowest pH, carrot anthocyanins were more stable than those from grape, as indicated by the more gradual anthocyanin decay in the former, retaining at the end of the experiment 40% in INTA43 and 53% in Purple 68, whereas the grape colorant evidenced complete degradation of these pigments at the end of the experiment, retaining only 6% of its initial content. At this pH, Purple 68 showed a more gradual decay in anthocyanin levels than INTA43. Although less evident, at pH 4.5, carrot anthocyanins were also more stable than grape anthocyanins, as suggested by their relative contents in the carrot (16-23%) and GRP (6%) solutions at the end of the incubation period. At pH 7, all three natural colorants were similarly and extensively degraded.

3.1.4. Concentration of Individual anthocyanins pigments

HPLC analysis of carrot anthocyanin extracts from Purple 68 and INTA43 revealed five and four major anthocyanin compounds, respectively (Supplementary Table S1). As reported in previous studies, two non-acylated [Cy-3-(2'-xylose-6-glucose-galactoside) (Cy3XGG) and Cy-3-(2'-xylose-galactoside) (Cy3XG)] and three acylated anthocyanins [Cy-3-(2'-xylose-6'-synapoyl-glucose-galactoside) (Cy3XSGG), Cy-3-(2'-xylose-6'-feruloyl-glucose-galactoside) (Cy3XFGG), and Cy-3-(2'-xylose-6'-(4-coumuroyl)-glucose-galactoside) (Cy3XCGG)] were detected (Kammerer et al., 2004; Kurilich et al., 2005). Cy3XFGG was the most abundant compound in Purple 68, representing 68.2% of the total anthocyanins content, whereas Cy3XSGG predominated in INTA43, accounting for 64.2% of the total. In both carrot

accessions, acylated forms predominated over non-acylated pigments, with the former accounting for 84.3% in Purple 68 and 83.2% in INTA43.

Analysis of the effect of pH and incubation time on individual anthocyanin pigments of Purple 68 and INTA43 revealed compound-, time-, and pH-dependent degradation of carrot anthocyanins (Figure 3). In general, anthocyanin degradation increased with time and pH value. Acylated anthocyanins (AA) suffered less degradation than non-acylated anthocyanins (NAA) at all pH values. In decreasing order, the stability of individual anthocyanin pigments was Cy3XFGG > Cy3XCGG > Cy3XSGG > Cy3XG > Cy3XGG.

3.1.5. Antioxidant activity

Variation for antioxidant activity, as affected by pH during the incubation period, was analyzed by ABTS and DPPH assays. In both cases, variation for antioxidant activity showed similar time-course patterns (Figure 4A and Supplementary Figure S4A). At pH 2.5, the natural colorant solutions from carrot and grape revealed minimal changes –throughout the incubation period- with respect to their initial antioxidant activity; whereas at higher pH values a significant decrease of activity was found, through time, in all the colorant sources. In absolute values, Purple 68 extracts had the highest antioxidant capacity among the three natural colorants (Supplementary Figure S5).

3.2. Thermal stability of the colorant solutions

The effect of the temperature on the stability of colorant solutions from the same four colorant sources was investigated. Based on results from the previous experiment on the effect of pH on colorants stability, revealing –generally- higher stability at low pH, a pH value of 2.5 was used for all the colorant solutions. Thus, thermal degradation of the colorant solutions

incubated (in the dark) at 4°C, 25°C, and 40°C, was monitored weekly for 56 days, by means of analysis of the same variables as in the pH stability experiment. Because the pH selected for preparing the colorant solutions was associated with relatively higher pigment stability, the incubation period was extended for two additional weeks, compared to the previous experiment.

3.2.1. Color intensity (CI)

In general, color intensity decreased with incubation time and temperature for all the colorant types, except for both carrot colorants and the synthetic dye at 4°C, which revealed no significant color degradation -relative to their initial CI- throughout the 56-days incubation period (Figure 1B). Conversely, at this temperature, the grape colorant suffered significant color degradation after 14 days of incubation, retaining at the end of the experiment ~62% of its initial color. At higher temperatures (25°C and 40°C) all the colorants exhibited some degree of degradation, with the carrot colorants retaining at the end of the incubation 21-47% of their initial CI, and the grape colorant 19-27%. At these temperatures, the synthetic dye exhibited early and extensive color degradation, reaching relative CI values below 5% after 21 days of incubation at 25°C and 3 days of incubation at 40°C.

3.2.2. Changes in visual color attributes

At 4°C, the visual aspect of all the colorants remained virtually unaffected throughout the incubation period, with the exception of GRP which exhibited a moderate decrease in color intensity after 21 days of incubation (Supplementary Figure S6). At temperatures of 25°C and 40°C, color degradation was more evident and occurred faster, especially for E131 and GRP, which evidenced a change in color intensity and hue; from dark blue to pale red, in E131; and

from purple to light brown, in GRP. In contrast, both carrot colorants exhibited little variation in their color aspects at 25°C, with only a slight decay in color intensity observed at the end of the experiment; whereas at 40°C, INTA43 gradually reduced its color intensity after 21 days of incubation, and a change in hue (to light-brown) was observed in the last time-point, while Purple 68 exhibited partial decay of its color intensity, with no change in hue (Supplementary Figure S6).

As expected, the rate of change in the Hunter parameters increased with temperature (Supplementary Figure S7). In agreement with results from the visual analysis, these temperature-dependent changes occurred at a faster rate in the synthetic dye (E131) and the grape colorant than in both of the carrot colorants. Generally, L* increased, and C* decreased with time and temperature, whereas h° increased for the carrot colorants INTA43 and Purple 68 and decreased for GRP and E131.

3.2.3. Total phenolics, anthocyanins, and chlorogenic acid

Results of the spectrophotometric analyses of total anthocyanins, phenolics, and chlorogenic acid concentrations performed in the three natural colorant solutions (Purple 68, INTA43, and GRP) during incubation at different temperatures, are presented in Figure 5. These compounds represent major antioxidants in the carrot root and grape fruits. In general, for all the colorants, the concentration of these compounds decreased with incubation time and temperature. At 4°C, no degradation of anthocyanins was observed in both carrot colorants throughout the experiment, whereas GRP showed a moderate yet significant decrease (of ~17%) in anthocyanin levels after the first week of incubation. At 25°C, both carrot colorants exhibited less anthocyanin degradation than the grape colorant, with the formers retaining at the end of the incubation 55% (INTA43) to 76% (Purple 68) of their initial anthocyanin contents, while GRP retained ~21% of its initial content. At 40°C, more extensive anthocyanin

degradation was found for all colorant sources, although a more gradual decay was observed in the carrot colorants reaching ≤ 50% of its initial content after 35 days (in Purple 68) and 42 days (in INTA43) of incubation, whereas in GRP this concentration was reached after 21 days of incubation. However, at the end of the incubation period, all the colorants retained a small percentage of its initial anthocyanin content (6-13% in carrot, and 4% in grape). Similar data for total phenolics and chlorogenic acid contents are presented in Figure 5.

3.2.4. Concentration of individual anthocyanin pigments

Monitoring –by HPLC analysis- the concentration of individual anthocyanin pigments in colorant solutions of Purple 68 and INTA43 during incubation at different temperatures, revealed compound-, time-, and temperature-dependent degradation of carrot anthocyanins (Figure 6). Anthocyanin degradation increased with incubation time and temperature. AA suffered less degradation than NAA in both carrot colorants, although such differences were clearly evident at higher temperatures, of 25°C and 40°C. In decreasing order, the thermal stability of carrot anthocyanin pigments was Cy3XFGG > Cy3XCGG > Cy3XSGG > Cy3XG > Cy3XGG.

3.2.5. Antioxidant activity

In general, antioxidant activity by both methods (ABTS and DPPH) in the three natural colorant solutions decreased with incubation time and temperature (Figure 4B and Supplementary Figure S4B). For the three colorant sources, the decay in activity was more evident and significant ($p<0.001$) at the end of the incubation period and for the higher temperatures evaluated.

3.3. Relationships among color physicochemical properties, compounds concentrations, and antioxidant capacity

Results from correlation analysis among all the variables analyzed in the two carrot colorants are presented in Supplementary Table S4. In both Purple 68 and INTA43, color intensity was significantly and positively correlated with concentration of total anthocyanins (r=0.70-0.79), total phenolics (r=0.59), and the individual acylated anthocyanin pigments Cy3XFGG (r=0.67-0.80), Cy3XSGG (r=0.75-0.77), and Cy3XCGG (r=0.62). The acylated anthocyanins were, generally, also significantly correlated the color parameters L, C and h°. Conversely, color intensity of the solutions and the Hunter parameters were weakly or not significantly correlated with the non acylated pigments Cy3XG and Cy3XGG. Antioxidant capacity was significantly and strongly correlated ($r \geq 0.60$) with concentration of total anthocyanins, total phenolics, chlorogenic acid, and all five individual anthocyanin pigments, in both carrot accessions.

4. Discussion

The present work examined the effect of pH and temperature on the stability of colorant aqueous solutions of two purple carrot accessions (Purple 68 and INTA43), a commercial natural food dye from grape (GRP), and a commercial synthetic food dye (E131), using side-by-side comparative analyses of various color-related and health-related traits. To this end, physical-chemical properties (e.g., color intensity and Hunter parameters L*, C*, and h°) and visual assessment of color intensity and hue, as well as concentration of the actual pigments (i.e., concentration of total and individual anthocyanins) and other compounds influencing colorant stability and nutraceutical value (e.g., other phenolics and chlorogenic acid), and

antioxidant capacity, were monitored weekly in the colorant solutions during incubation at different pH and temperature conditions. Thus, this study represents the most comprehensive evaluation of carrot anthocyanin extracts as potential food colorants and nutraceutical agents, published to date.

We observed -visually and analytically- that the color of the purple carrot solutions was markedly influenced by the pH, changing from red (at pH 2.5) to purple (at pH 4.5) to blue (at pH 7.0) as pH increases (Supplementary Figure S2). These color variations are associated with different molecular structures of the anthocyanins at different pH values (Tang et al., 2019). At low pH, the flavylium cation predominates, contributing to the red color of the solutions (Cevallos-Casals & Cisneros-Zevallos, 2004). When pH increases, a rapid proton loss occurs, which leads to the formation of purple or blue quinonoidal species (Choi, Lee, Lacroix, & Han, 2017). These quinonoidal structures are rather unstable and, with time, anthocyanin solutions tend to become brown and yellow, as observed in the carrot colorants incubated at pH 4.5 and 7.0 (Supplementary Figure S2), due to the formation of chalcones (Cevallos-Casals & Cisneros-Zevallos, 2004). Conversely, the flavylium cation is highly stable, and therefore the carrot solutions at pH 2.5 maintained their bright red color throughout the entire incubation period. These results suggest that red-colored carrot anthocyanins (rich in flavylium ions) used as colorant in food products with low pH may confer the best results in terms of color stability. On the other hand, there is a great need in the food industry for natural and stable blue colorants, as these are extremely rare in nature (reviewed by Houghton et al., 2021). However, the use of carrot anthocyanins at approximately neutral pH values, which exhibit blue hues, is discouraged due to their relatively low stability under these conditions, unless they are used for very brief periods and at low temperature.

The fact that both of the carrot solutions were more stable in their color properties than the grape colorant, especially at low pH, may be attributed to the fact that acylated anthocyanins predominate in the carrot colorants, with acylated forms accounting for 83.2-

84.6% of the total carrot anthocyanins (Supplementary Table S1), whereas in grapes non-acylated anthocyanins are largely predominant (Oh et al., 2008). Previous studies have shown that acylation and –to some extent- glycosylation can increase anthocyanin chemical stability (Jing, Bomser, Schwartz, He, Magnuson, & Giusti, 2008; Prior & Wu, 2006). Coincidently with our results, purple carrot colorants were previously reported to have higher stability at low pH than colorants from purple corn, purple-flesh potato, and grapes; the latter species containing mainly non-acylated anthocyanins (Cevallos-Casals & Cisneros-Zevallos, 2004; Kırca et al., 2007). The chemical structures of the five carrot anthocyanins are shown in Supplementary Figure S8.

Concomitantly with variations described above for color attributes, we found significant changes in L*C*h° parameters (Supplementary Figure S3), suggesting pigment degradation and confirming the changes in the colorant solutions estimated visually. Thus, an increase in L* and decrease in C* values are associated with color fading, whereas approximation of the h° parameter to ~90° is associated with the formation of yellow chalcone species, derived from anthocyanin degradation (Kammerer et al., 2004; Reyes & Cisneros-Zevallos, 2007). These changes in Hunter color parameter were less evident at low pH, at which the anthocyanin-based colorant solutions of carrot and grape had higher stability. Similar results were found previously for anthocyanins derived from purple- and red-flesh potatoes (Reyes & Cisneros-Zevallos, 2007). Interestingly, the synthetic dye E131 had an opposite performance to that observed in the natural anthocyanin-based colorants, revealing high stability at higher pH values (i.e., pH 4.5 and 7.0), whereas at pH 2.5 color degradation and change in hue was visually evident (Supplementary Figure S2), and also reflected by the sudden drop in h° values (Supplementary Figure S3). According to Reyes & Cisneros-Zevallos (2007), the decrease of h° is also a sign of color fading.

Temperature had a strong and differential effect on the stability of the colorant solutions. At 4°C, both of the purple carrot colorants remained stable throughout the 56-days

incubation period, showing no significant change in visual appearance (Supplementary Figure S6), color intensity (Figure 1B), Hunter color parameters (L*, C*, and h°) (Supplementary Figure S6), and total anthocyanins concentration (Figure 5A). In contrast, the grape-based colorant exhibited significant color degradation, as indicated by its fainter color aspect, as observed visually (Supplementary Figure S6), and the significant decrease (of 37%) in color intensity (Figure 1B) and anthocyanins concentration (Figure 5A). At higher temperatures, although more extensive degradation was found in all the natural colorants, a similar comparative trend between the two carrot colorants and GRP was observed, with the carrot colorants suffering substantially less degradation than the latter (Supplementary Figures S5 and S6, and Figures 1B and 5A). The different stability observed between both colorant sources may be due to the different chemical structure of the anthocyanins present in carrot and grape. Purple carrots are generally rich in acylated anthocyanins, and these forms of pigments account for over 80% of the total anthocyanins content in both carrot accessions used in this study (Purple 68 and INTA43), whereas grapes have no acylated anthocyanins (Oh et al., 2008). At a given pH, the stability of the anthocyanin structure depends on various components of the macromolecule, such as the type of aglycone (Keith & Powers, 1965), the type of sugar moieties (Attoe & Von Elbe, 1981), the complexity of the sugar residue (Dyrby, Westergaard, & Stapelfeldt, 2001), and the presence of acylating agents (Bridle & Timberlake, 1997). In particular, the extent of acylation has been shown to drastically increase anthocyanin stability, with the following decreasing order of stability reported for these pigments: diacylated anthocyanins > monoacylated anthocyanins > non-acylated anthocyanins (Malien-Aubert, Dangles, & Amiot, 2001). Thus, the predominance of monoacylated anthocyanins in the carrot solutions may partially explain their higher color stability, as compared to GRP.

In addition, the presence of other phenolic compounds in the solution may increase anthocyanins stability by inter and intramolecular ‘copigmentation’ (Del Pozo-Insfran, Brenes, & Talcott, 2004). For intermolecular copigmentation, the copigment/pigment molar ratio in the

plant extract is important, with more efficient copigmentation expected with higher ratios (Malien-Aubert et al., 2001). However, in the natural colorant solutions, the ratio of 'total non-anthocyanin phenolics/total anthocyanins' (TNAP/TA) and of 'chlorogenic acid/total anthocyanins' (CA/TA) did not vary substantially between the carrot colorants (TNAP/TA=2.8-3.6; CA/TA=1.4-2.8) and GRP (TNAP/TA=3.7; CA/TA=1.4) (Supplementary Table S5), suggesting that this is not the main mechanism underlying the observed differences in color stability. Alternatively, they could be related to intramolecular copigmentation. In a previous study, Malien-Aubert et al. (2001) reported that colorants rich in acylated anthocyanins displayed greater stability than colorants with non-acylated anthocyanins, and proposed that such differences may be due to intramolecular copigmentation of the formers. According to the authors, the aromatic acyl groups of acylated anthocyanins stack on the flavylium nucleus and thereby protect the pyrylium ring from the nucleophilic addition of water, which leads to the formation colorless forms (Brouillard, 1982). Consequently, the concentration of colorless forms in acylated pigments is lower than for the corresponding non-acylated pigments. Altogether, these data suggest that the predominantly acylated nature of carrot anthocyanins may account for most of their higher pH and thermal stability than the grape anthocyanins, with copigmentation perhaps playing a secondary role in the observed differences.

The thermal stability of both carrot colorants, Purple 68 and INTA43, was equal to (at 4°C) or greater than (at 25°C and 40°C) that of the synthetic dye E131, as indicated by comparative analysis of color intensity (Figure 1B) and visual color appearance (Supplementary Figure S6) of their respective colorant solutions. Greater color stability for both carrot colorants relative to E131 was also found at acidic pH (2.5) (Figure 1A and Supplementary Figure S2). These results suggest that carrot anthocyanins can be used as natural colorant alternatives to replace some of the synthetic dyes currently used in the food industry, thereby avoiding the health risks associated with the consumption of the latter

(Arnold, Lofthouse, & Hurt, 2012; Houghton et al., 2021). Food products with low pH may be particularly suitable for replacing synthetic dyes with purple carrot colorants, as their anthocyanins are most stable under these conditions (Figures 2A and 3). The greater degradation rate of E131 observed at low pH and relatively higher temperatures (25-40°C) can be explained by the fact that, under these conditions, its structure is more susceptible to degradation by oxidation processes (Yoe & Boyd, 1939). The chemical structure of E131 is presented in Supplementary Figure S9.

The present study is the first one to track changes in the concentration of individual anthocyanins (by HPLC analysis) as a means for evaluating pH and thermal stability of carrot pigments, for their potential use as food colorants. In both carrot colorants, Cy3XFGG was the most stable anthocyanin compound, followed -in decreasing order- by Cy3XCGG, Cy3SGG, Cy3XG, and Cy3XGG (Figures 3 and 6). These data suggest that purple carrots with high concentration of anthocyanins (exclusively) acylated with feruloyl and p-coumaroyl would be ideal for the production of chemically stable food dyes. However, in almost all of the purple carrot cultivars studied to date, Cy3XFGG and Cy3XSGG are the two major forms of acylated anthocyanins present in the storage root, with Cy3XCGG usually being present at very low concentrations (Cavagnaro et al., 2014; Kammerer et al., 2004; Montilla et al., 2011). In carrot root, acylated anthocyanins are believed to be formed from Cy3XGG, which is acylated through transesterification –by the activity of an acyltransferase- using sinapoyl, feruloyl, or p-coumaroyl esters as donors, to form cyanidin-3-(2”-xylose-6”-sinapoyl-glucose-galactoside) (Cy3XSGG), cyanidin-3-(2”-xylose-6”-feruloyl-glucose-galactoside) (Cy3XFGG), or cyanidin-3-(2”-xylose-6”-(4-coumuroyl)glucose-galactoside) (Cy3XCGG), respectively (Algarra, Fernandes, Mateus, de Freitas, da Silva, & Casado, 2014). With the rapid discovery and characterization of the genes involved in carrot anthocyanin biosynthesis and decoration, including specific glycosyltransferases and acyltransferases responsible for anthocyanin glycosylation and acylation (reviewed by Iorizzo, Curaba, Pottorff, Ferruzzi, Simon, &

Cavagnaro, 2020), carrot breeding programs may soon be possible to develop cultivars accumulating, exclusively, Cy3XFGG or Cy3XCGG for the production of stable food dyes.

The different stability found among individual anthocyanin pigments may also explain general differences observed in the colorant stabilities of Purple 68 and INTA43. While at low pH and temperature (pH 2.5, 4°C) both carrot extracts exhibited similar and highly stable colorant solutions; at higher temperatures, Purple 68 suffered somewhat less degradation than INTA43, as noted by a more gradual decay in the former for visual color attributes, color intensity, and concentration of anthocyanins and other phenolics. Such moderate differences in stability may be related to their anthocyanin composition. In Purple 68 Cy3XFGG is the major pigment, representing 68% of the total anthocyanin content, followed by Cy3XG (15.4%) and Cy3XSGG (10.7%); whereas INTA43 accumulates Cy3XSGG as the predominant pigment, accounting for 64% of the total, followed by Cy3XFGG (19%) and Cy3XG (14%). Therefore, in Purple 68, the most stable compounds, Cy3XFGG and Cy3XCGG, account (combined) for 73.6% of the total anthocyanins, whereas in INTA43 they only represent 19% of the total anthocyanins (Supplementary Table S1). In addition, Purple 68 colorants doubled the chlorogenic acid content of INTA43, as well as the ratio of 'chlorogenic acid/total anthocyanins' (Supplementary Table S5), and thereby copigmentation may be differentially influencing the stability of the two carrot colorants, in favor of Purple 68. According to Dangles & Brouillard (1992) intermolecular copigmentation can be very efficient in the presence of this cinnamic acid, especially at low pH.

Important antioxidant activity was found in the natural colorants from carrot and grape, being highest in Purple 68; and a significant proportion on their activity remained at the end of the incubation period under the pH and temperature conditions tested, suggesting that purple carrot extracts can be used as both food colorants and preservatives. Regarding the latter, it was previously reported that purple carrot extracts were highly effective in controlling the development of rancidity in sunflower oil, as compared to a synthetic antioxidant (BHT)

(Assous, Abdel-Hady, & Medany, 2014). The higher antioxidant capacity of the Purple 68 colorant, compared to INTA43 and GRP, may be due to the more than 2-fold higher chlorogenic acid content in the former (Supplementary Table S5), as this phenolic compound is a potent antioxidant (Naveed et al., 2018). Interestingly, antioxidant capacity -expressed in absolute values- increased with pH at the beginning of the incubation period, for all the natural colorants (Supplementary Figure S5). Such increase is likely due to an increase in electron-donating ability upon deprotonation of phenols and their stabilization in alkaline solutions, leading to polymerization reactions. Such polymerization reactions of phenolic antioxidants can form new oxidizable $^{-}$OH moieties in their polymeric products, resulting in a higher radical scavenging activity (Altunkaya, Gökmen, & Skibsted, 2016).

Correlation analysis among all the variables analyzed in the purple carrot colorants revealed relationships among their color properties, compounds concentrations, and antioxidant activities. The significant and relatively strong correlation ($r \geq 0.62$) found between color intensity (CI) and concentration of all the acylated anthocyanins, but not between CI and the non-acylated anthocyanins, may be due to the higher concentration and stability of the formers in the colorant solutions. In addition, antioxidant capacity was strongly and positively correlated with concentration of previously-known potent antioxidant phenolics, such as anthocyanins and chlorogenic acid (Akhtar, Rauf, Imran, Qamar, Riaz, & Mubarak, 2017). The association of antioxidant capacity in carrot extracts and individual anthocyanin pigments has not been reported before.

In conclusion, the present study performed comparative stability analysis of different color components (i.e., color intensity, color space parameters, and concentration of total and individual anthocyanin pigments), antioxidant capacity, and phenolics content in extracts of two genetically-unrelated purple carrot genotypes, a synthetic dye (E131) and a natural food colorant (GRP). In general, our results strongly suggest that pH and temperature management procedures are important for the maintenance of color stability, phenolics content, and

antioxidant activity in purple carrot –and other natural- anthocyanin-based colorants. Carrot anthocyanins exhibited greater stability to high temperatures and changes in pH than anthocyanin from GRP. Furthermore, our data suggest that carrot anthocyanins can be used as natural colorant alternatives to replace some of the synthetic dyes currently used in foods with low pH (e.g., yogurt, beverages, etc.), specially under refrigerated or ambient temperatures. The comparably-higher stability of the carrot colorants is likely due to their richness in acylated anthocyanins, particularly those with feruloyl and coumaroyl glycosides, as these were the most stable pigments. Copigmentation with other phenolics may also contribute -to a lesser extent- to color stability in the carrot extracts. Altogether, these data are relevant for carrot breeders developing suitable cultivars for the production of chemically-stable food dyes, as well as for food technologists and consumers aiming at replacing synthetic dyes with natural food colorants with antioxidant properties.

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Figure captions

Figure 1. Time-course variation for color intensity (CI), expressed as percentage (%) relative to the CI in the initial time, of colorant solutions incubated at different pH values (A) and temperatures (B). Colorant solutions were prepared from two purple carrots lines (Purple 68 and INTA43), a grape-based natural colorant (GRP), and a synthetic colorant (E131). Asterisks indicate significant changes ($p<0.05$) with respect to the initial time (day 0).

Figure 2. Time-course variation for relative anthocyanins (A) and total phenolics (B) content (%) in colorant aqueous solutions of Purple 68, INTA43, and GRP with different pH values incubated in the dark for 42 days. Asterisks indicate significant changes with respect to the initial time.

Figure 3. Time-course variation for concentration of individual carrot anthocyanin pigments in colorant solutions of Purple 68 (A) and INTA43 (B) with pH 2.5, 4.5, and 7.0. Asterisks indicate significant changes with respect to the initial time. Cy3XG: Cyanidin-3-(2″-xylose-galactoside). Cy3XGG: Cyanidin-3-(2″-xylose-6-glucose-galactoside). Cy3XFGG: Cyanidin-3-(2″-xylose-6″-feruloyl-glucose-galactoside). Cy3XSGG: Cyanidin-3-(2″-xylose-6″-sinapoyl-glucose-galactoside); Cy3XCGG.Cyanidin-3-(2″-xylose-6″-(4-coumaroyl)glucose-galactoside).

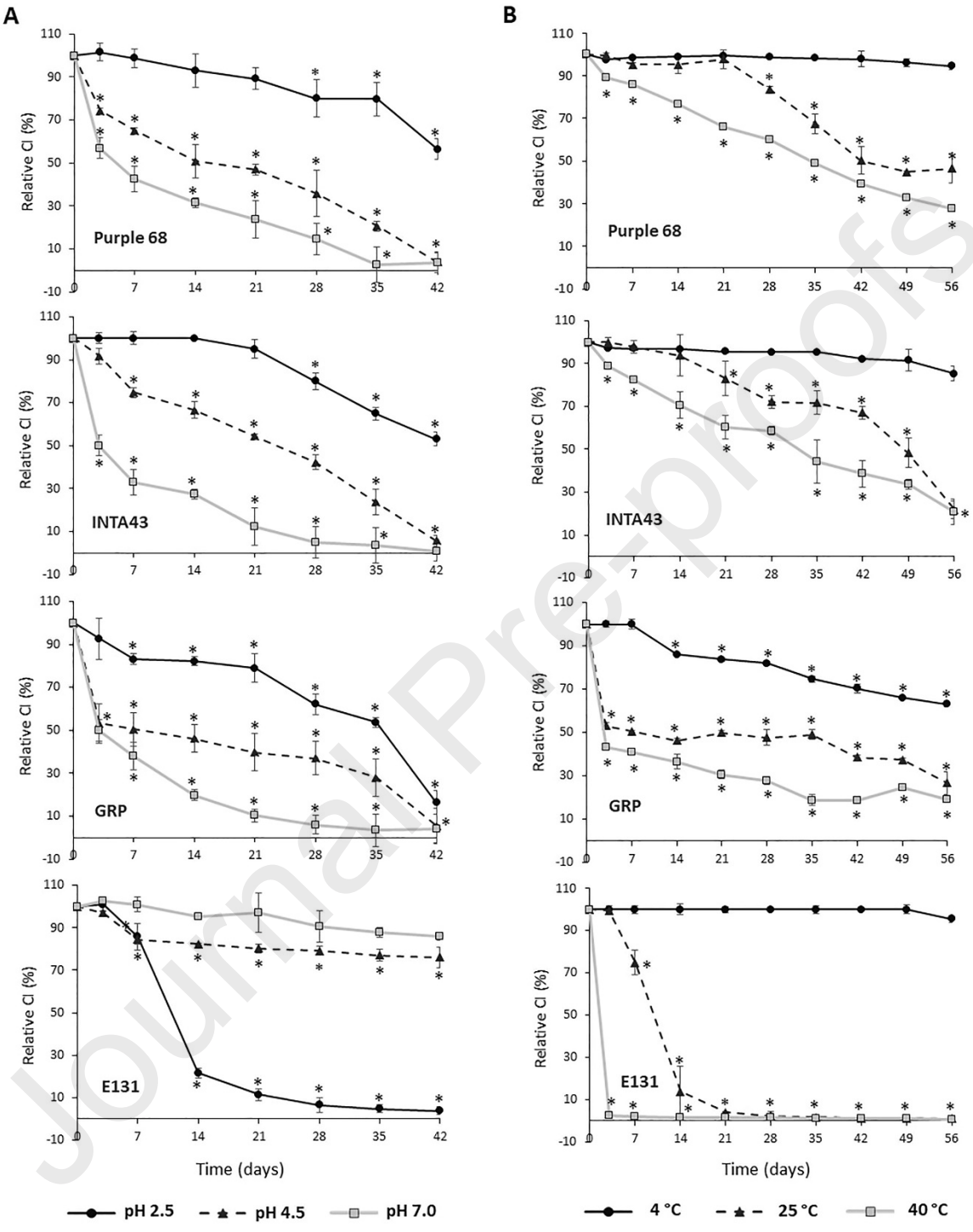
Figure 4. Time-course variation for antioxidant capacity by ABTS, expressed as percentage (%) relative to the antioxidant capacity in the initial time, of colorant solutions incubated at different pH values (A) and temperatures (B). Colorant solutions were prepared from two purple carrots lines (Purple 68 and INTA43) and a grape-based natural colorant (GRP).

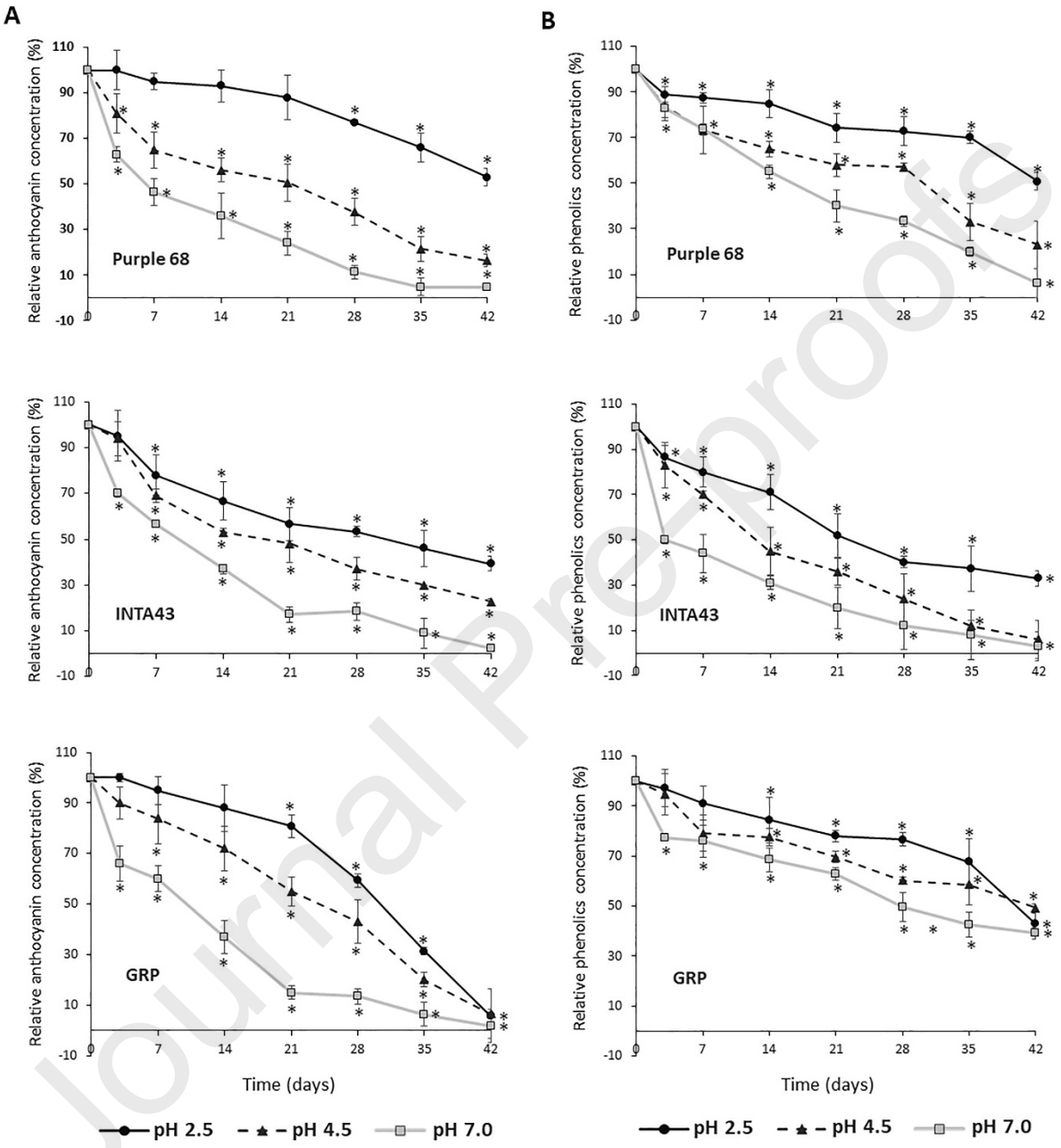
Figure 5. Time-course variation for total anthocyanins (A), phenolics (B), and chlorogenic acid content (C), expressed as percentage (%) relative to the initial content, of colorants solutions (pH =2.5) of Purple 68, INTA43, and GRP, incubated at 4°C, 25°C, and 40°C for 56 days. Asterisks indicate significant changes with respect to the initial time.

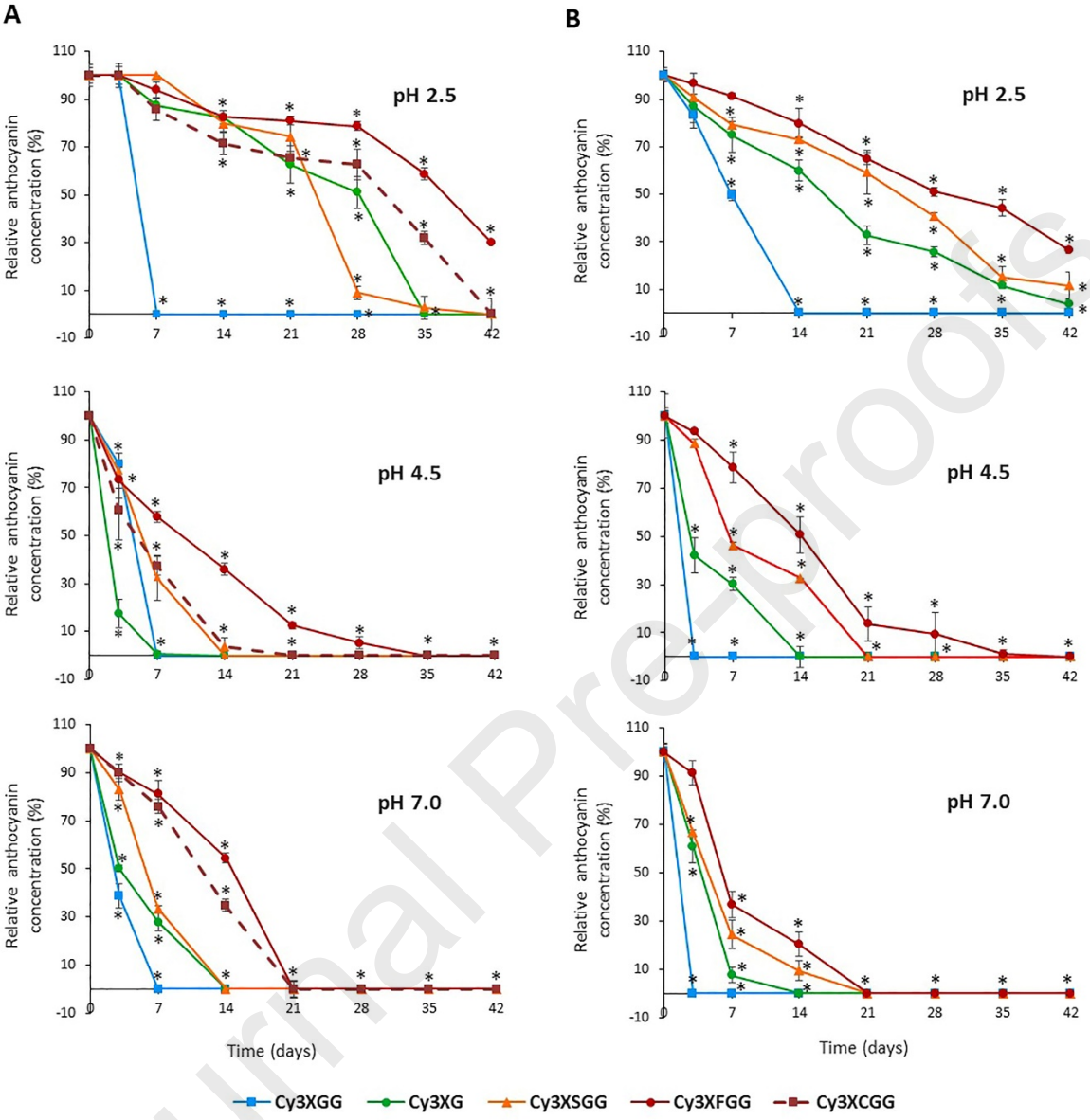
Figure 6. Time-course variation for concentration of individual carrot anthocyanin pigments, expressed as percentage (%) relative to the initial content, in colorant solutions of Purple 68 (A) and INTA43 (B) incubated at 4°C, 25°C, and 40°C for 56 days. Asterisks indicate significant changes with respect to the initial time. Cy3XG: Cyanidin-3-(2″-xylose-galactoside). Cy3XGG: Cyanidin-3-(2″-xylose-6-glucose-galactoside). Cy3XFGG: Cyanidin-3-(2″-xylose-6″-feruloyl-glucose-galactoside). Cy3XSGG: Cyanidin-3-(2″-xylose-6″-sinapoyl-glucose-galactoside); Cy3XCGG.Cyanidin-3-(2″-xylose-6″-(4-coumaroyl)glucose-galactoside).

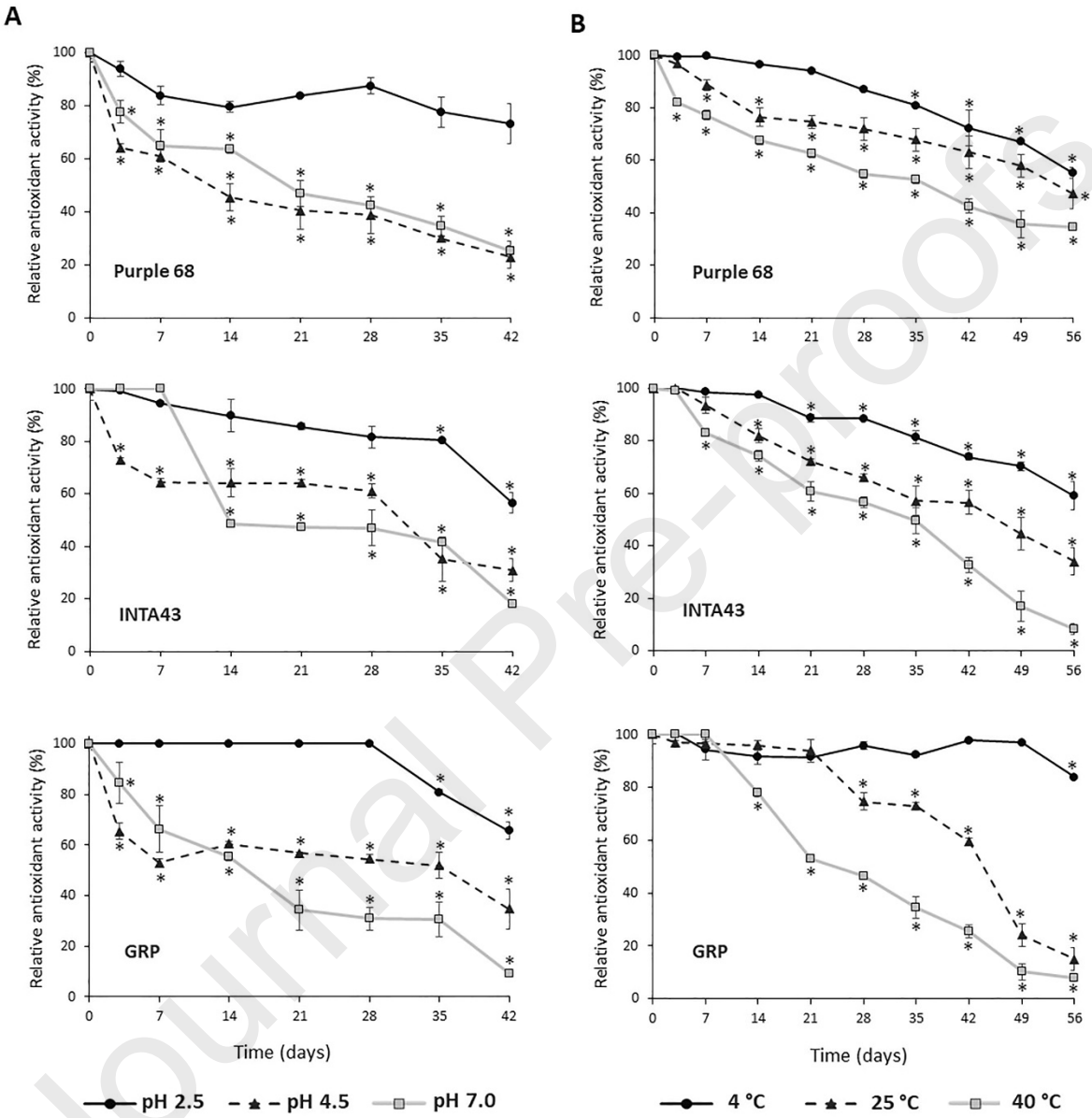
CRediT authorship contribution statement

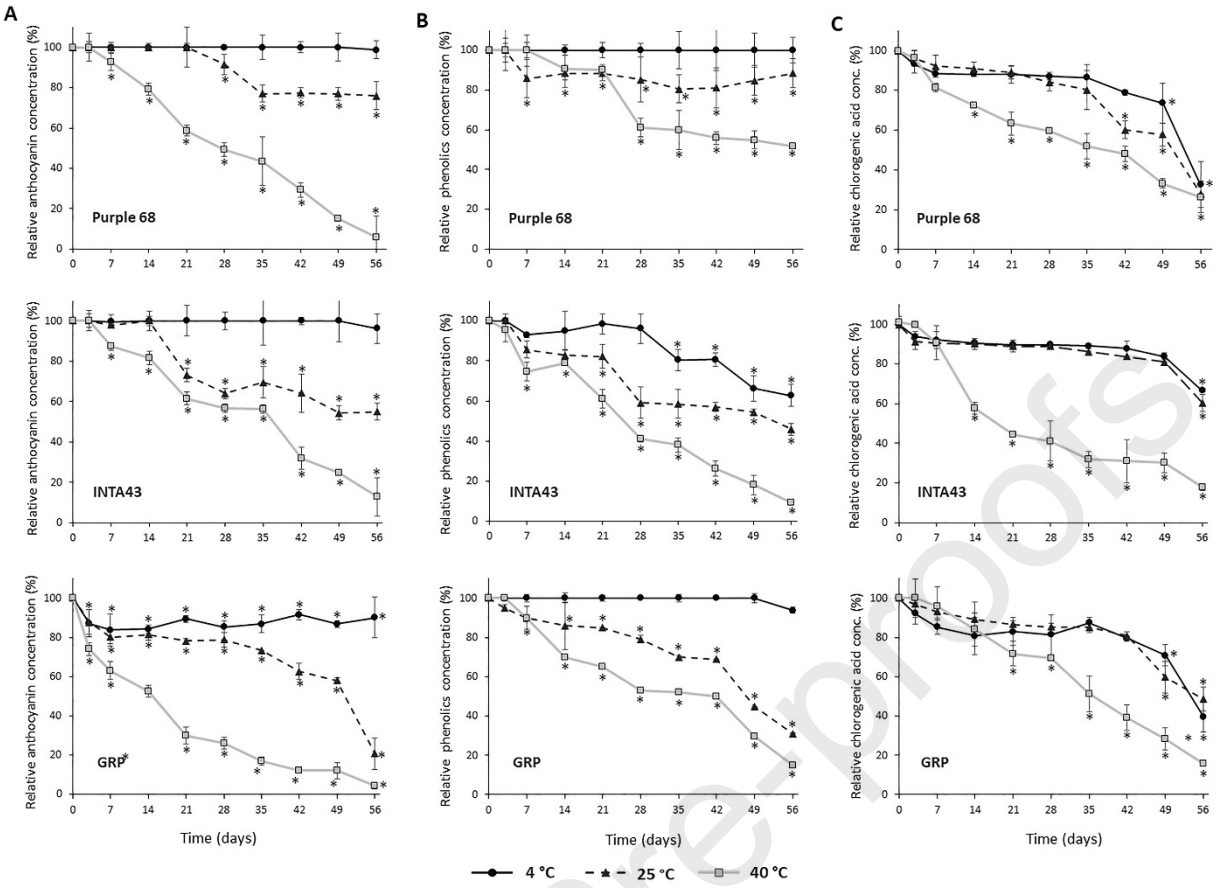
María B. Perez: Conceptualization, Investigation, Methodology, Validation, Writing - Preparation of original draft and final manuscript. **Da Peña Hamparsomian:** Methodology, Validation. **Roxana E. Gonzalez:** Methodology, Validation. **Gabriela I. Denoya**: Conceptualization, methodology. **Deolindo L. E. Dominguez:** Methodology. **Karina Barboza:** Methodology. **Massimo Iorizzo**: Writing - Review & Editing. **Philipp W. Simon:** Writing - Review & Editing. **Sergio R. Vaudagna**: Conceptualization, Writing - Review & Editing. **Pablo F. Cavagnaro:** Conceptualization, Writing - Review & Editing, Supervision, Project administration, Funding acquisition.

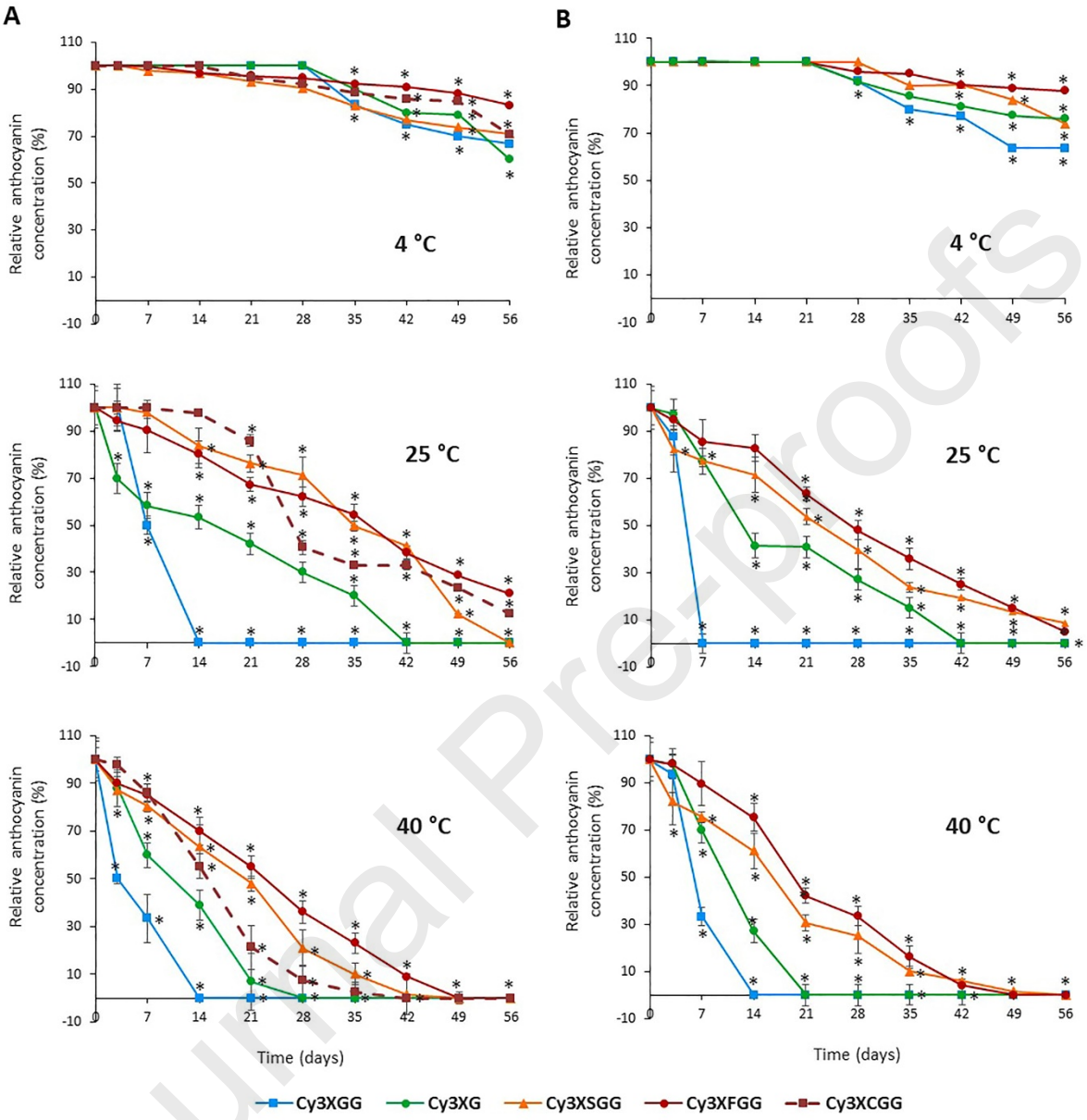












Highlights

- We evaluated carrot anthocyanins as food colorants and antioxidant agents
- Physicochemical stability analyzed in carrot, grape (GRP), and synthetic (E131) colorants
- Carrot colorants had higher thermal stability than GRP and E131

- Carrot anthocyanins were more stable at low pH and temperature conditions
- Acylated anthocyanins were more stable than non-acylated anthocyanins