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Application of betacyanins pigments from *Alternanthera brasiliana* as yogurt colorant

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ABSTRACT

Betacyanins are betalain derivatives showing a red-violet color suitable as a natural food colorant. An aqueous extract rich in betacyanin pigments and phenolic compounds from *Alternanthera brasiliana* was characterized using a high-performance liquid chromatography system with diode array and electrospray mass spectrometry detection. The major betacyanins (amaranthine, isoamaranthine, betanin and isobetanin, celosianin II and isocelosianin II) were identified and quantified. Eleven peaks were analyzed for phenolic compounds grouped into hydroxycinnamic acids and flavones. Different plant extract concentrations were added to freshly prepared set type yogurts (350, 700, and 1400 μL/80 g yogurt). Color properties in CIELAB space, pH, total phenolic content, and ferric reducing antioxidant power were analyzed on yogurts during storage time. The evolution of pigments and polyphenolic compounds during cold storage was studied by HPLC-ESI-QTOF-MS screening.

The extracted color showed to be stable at the yogurt pH range. When added to yogurts, a natural berry-like color was achieved. These results provide a natural plant pigment-based alternative for application in food products, being the first time that *A. brasiliana* is used as a colorant in a dairy product. Besides color, polyphenols input turns *Alternanthera brasiliana* extract into a desirable functional food ingredient.

1. Introduction

The recent trends in food production and consumption have spurred the search of novel food additives, either with consumer appeal, hedonic purposes (colorants, flavors), with capacity for food characteristics modification of preservation (antimicrobials), or with functional properties (antioxidant and prebiotic) (Ahmadiani et al., 2014).

Betalains are a "natural" alternative to the plant kingdom's ubiquitous anthocyanins. They can also be considered as a substitute when used as food colorants by the food industry (Davies, 2015; Tennant & Klingenberg, 2016). Both pigments' stability depends strongly on processing and storage conditions, namely, pH and temperature (Fischer et al., 2013; Kamiloglu et al., 2015).

Betalains are found in diverse plant sources (flowers, fruits, roots, leaves, stalks, seeds, grains) and are obtained from numerous species, although most are not grown for food purposes. The major commercial source of betalain colorants is the red beet crop (*Beta vulgaris*) (Strack et al., 2003). Betanin was approved as a red food colorant (EEC No. E162) by the European Union (Downham & Collins, 2000) and the USA Food and Drug Administration (Section 73.40, chapter 21, Code of Federal Regulations-CFR) (Griffith, 2005).

Betalains of different origins are currently being exploited and investigated for coloring of food, especially in the dairy and beverage sector, including prickly (cactus) pear, very popular in food research (Cejudo-Bastante et al., 2015; Moßhammer et al., 2005; Stintzing et al., 2003; Tesoriere et al., 2005, 2008) and red-purple pitaya (dragon fruit) (Herbach et al., 2007; Stintzing et al., 2002). Betalains are also appreciated for their natural antioxidant properties (Cai et al., 2005a; Escribano et al., 1998; Ravichandran et al., 2013; Zhang et al., 2014). Moreover, it has been shown *in vitro* that prickly pear betaxanthins are bio-accessible in post-intestinal digesta, retaining their antioxidant potential (Tesoriere et al., 2008). Betalains would protect the endothelium

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from oxidative stress-related diseases, such as inflammations (Gentile et al., 2004). It has also been shown, *in vitro*, that betalains have a promising potential for the prevention of cancer (Khan et al., 2012; Schwartz et al., 1983) and cardiovascular diseases (Delgado-Vargas et al., 2000).

Alternanthera brasiliana Kuntze (Amaranthaceae) is a perennial herbaceous plant, native to tropical and subtropical regions of Australia and South America (Kissmann & Groth, 1999), which grows easily on poor and deforested soil and has been traditionally used in popular medicine. Its morphology, anatomy, and extractable chemical compounds (betalains, polyphenolic compounds and triterpenoids) have been studied previously (Brochado et al., 2003; Deladino et al., 2017; Delaporte et al., 2001; Duarte & Debur, 2004; Macedo et al., 1999; Pereira et al., 2008; Reis et al., 2015).

Various reports on crude extracts, fractions, or isolated compounds of this plant, include antibiotic, antiviral, antioxidant, antimicrobial, insecticidal properties (Delaporte et al., 2001; Lagrota et al., 1994; Pereira et al., 2007), and the presence of therapeutically important secondary metabolites (Chandran, 2017). However, its potential application as a natural dye and source of antioxidants for the food industry is underexploited.

Green Chemistry and natural products global trend will favor natural pigments over synthetic colors. Accordingly, the aim of this work was to evaluate the potential of an aqueous leaf extract of *A. brasiliana* in set type yogurts, usually colored with synthetic food dyes, obtaining an added-value product. The effect of *A. brasiliana* extract concentration on yogurt color, antioxidant activity, and pH was monitored in refrigerated storage, assessing its behavior as a food colorant. The evolution of pigments and polyphenolic compounds was studied by HPLC-ESI-QTOF-MS screening.

2. Materials and methods

2.1. Plant material

Assays were carried out with material maintained in a greenhouse in the "Centro de Educação Ambiental Ernest Sarlet" (Lomba Grande, Novo Hamburgo, Brazil). Fresh leaves of *Alternanthera brasiliana*, of at least a month growth and approximately 70–80 mm long, were collected. Samples were cut apart from the plant with pruning shears and then cut in strips of approximately 2 mm. Then, they were dried using a forced air oven (GMX 9203A PEET LAB, USA) for 5 days at 35 °C, mimicking the traditional drying procedures of Brazilian agricultural communities.

2.2. Extract preparation and characterization

The vegetal material (10 g of leaves, dried weight (dw)) was extracted with 20 mL of mili Q water in an ultra-turrax T25 homogenizer (Janke & Kunkel, IKA-Labortechnik, Germany) at room temperature during 60 s. The supernatant was filtered through a 0.45 μm filter membrane (Millipore, Bedford, MA, USA). The filtrate was immediately characterized for betalain and polyphenol content and added to yogurt as described below.

2.3. Preparation of yogurts

Two batches of yogurt were prepared in a 100 mL glass container using reconstituted whole milk powder (15% w/w), 5% sucrose, and 80% of distilled water. Mixes were homogenized and heated to 85 °C for 30 min, cooled to ambient temperature and inoculated with 0.03% starter culture. The starter was a 1:1 mixture of *Streptococcus thermophilus* (Cp2, CIDCA collection 321) and *Lactobacillus delbrueckii* subsp. bulgaricus (Lbp, CIDCA collection 332) (Dello Staffolo et al., 2011). Samples were incubated at 43 °C in a forced air oven, to gelling pH (Ghafarloo et al., 2020), and stored at 7 ± 2 °C for 21 days.

A. brasiliana extracts were added to freshly prepared yogurts, at three different concentrations (350, 700 and 1400 μL/80 g yogurt), and thoroughly mixed.

The effect of extract concentration and storage time (0, 7, 14 and 21 days) on color parameters and pH was analyzed directly on yogurts, whereas for phytochemical analysis, yogurt was mixed with water and centrifuged (Jouki et al., 2021). Total phenolic content, ferric reducing antioxidant power and liquid chromatography were performed on clear supernatants.

2.4. Total phenolic content (TPC)

Total phenolic content was determined by Folin-Ciocalteau method (Jouki et al., 2021). Two mL of Na_2CO_3 (2 g/100 mL) (Anedra, Argentina) were mixed with 200 μL of the sample and 200 μL of Folin-Ciocalteu phenol reagent (Anedra, Argentina, 1:1 diluted). After 30 min, sample absorbance was measured at 725 nm in a spectrophotometer (Shimadzu, UV-mini 1240, Japan). Based on the curve equation of gallic acid standard (Sigma-Aldrich, USA, 0.05–0.9 mmol/L) the total phenolic content was expressed as mg of gallic acid equivalents (GAE) per gram of yogurt.

2.5. Ferric reducing antioxidant power (FRAP)

The assay was determined according to Hashemi et al. (2021). Briefly, the FRAP reagent was prepared by mixing 25 mL of 0.3 mmol/L acetate buffer (pH 3.6) with 2.5 mL 10 mmol/L TPTZ in 40 mM HCl and 2.5 mL 20 mM $FeCl_3$ at a ratio of 10:1:1 (v/v/v). For reaction, 50 μL of sample were mixed with 1.5 mL of freshly prepared reagent in a cuvette and incubated at room temperature for 30 min, after which the absorbance was read at 593 nm against the reagent blank. Based on the curve equation of Fe^{2+} ($FeSO_4.7H_2O$, Anedra, Argentina, 0.1–1.2 μmol Fe^{2+}/mL) results were expressed as μmol Fe^{+2} per g of yogurt.

2.6. Color extract stability along the pH scale

The effect of pH on color stability of *Alternanthera brasiliana* extract was determined qualitatively by preparing a pH scale with 13 tubes, according to Chang and Goldsby (2013). 5 mL of a 0.1 mol/L HCL solution (Sigma, USA) were placed in tube 1, 4.5 mL of distilled water were placed in each tube, from 2 to 12.5 mL of a 0.1 mol/L NaOH solution (Sigma, USA) were placed in tube 13. Subsequent dilutions were then prepared by taking 0.5 mL of tube 1 and transferring to tube 2, repeating this operation to tube 6, thoroughly mixing each tube before transferring to the next. On the other hand, successive dilutions were assembled, transferring 0.5 mL from tube 13 to tube 12, repeating this operation to tube 8. Tube 7 was left only with distilled water. Thus tube 1 has pH = 1, tube 2 pH = 2 and respectively until tube 13. Finally, 0.5 mL of extract was added to each tube. The pH scale colors obtained were qualitatively observed during 21 days of refrigerated storage.

2.7. Surface color stability and pH measurements in yogurts

pH values were monitored using a pH meter (EC-30 Hach, Broadley James Corporation, USA) during storage time. The surface color information was determined with a colorimeter (Minolta colorimeter, CR-400 Tokyo, Japan), to evaluate the evolution during cold storage of color quality of yogurts with different extract concentrations. The pH and color measurements were performed at room temperature. The pH of the yogurts was measured after performing a 2-point calibration. The chromameter was calibrated with a white porcelain reference plate ($L^* = 99.88$, $a^* = 0.01$, $b^* = 0.04$) (Alipoorfard et al., 2020). The evaluation of pigment performance was measured every seven days, in terms of the L^*, a^*, and b^* parameters of the CIE (Commission Internationale de l'Eclairage). L^* represents luminance or lightness. a^* and b^* chromatic components represent colors from green to red, and blue to yellow, respectively. L^* a^* b^* color space is the most used system in measuring

the color in foods due to the uniform distribution of colors and because it is very close to human perception of color (Lee et al., 2020). These color parameters were utilized to study pH-color relationships and calculate the total color differences (ΔE, equation (1)) of yogurts during refrigerated storage. In this equation, L_0*, a_0* and b_0* are values at zero time and L_t*, a_t* and b_t* are the values at final storage time.

$$\Delta E = \sqrt{\left[(L_t - L_o)^2 + (a_t - a_o)^2 + (b_t - b_o)^2\right]} \quad (1)$$

2.8. Liquid chromatography analysis and mass spectrometry conditions

Aliquots of 5 μL for the extract and 20 μL of supernatants from yogurt samples were injected in the HPLC-DAD-MS equipment. Analysis was performed on an Agilent 1200 series HPLC (Agilent Technologies, Waldbroon, Germany), comprised of a quaternary pump (G1311A) with integrated degasser (G1322A), an autosampler (G1367B), a thermostated column compartment (G1316A) a diode array detector (DAD) (G1315B) and a hybrid mass spectrometer quadrupole-time of flight via an electrospray ionization source (ESI) with JetStream technology (Agilent Accurate Mass QTOF LC-MS, Waldbronn, Germany) in series in the same chromatographic line.

The chromatographic separation was carried out in a 150 mm × 4.6 mm i.d., 5 μm, C18 Agilent Zorbax Eclipse XDB-C18 analytical column, eluted with a mobile phase made up of a mixture of deionized water (solvent A) and acetonitrile (solvent B), both acidified with 0.1% formic acid. Solvent gradient for positive and negative mode was: 70% (A) and 30% (B), from 30 min; 100% (B), from 30 to 40 min; 95% (A) and 5% (B), from 40 to 45 min and 95% (A) and 5% (B), from 45 to 50 min at a flow rate of 0.8 mL/min. The compounds were monitored at 280 nm (phenolic compounds), 360 nm (flavonoids), 520 and 430 nm (flavonols) and 538 nm (betalains), while mass spectra were acquired with electrospray ionization and the TOF mass analyzer in both positive (betalains) and negative (polyphenols) mode over the range m/z: 100–1000. Ultrahigh pure nitrogen was used as the collision gas and high-purity nitrogen as the nebulizing gas. The capillary voltage was set at 3500 V (negative and positive mode) and fragmented, 100 V. The ESI Jetstream nitrogen pressure and flow rate on the nebulizer were 45 psi and 10 L/min, respectively, with a drying gas temperature of 350 °C; sheath gas temperature, 350 °C; sheath gas flow, 11 L/min; and MS/MS collision energies were set at 20 V. Samples were analyzed in duplicate.

The MS and MS/MS data were processed through Masshunter data Acquisition and Masshunter Qualitative Analysis software (version B.05.01 and B. 07.00 respectively, Agilent Technologies, Waldbronn, Germany) which provides a list of possible elemental molecular formulas by using the Generate Molecular Formula™ editor according to the accurate masses and isotopic pattern. Besides the observed MS and MS/MS spectra and data obtained by QTOF-MS analysis, the main tools for betalains and phenolic compound identification were the interpretation of the observed MS/MS spectra in comparison with those found in the literature and the online database Phenol-Explorer (Rothwell et al., 2012) and also the comparison of chromatographic behavior, DAD (UV–vis) data [280 nm (polyphenols) and 538 nm (betacyanins)] and mass spectral data generated by authentic standards (betanin, caffeic acid, caftaric acid, coumaric acid, ferulic acid, vitexin, 2-O-rhamnosylvitexin purchased from Sigma (St. Louis, MO, USA)) and/or related structural compounds. Quantitative data for compounds, derivatives or related compounds were obtained by calibration curves of related structural compounds.

2.9. Statistical analysis

The experiment was performed following a bifactorial design with the extract concentration (350, 700 or 1400 μL/80 g yogurt) and the storage time (0, 7, 14 and 21 days) as the factors, and three batches of each combination were executed to analyze the interaction effect. ANOVA was conducted to evaluate the influence of variables. Fisher test was used for mean comparison. Pearson correlation analysis was carried out to appraise correlations between color parameters and yogurt pH. These statistical methods were applied using the software Systat 12.0 (Systat Inc., Evanston, IL, USA).

3. Results and discussion

3.1. Separation and characterization of betalain in the extract

The tentative structural identification of each compound found in *A. brasiliana* extract is shown in Table 1 and was carried out based on their retention time, accurate mass, molecular formula, and MS/MS fragmentation ESI spectra. Also, by comparing their chromatographic and mass spectra characteristics with a betanin standard and data found in the literature, including a previous work (Cai et al., 2005b; Deladino et al., 2017; Jerz et al., 2014; Li et al., 2015).

Betalains are relatively rare in nature, when compared to the omnipresent anthocyanin and carotenoid pigments. Within the Caryophyllales order, betalains occur in a mutually exclusive fashion with anthocyanins, standing out that no plant has been found to naturally synthesize both types of pigments (Aguirre-Joya et al., 2020; Corrêa et al., 2019). This is the case of *A. brasiliana*, which contains exclusively betalains pigments, while only the subgroup of betacyanins was found with the extraction method used in the present work.

The aqueous extract showed the following descending concentration order: amaranthine > isoamaranthine > betanin ~ celosianin II > isobetaniñ isocelosianin II, with a total betacyanin content of 4.6 mg/g (dw). The same decreasing order has been found in a previous work, when studying extracts of *A. brasiliana* leaves obtained by maceration in acidic media (Deladino et al., 2017).

However, celosianin II (m/z 903.2308) and its isomer were only found in the present extract. This could be attributed to the higher polarity of the extraction solvent and the partial cell rupture by ultra-turrax processing that assists the betacyanins to be driven out from cell walls. Cai et al. (2001, 2005b) found these acylated betacyanins when studying methanolic extracts of *Amaranthus* plants, the highest content being present in *Iresine herbstii* (80% of the total peak area), *Gomphrena globosa* (68%), and *Celosia cristata* (40%). It is worth mentioning that these authors found a total betacyanin content ranging from 0.08 to 1.36 mg/g fresh weight, values very close to those found at the present work which was 0.7 mg of betacyanin per g of fresh weight (water content in the fresh plant material was 85%). Hasli et al. (2019), found a lower amount of betacyanins (0.03 mg (betanin + amaranthin)/g extract in fresh weight) for their aqueous extract of *Alternanthera sesillis* (red).

Betacyanins can be further classified by their chemical structures into four kinds: betanin, amaranthin, gomphrenin and bougainvillein-type (Strack et al., 1993). Hence, the betacyanins found in this extract can be all associated with the amaranthin-type.

3.2. Separation and characterization of phenolic compounds in the extract

Looking forward to the objective of monitoring the compound's evolution during yogurt storage, only the most abundant phenolic compounds were identified in the extract, based on the same criteria as for pigments. However, MS/MS fragmentation was performed in negative ion ESI spectra. The phenolic compounds identified are summarized in Table 2, where the compounds abbreviations used in this manuscript are also listed. Eleven peaks were analyzed and grouped, seven into hydroxycinnamic acids and four into flavones. Among the hidroxycinnamic acids, CTA and CMA were quantified as caffeic acid (0.9 mg/g of the dry weight of leaves), FTA and FMA were quantified as ferulic acid (0.9 mg/g dw) and pCMA were quantified as cumaric acid (0.7 mg/g dw). Whereas the flavones VIC2, AP, and VIT were all quantified as vitexin with a total content of 1.9 mg/g dw, RV was quantified from its standard (1.7 mg/g dw).

Table 1
Identification of betacyanins of A. *brasiliana* from leaves extracts by HPLC-ESI-QTOF-MS.

Proposed Compounds	N°	$T_R$min	Formula	Score[a]	$(M+H)^+$	MS/MS	Id[b]	Ref.[c]
Betacyanins								
Amaranthine (Betanidin-5-*O*-β-glucuronosylglucoside)	1	3.9	$C_{30}H_{34}O_{19}N_2$	90.4	727.1829	551(5)389(100)	2	d
Isoamaranthine (Isobetanidin-5-*O*-β-glucuronosylglucoside)	2	5.29	$C_{30}H_{34}O_{19}N_2$	83.2	727.1829	551(5)389(100)	2	d
Betanin (Betanidin-5-*O*-β-glucoside)	3	5.87	$C_{24}H_{26}O_{13}N_2$	96.6	551.1508	389(100)	1	d
Isobetanin (Betanidin-5-*O*-β-glucoside)	4	7.34	$C_{24}H_{26}O_{13}N_2$	95.2	551.1508	389(100)	2	d
Celosianin II (Feruloyl-amaranthine)	5	10.25	$C_{40}H_{42}O_{22}N_2$	96.7	903.2302	389 (100)	2	e
Isocelosianin II (Feruloyl-amaranthine)	6	11.07	$C_{40}H_{42}O_{22}N_2$	97.3	903.2302	389(100)	2	e

[a] Percentage of proximity of the molecular formula generated by Masshunter software with the exact mass and the isotopic distribution.
[b] **Id**: (1) Identification by comparison with authentic standards and databases and (2) identification by comparison with literature data and databases (2).
[c] **Ref**. Confirmed with reference.
[d] Confirmed with references Cai et al. (2005b) and Li et al. (2015).
[e] Confirmed with reference Jerz et al. (2014).

Table 2
Identification of phenolic compounds in A. *brasiliana* leaves extract by HPLC-ESI-QTOF-MS.

Proposed Compounds	Abbr.	T_R (min)	Formula	Score[a]	$(M-H)^-$	MS/MS[b]	Id[c]	Ref.[d]
Hydroxycinnamic acids								
Caffeoyl tartaric acid	CTA	10.8	$C_{13}H_{12}O_9$	**98.1**	311.0400	149(100) 149(34)135(68)179(100)	2	[e,f]
		11.6		**97.3**			2	[e,f]
Caffeoyl malic acid	CMA	15.8	$C_{13}H_{12}O_8$	**99.9**	295.0400	115(54)133(100)179(17)	2	f
Feruloyl tartaric acid	FTA	16.5	$C_{14}H_{14}O_9$	**89.5**	325.0586	135(40)193(100)	2	g
p-Coumaroyl malic acid	pCMA	19.4	$C_{13}H_{12}O_7$	**95.4**	279.0526	119(100)163(77)	2	h
		20.0		**95.3**		119(100)163(80)	2	h
Feruloyl malic acid I	FMA	21.00	$C_{14}H_{14}O_8$	**90.7**	309.0600	134(100)193(85)	2	h
Flavones								
Apigenin 6-8 di glucoside (vicenin II)	VIC II	17.9	$C_{27}H_{30}O_{15}$	**93.2**	593.1519	593(100)473(20)429(15)	2	i
2″-*O*-Rhamnopyranosyl-vitexin	RV	19.6	$C_{27}H_{30}O_{14}$		577.1563	413(100)293(37)	1	
2″-*O*-Pentosil-6-*C*-hexosyl-apigenin (2″-*O*-Pentosyl-isovitexin)	AP	19.7	$C_{26}H_{28}O_{14}$	**97.9**	563.1406	413(100)293(24)	2	e
Vitexin (apigenin-8-C-glucoside)	VIT	19.87	$C_{21}H_{20}O_{10}$		431.0984	473(100)311(53)	1	

[a] Percentage of proximity of the molecular formula generated by Masshunter software with the exact mass and the isotopic distribution.
[b] Identification with MS fragmentation of the standard and database.
[c] **Id**: Identification by comparison with authentic standard (1) and with references (2).
[d] Confirmed with reference.
[e] Confirmed with reference Santos et al. (2014).
[f] Confirmed with reference Viacava et al. (2017).
[g] Confirmed with reference Contreras et al. (2018).
[h] Confirmed with reference Lu et al. (2019).
[i] Confirmed with reference Simirgiotis et al. (2013).

In previous work, an acetonitrile: water acidified with 0.1% formic acid (1:1, v/v) extract was obtained and characterized (Deladino et al., 2017). In this case, flavonols (kaempferol, quercetin, rutin and its derivatives) were the major phenolic compounds identified with a total content of 34 mg polyphenols/g dw, including hydroxybenzoic and hydroxycinnamic acids, flavones and flavonols. The aqueous mechanic extraction performed in the present work led to an extract rich in hydroxycinnamic acids and flavones (6 mg/g dw). RV content was 10-fold in the aqueous extract whereas VIT and AP were 50-fold in the present extract concerning the acetonitrile: acidified water extract. Nevertheless, TPC content in extract was 2.40 ± 0.01 mg GAE/g dw, these values are in tune with the scarce data found for *Alternanthera* species, i.e., Halsi et al. (2019) found close TPC values for *Alternathera sesillis* (1.66 mg GAE/g dw) using water as solvent.

3.3. Color stability of extract at different pH during refrigerated storage

Fig. 1 shows the raw extract color under the whole pH scale at t = 0 and t = 21 days of refrigerated storage. As can be observed in Fig. 1A, color is strong red-purple at 1–9 pH range that included the yogurt pH range (4–5). This is in good agreement with bibliography reviews claiming that betalains show the greatest stability in the pH range 4–6 and when stored at 4 °C (Khan, 2016; Li et al., 2019). In addition, at pH > 10 the solution rapidly turns yellow (Li et al., 2019); because strongly acidic or alkaline conditions cause structural changes in the molecules of betalains (Skalicky et al., 2020). A lack of red-purple color at pH 2 and 3 was exhibited at 21 days of storage (Fig. 1B). Stintzing et al. (2006) found the start of the betalain stability range at pH 3, when studying reed beet extracts, with maximum chroma values at pH 4.5. Huang and von Elbe (1987) also observed in red beet that below pH 3.5, λ shifts toward a lower wavelength and the intensity of the visible spectra decreases. Gengatharan et al. (2017) observed the lowest betacyanin content of colorant preparations from red pitahaya and betanin at pH 3. von Elbe et al. (1974) stated that degradation rates of betacyanins, such as betanins, are three-fold higher in pH 3. The modification of betacyanin structures, such as dehydrogenation and decarboxylation, takes place at low pH, causing a shift in the maximum absorption wavelength which, in turn, reduces betacyanin content (Herbach et al., 2006). Tsai et al. (2010) evaluating the pH stability of betacyanins from the cereal djulis (*Chenopodium fromosanum*), observed that the highest pigment stability and half-life was achieved at pH 5, with a characteristic red color. At pH 4–7, the purple-red color was the most prevalent, while at pH over 9 or lower than 3, an orange or pale-yellow color was found. Likewise, *Alternanthera brasiliana* extract presented an extended pH range of purple-red color (pH 4–10) and exhibited a pale-yellow color at pH ≥ 11 under 21 days of cold storage (Fig. 1B).

3.4. Stability of betacyanins and polyphenols in yogurt

Fig. 2 shows the evolution of the HPLC profiles of betacyanins in

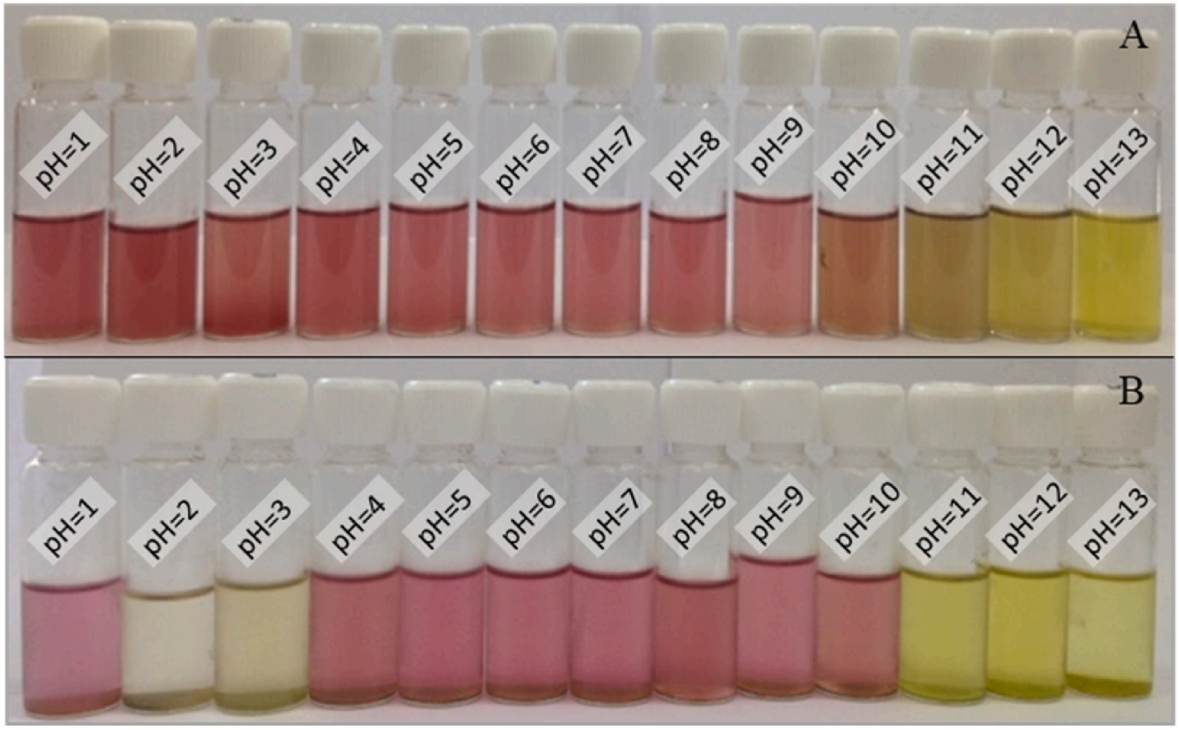

Fig. 1. Stability of Alternanthera brasiliana extracted pigments towards pH scale. A: at t = 0 refrigerated storage days. B: at t = 21 refrigerated storage days.

yogurt added with different concentrations of extract (350, 700 and 1400 μL/80 g yogurt), between time = 0 and time = 21 days of cold storage. All the *Alternanthera brasiliana* pigments identified in Table 1 were monitored, and the relative abundance of individual peaks was estimated. The summation of all the betacyanin compounds, at the initial time and at 7 and 21 days of storage is shown in Table 3. Fig. 3 shows the remaining betacyanin content at each storage time, calculated as the proportion of each betacyanin with respect to its initial concentration in yogurt (t = 0). At 21 days, the betacyanin content seemed to decrease for the three extract concentrations assessed. Indeed, looking at the temporal evolution in Fig. 3, the betacyanin content significantly decreased ($p < 0.05$) at 21 days. The interaction between the concentration of the six analyzed betalainic pigments in the yogurts and the storage time was significant ($p < 0.05$). In particular, the extract concentration of 1400 μL showed a raised remaining pigment percentage at 7 and 14 days for all the six compounds (Fig. 3). These results agree with that found by Moβhammer et al. (2005) and Herbach et al. (2006) who, despite working with different origin pigment extracts, concluded that betacyanin stability increased with pigment concentration.

The high degree of preservation of color observed at 7 and 14 days can be explained considering that betacyanins can be regenerated in the presence of different organic acids, such as lactic, citric or acetic acids (typical of yogurt) or gallic and gluconic acids. In addition, other acids such as ascorbic, isoascorbic, gluconic, metaphosphoric and phosphoric acid can accomplish the same function (Herbach et al., 2006). Furthermore, the quantity of regenerated betacyanins is affected by pH, storage temperature, type of additives and oxygen (Ngamwonglumlert et al., 2017). As mentioned earlier, betalains pigments are most stable in a pH range of 3–7, being betacyanins more resistant to acidic conditions and betaxanthins most stable at neutral pH (Stintzing & Carle, 2008).

However, after 21 days of storage, there were no significant differences between the three concentrations for each betalain pigment (Fig. 3). Pigments concentration decreased to around 20% from the initial values. Gengatharan et al. (2017) also observed in yogurts added with betacyanins, a rise in pigment contents, followed by a decrease during refrigerated storage at 4 °C. They presume that this depletion can be attributed to the normal hydrolysis process of betacyanins that occurs during refrigerated storage.

Fig. 4 shows the extract concentration effect on polyphenols content in yogurt at initial storage time. In the same way that in the raw extract, ramnovitexin was the major compound, representing about 25–30% of the total amount of polyphenols. Whereas vicenin II was reduced from 22% in the original extract to half in yogurt and *p*-coumaroyl malic acid isomers almost triplicates in yogurt concerning the 11% present in the raw extract. This behavior was observed for the three studied concentrations. The other monitored compounds presented slight variations when comparing raw extract with yogurts. These changes in polyphenols proportions when incorporating the extract to yogurt were attributed to milk–polyphenol interaction, as reported by Muniandy et al. (2016) and Dubeau et al. (2010). Lamothe et al. (2014) determined the concentration of bioaccessible polyphenols from tea extracts in yogurts after simulated gastrointestinal digestion. They attributed the low recoveries to the formation of complexes between polyphenols and milk proteins. Hasni et al. (2011) concluded that the binding capacity between tea polyphenols and proteins rises as the number of hydroxyl groups increases in phenolic compounds. Accordingly, vicenin II has an elevated number of hydroxyl groups.

As the polyphenol compounds proportion in yogurts remained unchanged with storage time, the summation of all the compounds, at 7 and 21 days of storage is shown in Table 3. Although a decline in polyphenol content after storage was evidenced, an acceptable remaining percentage was observed (ranging from a 37% for 350 μL/80 g yogurt to 19% for 1400 μL/80 g yogurt ones). Namely, phenolic remaining content increased with decreasing extract concentration.

However, the Folin Ciocalteau method did not reflect the reduction in polyphenols detected by HPLC. For TPC, significant effects of the extract concentration were observed (Fig. 5A). The values for 350 and 700 μL/80 g yogurt samples were not different, whereas the 1400 μL/80 g yogurt sample value resulted significantly higher ($p < 0.05$). In addition, TPC was significantly different at each storage time. Akgün et al. (2020) found around 12 mg GAE/100 g yogurt, when studying the incorporation of sour cherry extract in chitosan-coated liposomes, in

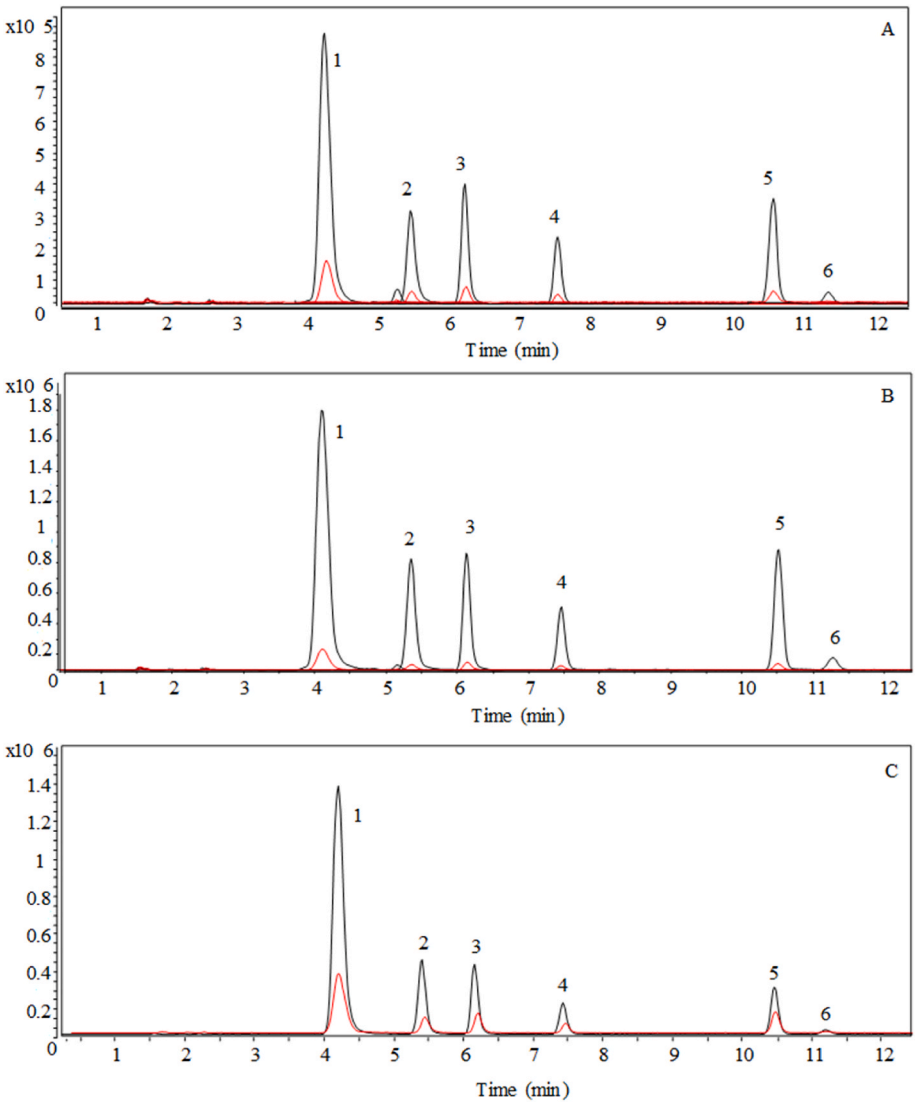

Fig. 2. Changes in HPLC profiles of betacyanins extracts of Alternanthera brasiliana before storage (black line, 0 days) and after storage (red line, 21 days) at 4 °C. Detection was performed at 538 nm. Peak 1, Amaranthine; 2, Isoamaranthine; 3, Betanin; 4, Isobetanin; 5, CelosianinII and 6, IsocelosianinII. Extract concentration A: 350, B: 700 and C: 1400 μL/80 g yogurt. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 3
Total betacyanins (μg of betacyanins/g of yoghurt) and phenolics content (μg of phenolic compounds/g of yoghurt) during storage time (days) determined by HPLC.

		Extract concentration (μL/80 g yogurt)		
	Storage time	1400	700	350
Total betacyanin content	0	486 ± 46 ^{Ac}	480 ± 32 ^{Bc}	230 ± 17 ^{Cc}
	7	290 ± 54 ^{Ab}	103 ± 5 ^{Bb}	77.3 ± 6.6 ^{Cb}
	21	55.1 ± 7.4 ^{Aa}	40.4 ± 4.5 ^{Ba}	35.8 ± 4.4 ^{Ca}
Total phenolic content	0	3.03 ± 0.18 ^{Ac}	1.70 ± 0.13 ^{Bc}	0.93 ± 0.10 ^{Cc}
	7	2.07 ± 0.19 ^{Ab}	0.49 ± 0.36 ^{Bb}	0.30 ± 0.02 ^{Cb}
	21	0.57 ± 0.10 ^{Aa}	0.49 ± 0.16 ^{Ba}	0.35 ± 0.08 ^{Ca}

*Different capital letters within the same row indicate significant differences and different lower case letters within the same column indicate significant differences ($p < 0.05$).

stirred-type yogurt, stored for 21 days. This value is very close to those determined in this work (Fig. 5A).

Antioxidant activity assessed by FRAP (Fig. 5 B), revealed a significant interaction between extract concentration and storage time ($p < 0.05$). The more relevant result was the decrease of the antioxidant activity at 7 days of storage, matching with the total phenolic content, and a significant increase at longer times.

It is worth mentioning that betacyanins also react to Folin Ciocalteau, contributing to the overall total phenolic content and antioxidant activity results observed by TPC and FRAP, which would be the sum of both polyphenols and betacyanins contributions. In this sense, Mosquera et al. (2020) found a positive correlation between phenolic content, determined by the Folin Ciocalteau method, and the antioxidant activity towards ABTS• of betalains extracted and purified from

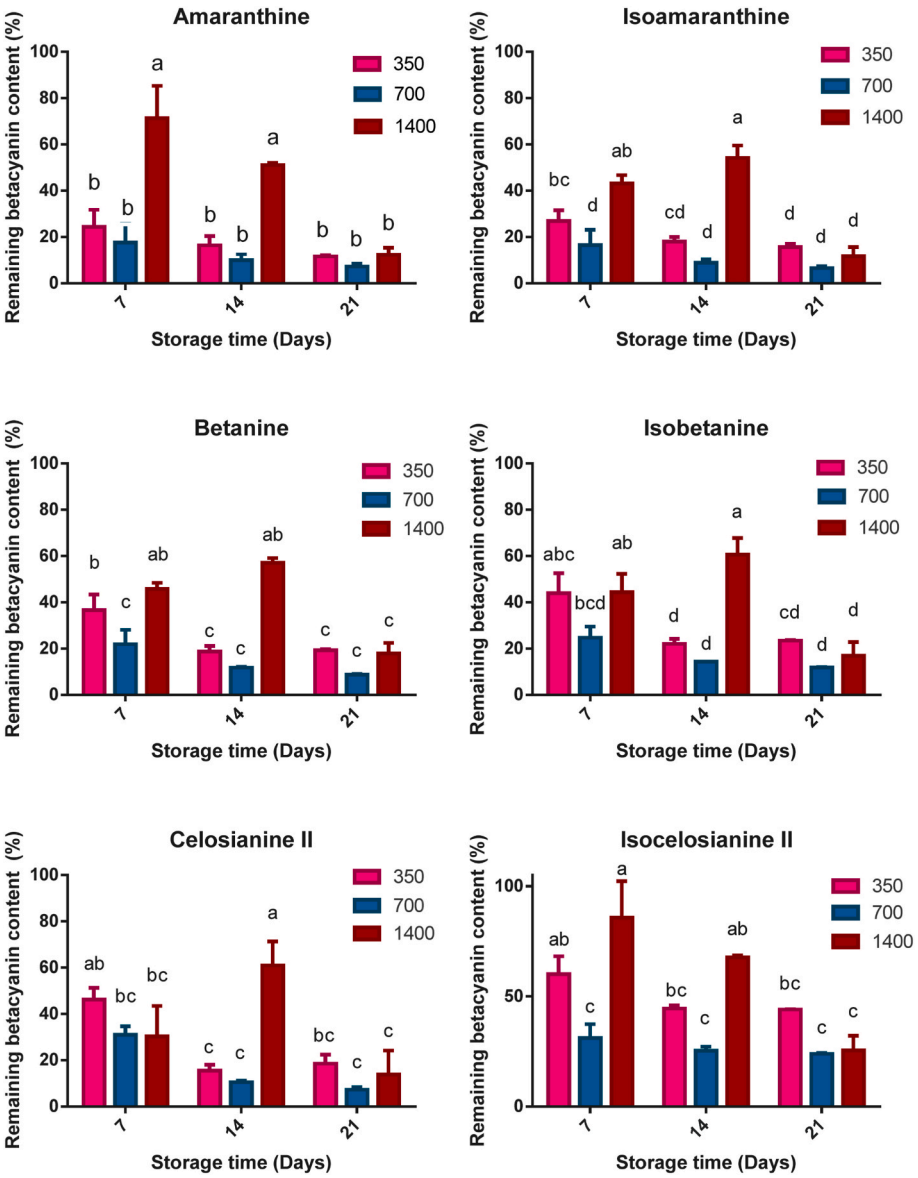

Fig. 3. HPLC profile of betacyanins in stored yogurt samples. Effect of extract concentration (350, 700 or 1400 μL/80 g yogurt) and storage time (7, 14 or 21 days) on remaining betacyanin content. Different letters over bars imply significant differences, bars indicate standard deviation, n = 3).

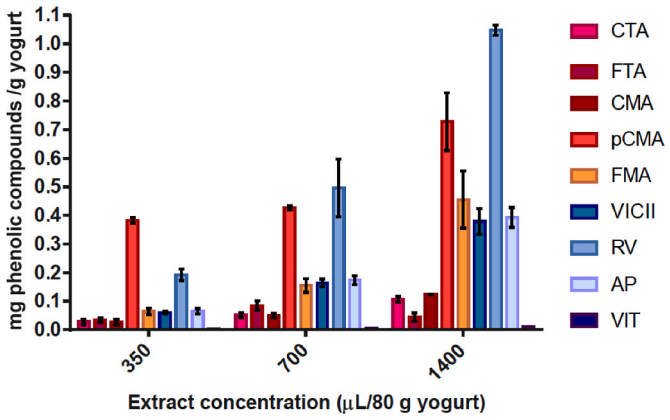

Fig. 4. Effect of extract concentration on polyphenol content at time = 0, by HPLC-MS. Nomenclature is referred in Table 2, bars indicate standard deviation, n = 2.

Ullucus tuberous, exhibiting the fraction rich in betacyanins a greater antioxidant activity and phenolic content than the fraction rich in betaxanthins. Cai et al. (2003) evaluated the antioxidant activity of betalain pigments from plants of the family Amaranthaceae by the DPPH• method. They determined that the EC_{50} values for betacyanins were stronger in the case of amaranthine and isoamaranthine, and close to the antioxidant activity of rutin or cathequin, for the other betacyanins. In the same way, Gandía et al. (2012) found that the free radical scavenging activity of betalains was highly affected by pH (an increased activity was observed at pH > 5.5). Although pH in yogurt was lower than these values, these authors also demonstrated that the activity of betalains at more acidic pH values was higher than that exhibited by Trolox.

3.5. Surface color stability and pH measurements in yogurts

Color is considered one of the first attributes of quality that consumers perceive which may influence their judgment of other attributes such as flavor (Zhang et al., 2021). However, changes of color and time-stability due to interferences with other food ingredients or storage

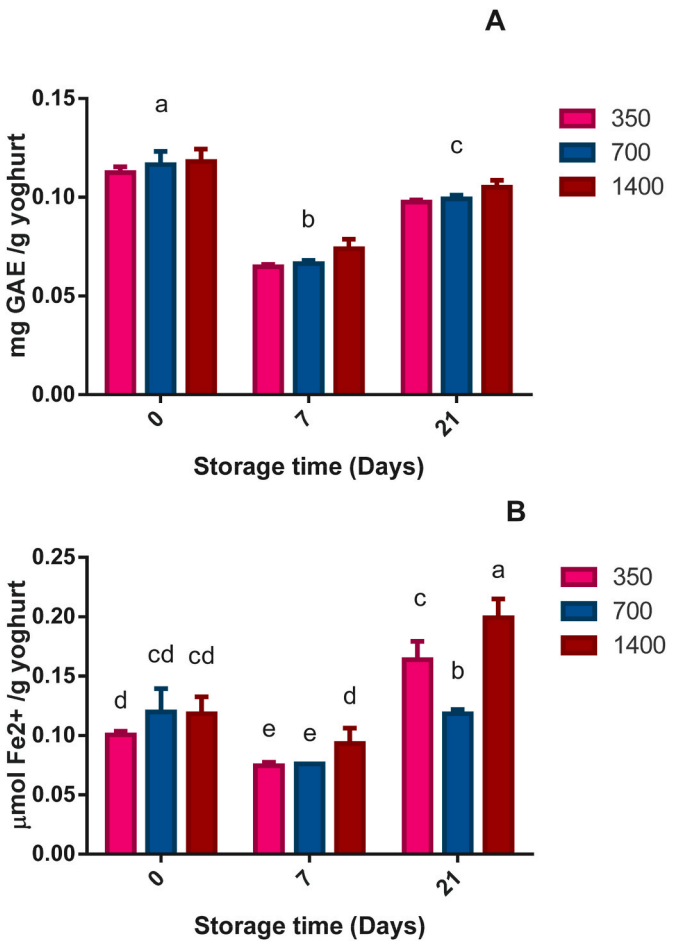

Fig. 5. **A**) Total phenolic content (mg GAE/g yogurt) and **B**) Ferric Reducing Power (µmol Fe^{+2}/g yogurt). Extract concentration pink: 350, blue: 700 and red: 1400 µL/80 g yogurt. Different letters indicate statistical differences between storage time in A-frame, and between samples in B-frame (LSD, $\alpha = 0.05$, $n = 2$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

conditions may occur (Teribia et al., 2021).

Betacyanins have been defined as condensation products of betalamic acid with cyclo-DOPA, usually glycosylated (Piattelli, 1981). Glycosylation refers to the multiple possibilities of derivatization of the resulting condensation compound, betanidin (Strack et al., 2003). The simplest and best-known glycosylated derivative of betanidin is betanin (betanidin-5-O-β-glucoside), the main pigment in beetroot (Rodriguez-Amaya, 2018; Skalicky et al., 2020).

The betalain color is attributable to its resonating double bonds (Delgado-Vargas & Paredes-López, 2003). Specifically, betacyanin colorant properties are linked to the aromaticity and extra intramolecular cycle in resonance with both nitrogen atoms of the molecule (Gandía-Herrero, 2010).

The CIELAB parameters of yogurt formulations at each storage time are shown in Fig. 6. Considering the obtained a^* and b^* values, yogurts with the three betacyanins extract concentrations presented a soft red-purple color, compatible with berry yogurts, highly appreciated by consumers. The color parameters of the freshly prepared betacyanin yogurts (at the initial storage times) were between those of strawberry yogurts ($L^* = 68.96 \pm 0.09$; $a^* = 7.83 \pm 0.02$; $b^* = 1.77 \pm 0.02$) and wild berry yogurts ($L^* = 64.15 \pm 0.02$; $a^* = 5.74 \pm 0.01$; $b^* = -2.73 \pm 0.02$) found in the market. Significant differences ($p < 0.05$) in color (ΔE) among freshly prepared yogurts with the three extracts concentrations were observed ($\Delta E_{1400\text{-}350} = 11.87$; $\Delta E_{1400\text{-}700} = 5.98$; $\Delta E_{700\text{-}350} = 5.96$). The redness ($+a^*$) increased as the concentration of added

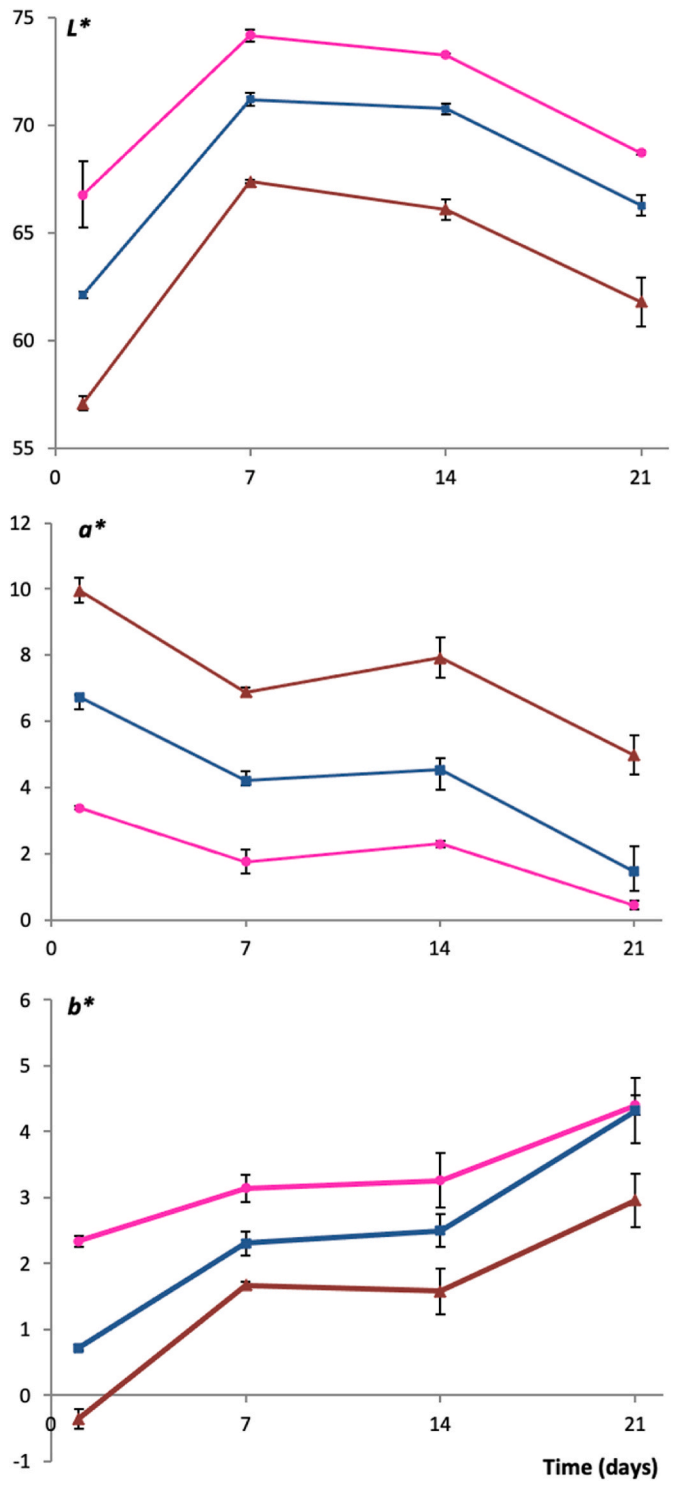

Fig. 6. Surface color parameters (L^*, a^*, and b^*) vs. storage time [days]. Extract concentration: 1400, 700, 350 µL/80 g yogurt. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

extract increased ($p < 0.05$). The yogurts exhibited an initial negative b^* value (blue color) for the 1400 extract content, that turned towards positive b^* values, when the extract content diminished ($p < 0.05$). The luminosity (L*) decreased as the concentration of added extract increased ($p < 0.05$), as expected, due to the rising preponderance of pigments.

Storage caused a decrease in a^* and an increase in $b^*$and L^* in all

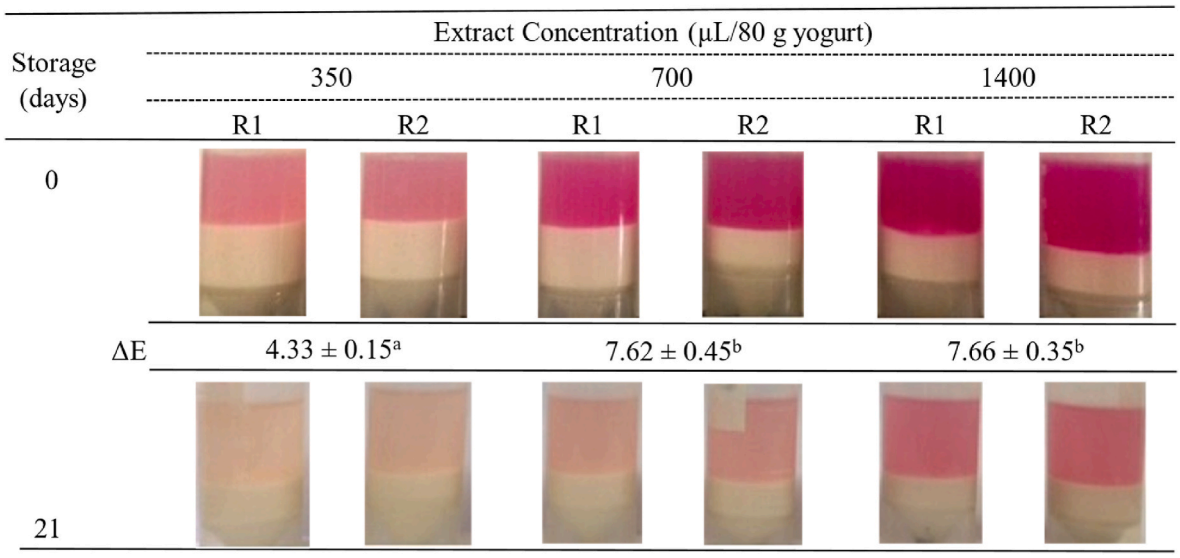

Fig. 7. Effect of extract concentration on total color differences (ΔE) in yogurts supernatants (pink upper stripe) after 21 days of refrigerated storage. R1 and R2 are different yogurt batches, the white lower stripe is yogurt after centrigugation. Different letters indicate statistical differences between samples (LSD, α = 0.05). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

concentrations assayed, indicating a modification of the initial color (Fig. 6). The *a** and *b** trends were stabilized at 14 days of storage (Fig. 6), which could be related to the significant increase in pigment contents that was detected in those days of refrigerated storage (Fig. 3).

Total color differences (ΔE) among yogurts colored with the three different extract concentrations are presented in Fig. 7. According to Mokrzycki and Tatol (2011) when $3.5 < \Delta E < 5$, a clear color difference can be noticed by a standard observer. Although a slight difference in color could be perceptible to human eyes, no significant differences were found in ΔE values between yogurts with the addition of 700 or 1400 μL μL/80 g yogurt of betacyanin aqueous extract. These results agree with data of HPLC pigment quantification (Fig. 3), where no significant differences were detected at 21 days of storage between 700 and 1400 μL/80 g yogurt samples.

Structural modifications of betacyanins due to dehydrogenation, decarboxylation, and hydrolytic cleavage (Herbach et al., 2006) may have caused the loss of betacyanins and the vanishing of color during storage (Gengatharan et al., 2017). In addition, the enzymes present in plant tissues can be extracted together with the pigments and their activity can contribute to discoloration over time. It has been observed that β-glucosidases, polyphenol oxidases and peroxidases are involved in this degradation (Stintzing & Carle, 2004).

A small decrease in pH was observed from day-0 up to day-21 of cold storage, for all yogurt samples (Table 4). This behavior is associated to the fermentation of lactose and the post-acidification of yogurt by *S. thermophilus* and *L. bulgaricus* during refrigerated storage (Damunupola et al., 2014; Dello Staffolo et al., 2004).

Significant correlations were observed between *a** and *b** values and yogurt pH (Table 4), which support the data found in previous studies, regarding the pH influence on the betacyanin color stability. This effect is associated with the presence of charged groups in betacyanin molecular structure (Stintzing & Carle, 2004; Gandía-Herrero, 2010). The redness diminished with the decrease in pH ($r > 0.815$; $p < 0.014$). It is recognized that isomerization, decarboxylation or cleavage of betacyanins by acid may occur (Schweiggert, 2018; Stintzing & Carle, 2004). Whereas, b* values increased towards positive values (yellow color) when pH decreased ($r > -0.783$; $p < 0.022$), probably due to the formation of yellow neobetanin (Skalicky et al., 2020).

In summary, results evidenced that yogurt pH remained within the interval where betacyanins stability is maximum. However, the noticeable decrease of betacyanins and polyphenols at 21 days of storage makes it inevitable to look forward to strategies for stabilizing this kind of compound before their application as colorant an antioxidant in food systems. Encapsulation (Aguirre-Calvo et al., 2020; Deladino et al., 2008; Matencio et al., 2021), the addition of anionic polysaccharides (Marchuk et al., 2019) and the presence of metals and organic acids

Table 4
YogurtpH during storage time and correlationcoefficients between*a** and*b** color parameters andpH.

		Extract concentration (μL/80 g yogurt)		
		1400	700	350
		pH		
Storage time (days)	0	4.99 ± 0.01 ^{Cc}	4.89 ± 0.00 ^{Ac}	4.97 ± 0.02 ^{Bc}
	7	4.93 ± 0.03 ^{Cbc}	4.95 ± 0.00 ^{Abc}	4.89 ± 0.00 ^{Bbc}
	14	4.99 ± 0.00 ^{Cb}	4.85 ± 0.00 ^{Ab}	4.92 ± 0.04 ^{Bb}
	21	4.82 ± 0.01 ^{Ca}	4.77 ± 0.03 ^{Aa}	4.85 ± 0.00 ^{Ba}
		Correlation between color parameters and yogurt pH		
*a**	***r***	0.889	0.815	0.841
	p	0.003	0.014	0.009
*b**	***r***	-0.914	-0.849	-0.783
	p	0.001	0.008	0.022

* Different capital letters within the same row indicate significant differences and different lower case letters within the same column indicate significant differences ($p < 0.05$), *r* is the correlation coefficient and *p* is the probability outputted from the Pearson correlation statistical analysis.

(Khan & Giridhar, 2014), are the most promissory solutions.

4. Conclusions

The aqueous extract of *Alternanthera brasiliana* showed the presence of betacyanins from amaranthine type and polyphenols grouped into hydroxycinnamic acids and flavones. The extraction method and solvent used have a strong influence on the extracted compounds. The color of the extract was stable in the pH range 4–11 during 21 days of refrigerated storage. However, different factors would have an effect on the different compound temporal evolution.

This is the first attempt to evaluate the incorporation of betalains from *Alternanthera brasiliana* into a food product. The aqueous extract provided a berry-like color to the yogurts. Significant correlations between a^* and b^* instrumental color parameters and yogurt pH were found. These results reinforce the knowledge built up in previous studies for other plant species, regarding the pH influence on the betacyanin color stability.

Diverse changes for pigment and phenolic compounds profile were detected along with cold storage. Both the partial color fading and the persistence of phenolic compounds at 21 days were linked to extract concentration.

Further studies are necessary to extend color stability for a longer time. However, these results provide an alternative to the use of red beet pigments. *Alternanthera brasiliana* can become a potential source of the relatively scarce betacyanin pigments for food applications.

CRediT authorship contribution statement

Aline Schneider-Teixeira: Conceptualization, Methodology, Formal analysis, Writing – original draft, Preparation. **Antonio D. Molina-García:** Funding acquisition, Writing – review & editing. **Inmaculada Alvarez:** Formal analysis, and Interpretation. **Marina Dello Staffolo:** Methodology, Formal analysis, Writing – review & editing, and. **Lorena Deladino:** Conceptualization, Methodology, Formal analysis, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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