



# Synergistic effects of ascorbic acid, low methoxy pectin, and EDTA on stabilizing the natural red colors in acidified beverages

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## ARTICLE INFO

### Keywords:

Color stabilization  
Betacyanins  
Pectin  
Ascorbic acid  
EDTA

## ABSTRACT

Betacyanins are one of the reddish to violet betalain pigments that are unstable under an oxygen atmosphere, greatly limiting their application and commercial potential. To improve the betacyanins color stability, we explored synergistic effects between ascorbic acid (AA), polysaccharides, and Ethylenediaminetetraacetic acid (EDTA) to improve the color stability of betacyanins in acidified conditions. We found that alginate and low methoxy pectin (LMP), among the thirteen studied polysaccharides, increased the red color stability of beetroot extract at pH 3.2 during thermal treatment. Our results proved that there is a synergistic effect between polysaccharides, AA, and EDTA, for enhancing the betacyanins color stability. Further, the red color in a model sports beverage recipe protected by the LMP, AA, and EDTA was stable for up to 45 days at room temperature and under natural light. The synergistic stabilization of betacyanins in acidified beverages was confirmed through ATR-FTIR and quartz crystal microbalance with dissipation analyses.

## 1. Introduction

Food products formulated with natural colorants have become increasingly in demand (Tuli et al., 2015). Currently, FD&C Red 40, a synthetic dye is used in many sports drinks, and both consumers and manufacturers have been seeking a non-synthetic alternative. Synthetic dyes offer significant stability toward many factors such as pH and light, that natural dyes do not (Cevallos-Casals and Cisneros-Zevallos, 2004). Therefore, we seek to find methods to stabilize natural pigments for use in acidic sports drinks.

Betacyanins, typically extracted from beetroot, are getting more attention as a type of natural red colorant. Betacyanins (reddish to violet) are one major category of betalains, which are a class of red and yellow indole-derived pigments found in plants of the Caryophyllales family. Beetroot red has been approved as a food/color additive in the US (Esatbeyoglu et al., 2015). However, betacyanins are unstable under light and oxygen atmosphere, which greatly limits their application and commercial potential. Temperature, water activity, light, pH, and the presence of oxygen and metal cations can all influence the degradation rate of betacyanins (Herbach et al., 2006).

A variety of stabilization methods have been proposed to stabilize the betacyanins (Khan, 2016). Among them, the encapsulation of

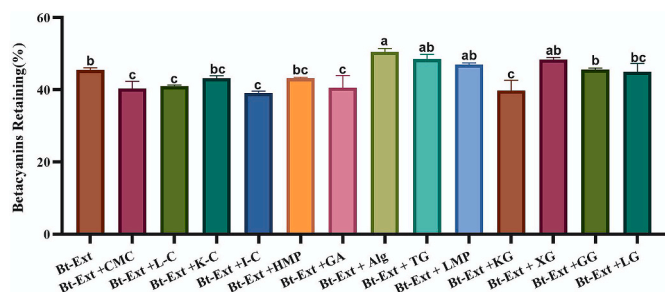
betacyanins using various shell materials has been broadly investigated (Chronakis, 1998). For example, encapsulation by polysaccharides (e.g., Xanthan gum, Guar gum, and gum Arabic) and gelatin increases the stability of betacyanins and makes them less hygroscopic (Barbosa et al., 2005; Castro-Muñoz et al., 2015; Fang and Bhandari, 2010; Ravichandran et al., 2014). Although some improvements have been achieved to stabilize the natural colorant through encapsulation, however spray drying and freeze-drying are energy-intensive and therefore not ideal methods for encapsulation (Nath and Satpathy, 1998). Our previous study showed that some polysaccharides stabilized betacyanins in aqueous solutions at different pHs through complex formation (Marchuk et al., 2019). The role of polysaccharides to enhance the stability of betacyanins has not been explored considering a wide range of available polysaccharides.

In addition, antioxidants have been reported as able to stabilize betacyanins. Ascorbic acid and iso-ascorbic acid can remove oxygen and protect betacyanins from oxidation (Ernest L. Attoe and Von Elbe, 1982; Strack et al., 2003). As chelating agents, citric acid and EDTA also help with betacyanins stabilization by making the heavy metal ions unavailable (Pasch and Von Elbe, 1979). In many cases have these agents have been found to stabilize betacyanins in real food systems. For instance, previous studies reported that encapsulated betacyanins can maintain

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**Fig. 1.** Betacyanin remaining (%) from 40 °C accelerated shelf stability tests after 16 h for 0.2% (w/w) Bt-Ext with or without 0.25% (w/w) of carboxymethyl cellulose (CMC), Lambda-carrageenan (L-C), Kappa-carrageenan (K-C), Iota-carrageenan (I-C), high methoxy pectin (HMP), gum Arabic (GA), sodium alginate (Alg), tara gum (TG), low methoxy pectin (LMP), and Konjac Gum (KG), xanthan gum (XG), gum guar (GG), and locust bean gum (LG) at pH 3.2. Similar letters on the bar indicate an insignificant change ( $p$ -value>0.05).

their stability after storage in gummies and chewing gums (Chranioti et al., 2015; Otálora et al., 2019). To date, however, research lacks any study on synergistic effects of ascorbic acid (AA), Ethylenediaminetetraacetic acid (EDTA), and polysaccharides to further

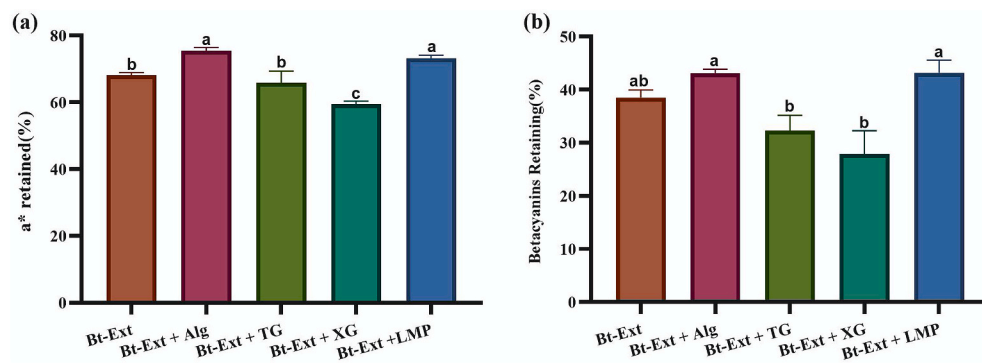
stabilize the betacyanins.

In this study we screened thirteen polysaccharides that have the potential to protect betacyanins from thermal and light degradation. Second, we designed different combinations of polysaccharides and antioxidants to explore synergistic effects of ternary mixtures on betacyanins stability. We then used FTIR and QCM-D to confirm the protection of betacyanins from these additives. The overarching goal of this study was to find a natural substitute for FD&C Red 40 that is stable in aqueous solutions within a pH range of 2.5–3.5. To this end, a simulated model sports beverage recipe was used to further validate the aqueous solution results of betacyanins color stability.

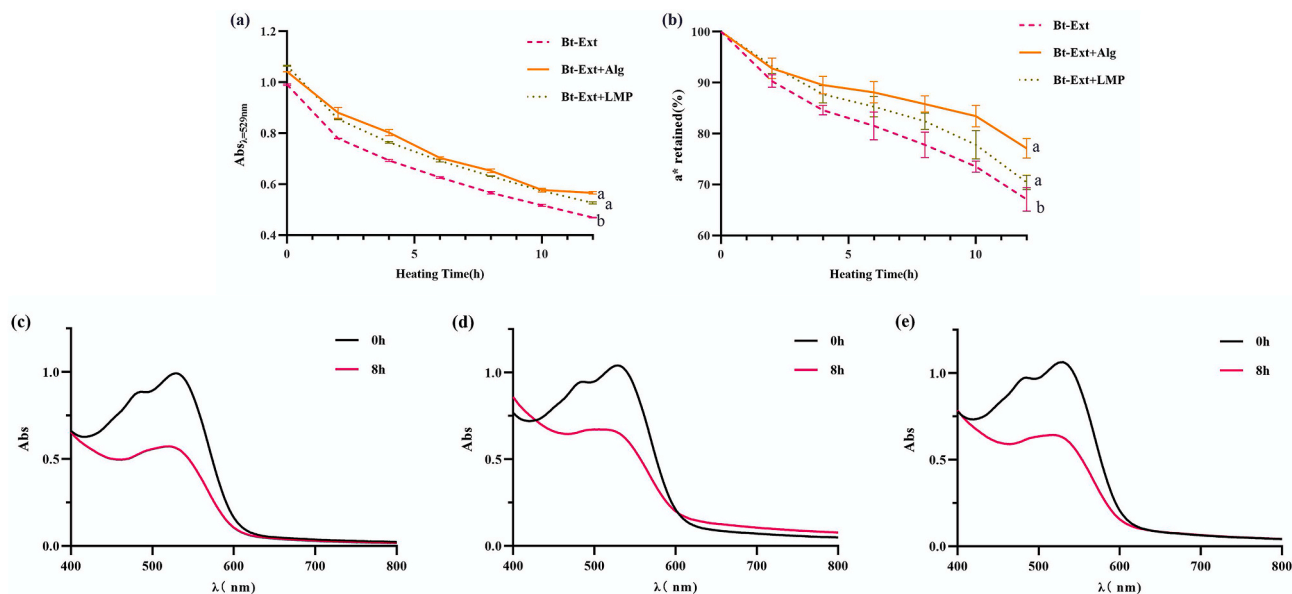
## 2. Materials and methods

### 2.1. Materials

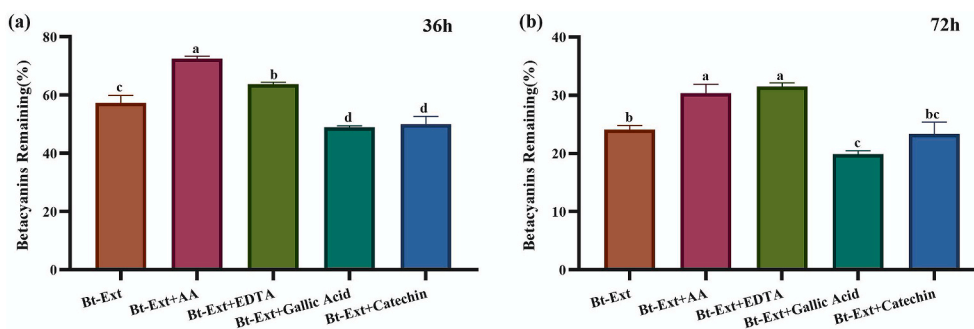
Beetroot powder was purchased from Bulk Supplements (Henderson, NV, US). Ticaloid®710 H-96 Powder (Lambda-carrageenan), Ticaloid®750 (Kappa-carrageenan), Ticaloid®881 M Powder (Iota-carrageenan), Ticaxan®Xanthan (Xanthan gum), TIC Pretested® Pectin 1400 (High Methoxy Pectin; HMP), TIC Pretested® Pectin LM 35 Power (LMP), TIC Pretested® Gum Arabic Spray Dry Powder (gum Arabic), TIC Pretested® Tara Gum 100 (Tara gum), Ticagel® Konjac High Viscosity



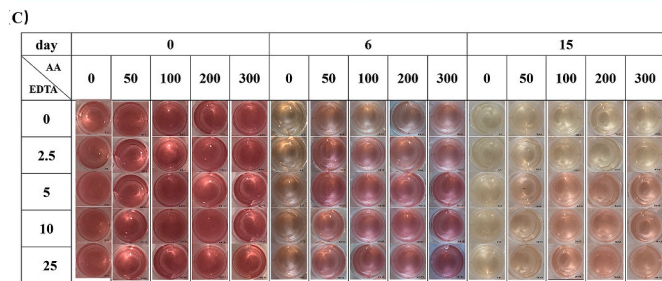
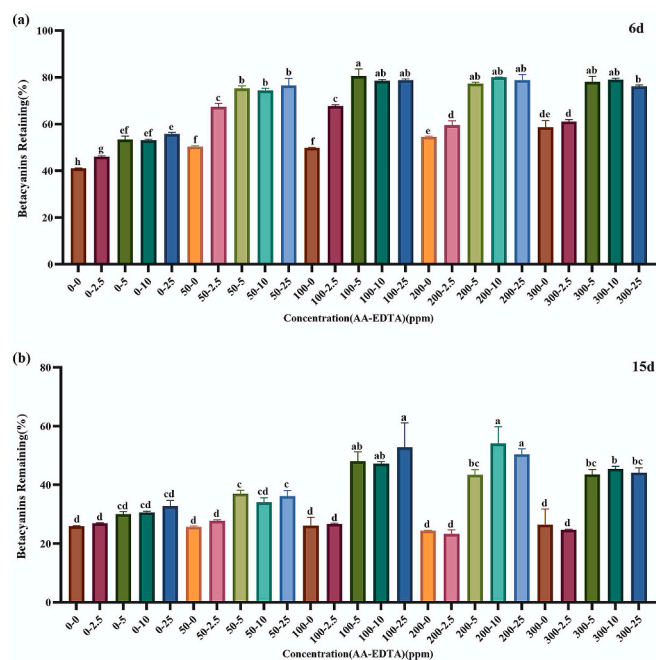
**Fig. 2.** Accelerated shelf stability tests at 40 °C after 16 h for 0.2% (w/w) of beetroot extracts (Bt-Ext) with or without 0.25% (w/w) of sodium alginate (Alg), tara gum (TG), xanthan gum (XG), and low methoxy pectin (LMP) at pH 3.2: (a) Percentage of red color retained (% a\* retained) using CIE L\* a\* b\* colorimeter; (b) Betacyanin remaining (%) using Spectra-Max iD3 Multi-Mode Microplate Reader. Similar letters on the bar indicate an insignificant change ( $p$ -value>0.05). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)



**Fig. 3.** Accelerated shelf stability tests at 40 °C for 0.2% (w/w) Bt-Ext with or without 0.25% (w/w) Alg and LMP at pH 3.2: (a) Declining absorbance at 529 nm every 2 h; (b) Declining red color retained (% a\* retained) using CIE L\* a\* b\* colorimeter; (c, d, and e) Absorption curve of Bt-Ext, Bt-Ext + Alg, and Bt-Ext + LMP from 40 °C accelerated shelf stability tests before and after 8 h. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)



**Fig. 4.** Betacyanin remaining (%) with UV light exposure for 0.2% (w/w) beetroot extracts (Bt-Ext) with or without 100 ppm Ascorbic Acid (AA), Ethylenediaminetetraacetic acid (EDTA), Gallic acid, and Catechin: (a) 36 h (b) 72 h. Similar letters on the bar indicate an insignificant change ( $p$ -value>0.05).



**Fig. 5.** Betacyanin remaining (%) with daylight exposure for 0.2% (w/w) beetroot extracts (Bt-Ext) with or without the different concentrations of Ascorbic Acid (AA) and Ethylenediaminetetraacetic acid (EDTA): (a) 6 days, (b) 15 days, and (c) Images taken at each time interval. In plots, the “0-0” means the solution contains just Bt-Ext. Concentration of EDTA varies 0, 2.5, 5, 10, and 25 ppm. Concentration of AA varies 0, 50, 100, 200, and 300 ppm. Similar letters on the bar indicate an insignificant change ( $p$ -value>0.05).

(Konjac gum), TIC Pretested® Gum Guar 8/22 Powder (Guar gum), and TIC Pretested® Locust Bean Gum PORIA Power (Locust Bean gum) were provided by TIC gum (White Marsh, MD, US). Sodium alginate (Manugel GHB; Alg) was purchased from FMC Biopolymer (Philadelphia, PA, US). (Ethylenediaminetetraacetic acid, Reagent ACS (EDTA), and Gallic acid ethyl ester (Gallic acid) were purchased from Acros Organics (Belgium, US). Carboxymethyl cellulose, L-ascorbic acid (AA), Catechin hydrate

(Catechin), citric acid (food graded), and other chemicals used for beverage were purchased from Sigma Aldrich (St. Louis, MO, US). A commercially available sports beverage containing FD&C Red 40 dye was purchased from the local market as a reference sample.

### 2.2. Extraction of red colorants from beetroot

Beetroot powder 0.2% (w/w) was dissolved in deionized water for the extraction of red colorants. The mixture was kept under constant mixing for 60 min to allow the extraction followed by centrifugation at 3000 rpm for 15 min to remove the insoluble matter. Such aqueous beetroot extracts (Bt-Ext) were then filtered by filter paper and kept in the dark at  $-18\text{ }^{\circ}\text{C}$  until used.

### 2.3. Screening of polysaccharides for betacyanins stabilization

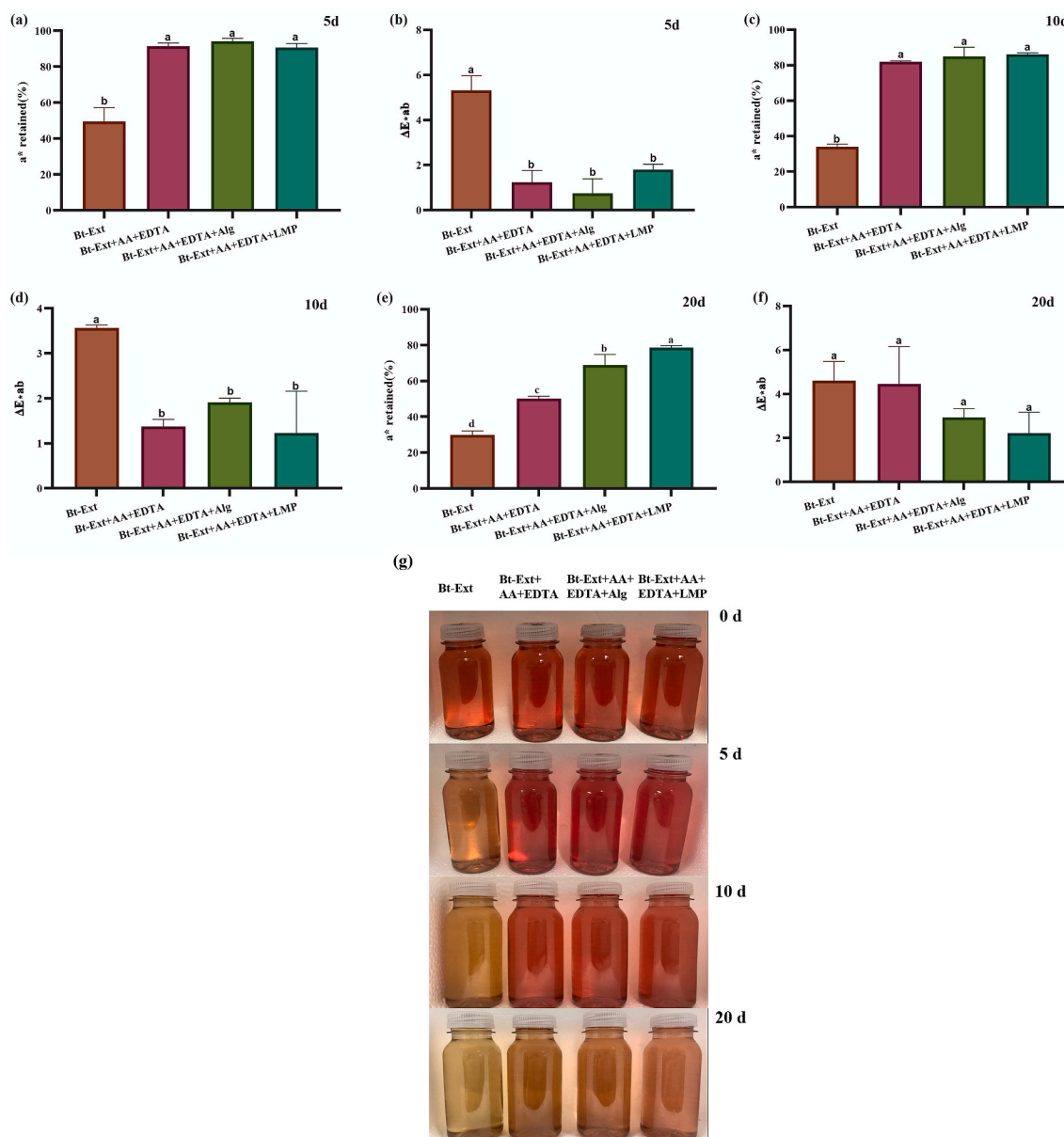
Thirteen different polysaccharides such as carboxymethyl cellulose, Lambda-carrageenan, Kappa-carrageenan, Iota-carrageenan, HMP, gum Arabic, Tara gum, Xanthan gum, Konjac gum, Guar gum, Locust Bean gum, LMP, and Alg were screened, targeting betacyanins stabilization. An aqueous solution of 0.25% (w/w) of each polysaccharide was prepared and combined with the Bt-Ext at a 1:1 ratio, respectively. To eliminate the influence of the native pH of each polysaccharide solution, the final pH of the mixed solutions was adjusted to 3.2 with citric acid. The accelerated stability study was performed at  $40\text{ }^{\circ}\text{C}$  for 16 h. The red color intensity was measured in a 24-well plate at 529 nm by a SpectraMax iD3 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA, US).

### 2.4. Light stability studies of antioxidants in acidic solution

Investigating the influence of antioxidants on light stability of Bt-Ext, four different antioxidants such as AA, EDTA, Gallic acid, and Catechin were studied. A solution of 50 ppm of each antioxidant was prepared and separately added into Bt-Ext. The final pH of each mixture was adjusted to 3.2 using citric acid. The samples were exposed to UV light for 72 h for accelerated studies. The betacyanins' color intensity was measured every 36 h in a 24-well plate at 529 nm using SpectraMax iD3 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA, US). Antioxidants that help preserve more betacyanins were further investigated through the effects of different concentrations and under natural light.

### 2.5. Short-term storage stability of Bt-Ext: effect of polysaccharides and antioxidants

Selected polysaccharides and antioxidants aqueous solutions were mixed with Bt-Ext, and the pH was adjusted to 3.2 with citric acid. The mixed solutions were pasteurized ( $95\text{ }^{\circ}\text{C}$ , 10 min) and stored under natural light (Ithaca, NY) for 15 days (September through October) at



**Fig. 6.** Percentage of red color retained (% a\* retained) and ΔE\*ab with daylight exposure for 0.2% (w/w) of Bt-Ext with or without the combination of AA-EDTA, Alg, and LMP: (a, b) 5 days, (c, d) 10 days, (e, f) 20 days, and (g) Actual images were taken at 0, 5, 10, and 20 days. Similar letters on the bar indicate an insignificant change (*p*-value>0.05). This study was performed based on the aqueous solution at pH 3.2. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

room temperature. The degradation of betacyanins was measured on day-6 and day-15.

### 2.6. UV-vis spectrophotometry

Spectroscopy has been used for detecting betacyanins in a solution. The absorbances of the solutions at 529 nm wavelength were compared over time using a SpectraMax iD3 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA, US). The subscripts 1 and 2 denote pre- and post-storage measurements. Betacyanins remaining (%) is calculated based on the followed equation.

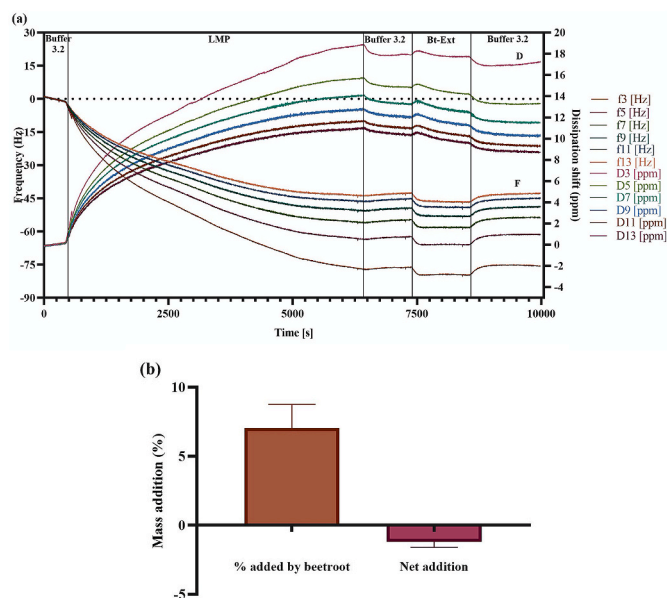
$$\text{Betacyanin remaining (\%)} = \frac{Abs_2}{Abs_1} 100\% \quad \lambda = 529 \text{ nm} \quad (1)$$

### 2.7. Colorimetry

To access color changes of prepared solutions, we followed the CIE system (Francis and Clydesdale, 1975). L\* (lightness), a\* (red-green axis), and b\* (blue-yellow axis) values were collected using a Konica Minolta Chroma CR400/410 portable colorimeter (Osaka, Japan). The subscripts 1 and 2 indicate the initial and final stages of measurements, respectively. Color changes (ΔE\*<sub>ab</sub>) and red color remaining (% a\* remaining) were assessed according to the following equations.

$$\Delta E_{ab} = \sqrt{(L_2 - L_1)^2 + (a_2 - a_1)^2 + (b_2 - b_1)^2} \quad (2)$$

$$a\% = \frac{a_2}{a_1} 100\% \quad (3)$$



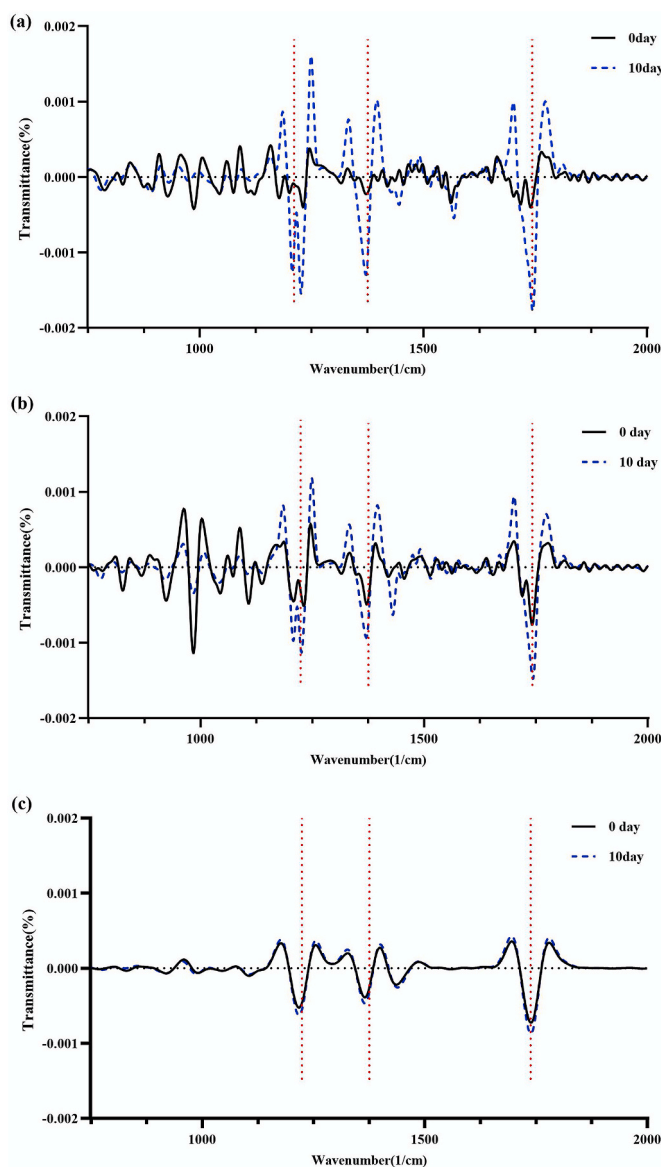
**Fig. 7.** (a) QCM-D frequency shift and corresponding dissipation for Bt-Ext binding events at pH 3.2 and (b) The initial adsorbed mass of Bt-Ext over LMP and final mass removal at final buffer wash step. The error bar is the standard deviation calculated for the triplicated experiment.

## 2.8. Quartz crystal microbalance with dissipation monitoring (QCM-D)

The real-time interaction between Bt-Ext and polysaccharide at pH 3.2 was evaluated by the QCM-D technique. In this study, we used a QSense Explorer machine (Biolin Scientific, Gothenburg, Sweden) equipped with a gold sensor (QX3 301). Briefly, we employed this machine to monitor changes due to the frequency ( $\Delta f$ ) and dissipation ( $\Delta D$ ) of the sensor (Lee et al., 2020; Wang et al., 2020). This information can be further post-processed through DFind software using the composite Sauerbrey equation to quantify the mass (Marchuk et al., 2019; Rodahl et al., 1997; Wang et al., 2021; Yan et al., 2021; Zhang et al., 2021). Through the flow module of the machine, the solution can be injected, and real-time frequency and dissipation data can be recorded simultaneously. To do this, first LMP solution (0.1% w/w) and Bt-Ext (0.2%) were prepared. As pH is a key parameter, we adjusted all the solutions' pH to 3.2 prior to experiments. The QCM-D experiments were performed at room temperature ( $21 \text{ }^\circ\text{C} \pm 2$ ) and at a flow rate of 0.3 ml/min. Due to the detrimental influence of bubbles in this experiment, all the solutions were carefully degassed (20 min in degassing bath). As a second step, to prime the flow module and the sensor, after rinsing with Milli Q water for 30 min, the corresponding citrate buffer at pH 3.2 for the experiment was injected for around 10 min to establish a baseline for the measurement. In the third step, the substrate, LMP solution, was injected into the flow module. This is a key step in this experiment, forming a stable substrate over the sensor. Allowing enough time for LMP to be adsorbed over the sensor and to saturate the sensor active area, the buffer was flowed over, making sure that a loose LMP-sensor interaction layer (*i.e.*, fluffy layer and unbound LMP) was removed. This assures the formation of a uniform and stable LMP layer on the sensor. Then, Bt-Ext was run over the coated sensors at the same flow rate. Finally, a citrate buffer (pH 3.2) was again used to wash the unattached Bt-Ext. This procedure was repeated in triplicate.

## 2.9. Attenuated total reflectance-fourier transform infrared spectroscopy (ATR-FTIR)

Bt-Ext with or without AA, EDTA, and LMP at pH 3.2 was prepared and stored for 10 days in daylight. Samples of solutions at 0 day and 10 days were freeze-dried for the measurements. ATR-FTIR spectra of



**Fig. 8.** ATR-FTIR spectra of freeze-dried samples in 0 day and 10 days: (a) Bt-Ext, (b) Bt-Ext with AA and EDTA, (c) Bt-Ext with AA, EDTA, and LMP.

samples were taken using an IRAffinity-1S spectrometer equipped with a single-reflection ATR accessory (Shimadzu Corporation; Kyoto, Japan). All spectra were averages of triplicate, 32 scans each from 500 to 4000  $\text{cm}^{-1}$  with a resolution of  $2 \text{ cm}^{-1}$ .

## 2.10. Validation of the method in a sports beverage model

We prepared a model sports beverage using sodium citrate dihydrate (1.95 g/1000 ml), monopotassium phosphate (0.44 g/1000 ml), citric acid monohydrate (3.70 g/1000 ml), dextrose (57.53 g/1000 ml) in distilled water. We based this approximate formulation on the ingredients listed on the purchased sports beverage and adjusted the pH to match the measured pH of the purchased drink. The solutions of 0.2% (w/w) Bt-Ext with or without AA, EDTA, and LMP, were incorporated in the beverage model. The final pH was adjusted to 3.2 with citric acid, and the beverages were pasteurized for storage at  $95 \text{ }^\circ\text{C}$  (internal solution temperature  $90 \text{ }^\circ\text{C}$ , 2.5 min). Final products were bottled in plastic containers and stored under daylight. Photos were taken periodically to monitor color change during storage.



**Fig. 9.** Images of storage studies conducted in a model sports beverage recipe at pH 3.2: The recipe and red color were formulated with different antioxidant combinations. This study was performed based on actual model sports beverage formulation at pH 3.2. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

### 2.11. Statistical analysis

All analyses were performed in triplicate, and data were reported as mean  $\pm$  standard deviation (SD). One-way and two-way Analysis of variance (ANOVA) with Bartlett's test ( $p < 0.05$ ) were performed to determine the statistical significance.

## 3. Results & discussion

### 3.1. Effect of polysaccharides on Bt-Ext stability in acidic solutions

Thirteen mixtures of Bt-Ext/polysaccharides (at pH 3.2) were prepared as stated above. The samples were kept at 40 °C for 16 h with light exposure and tested by the microplate reader at 529 nm. The betacyanins remaining (%) were calculated and used to indicate the protective effect of these polysaccharides. Fig. 1 shows the betacyanins remaining (%) of Bt-Ext with or without polysaccharides after 16 h of storage. The mixture of Bt-Ext with Alg, Tara gum, LMP, and Xanthan gum retained more red colors after storage, which is  $50.50 \pm 0.95\%$ ,  $48.50 \pm 1.35\%$ ,  $47.03 \pm 0.45\%$ , and  $48.43 \pm 0.55\%$ , respectively. The Bt-Ext with Alg presents the highest betacyanins retention. The statistical analysis showed that these four polysaccharides have a significant influence ( $p$ -value  $< 0.05$ ) on red color stability when compared to the other studied compounds. Only Alg showed a significant difference compared to Tara gum, LMP, and Xanthan gum. Thus, four polysaccharides, i.e., Alg, Tara gum, LMP, and Xanthan gum, were selected and subjected to acidic conditions, over time and at slightly higher than ambient temperature (pH 3.2, 16 h, and 40 °C).

Fig. 2a presented the red color of Bt-Ext with or without Alg, Tara gum, LMP, and Xanthan gum polysaccharides in acidic solutions. The mixture of Bt-Ext with Alg and LMP show higher red color retention ( $a$ -value of  $75.40 \pm 1.05\%$  and  $73.23 \pm 0.85\%$ , respectively) compared to the Bt-Ext only solution ( $a$ -value of  $68.13 \pm 0.74\%$ ). Consistent with the reported in Fig. 1, both Bt-Ext + Alg and Bt-Ext + LMP showed significant improvement ( $p$ -value  $< 0.05$ ) in  $a$ -value compared to Bt-Ext + TG and Bt-Ext + XG mixtures. A similar trend is confirmed by spectrophotometric readings (Fig. 2b). Lower red color retentions and absorbances have been recorded on Tara gum and Xanthan gum compared to the mixtures of Bt-Ext with LMP and Alg. Tara gum and Xanthan gum showed insignificant impact ( $p$ -value  $> 0.05$ ) on betacyanins remaining (%); however, compared to LMP and Alg, the difference is significant ( $p$ -value  $< 0.05$ ) in protecting the red color. Previous research has shown that pectin and alginate can be used as effective shell material in the encapsulation of betacyanins (Lejeune et al., 1983; Rodríguez-Sánchez et al., 2017). However, it is uncertain why pectin and alginate can help protect the betacyanins in Bt-Ext. It is believed that binding interactions between beet pigment and two of the polysaccharides may contribute to color stability (Marchuk et al., 2019).

To study this red color change during storage more precisely, mixtures of Bt-Ext with Alg and LMP were prepared, and the absorbance and  $L^*$ ,  $a^*$ , and  $b^*$  of the two samples were monitored every 2 h during thermal treatment (12 h, 40 °C). Absorbance decreases of the three samples showed a relative betacyanins degradation rate of: Bt-Ext  $>$  Bt-Ext + LMP  $>$  Bt-Ext + Alg (Fig. 3a). The red color ( $a$ -value) retention curves also showed similar results (Fig. 3b). ANOVA analysis showed a significant ( $p$ -value  $< 0.05$ ) difference between the mixtures of Bt-Ext/polysaccharides and Bt-Ext, but no significant difference ( $p$ -value  $> 0.05$ ) between Bt-Ext + Alg and Bt-Ext + LMP. The UV-vis spectra of Bt-Ext, Bt-Ext + Alg, and Bt-Ext + LMP, at 0 h and 8 h, which indicates smaller absorbances change when Bt-Ext + Alg + LMP (Fig. 3c–e). Considering these results, we concluded that Alg and LMP improve the betacyanins stability of Bt-Ext in acidic conditions (pH 3.2) during a 40 °C storage.

### 3.2. Effect of antioxidants on Bt-Ext stability in acidic solutions during light treatment

Four antioxidants, including AA, EDTA, Gallic acid, and Catechin, were selected to inhibit the oxidation of betacyanins during storage. The mixtures of Bt-Ext and antioxidants at pH 3.2 were prepared and exposed to UV light for 36 and 72 h. The absorbances of the samples were evaluated in the microplate reader at 529 nm. Fig. 4a shows betacyanins remaining (%) of Bt-Ext with or without antioxidants in 36

h. Lower betacyanins remaining (%) was found in Bt-Ext with Gallic acid and catechin ( $48.90 \pm 0.52\%$  and  $49.97 \pm 2.75\%$ , respectively) when compared with Bt-Ext itself ( $57.33 \pm 2.55\%$ ), which is consistent with previous studies (E. L. Attoe and Von Elbe, 1985; Khan and Giridhar, 2014). The extent of influence of Gallic acid and catechin on betacyanins remaining (%) is insignificant ( $p$ -value $>0.05$ ). However, Bt-Ext with AA and EDTA showed much higher betacyanins remaining (%) ( $72.50 \pm 0.87\%$  and  $63.67 \pm 0.76\%$ , respectively,  $p$ -value $<0.05$ ). The differences between AA and EDTA is significant ( $p$ -value $<0.05$ ), as well as their difference compared to Gallic acid and catechin. In fact, previous studies have proved that AA helps stabilize betacyanins in a real food system (Herbach et al., 2006; Khan and Giridhar, 2014; Leong et al., 2018; Mofhammer et al., 2007). EDTA has also been reported as a stabilizer of betanin (Pasch and Von Elbe, 1979). A similar trend was found after 72 h (Fig. 4b). Interestingly, EDTA preserves nearly the same content of betacyanins as AA after 72 h ( $p$ -value $>0.05$ ), which suggests that both EDTA and AA are essential in the preservation of betacyanins for long term storage (Ernest L. Attoe and Von Elbe, 1982; Pasch and Von Elbe, 1979). However, the AA and EDTA protect betacyanins through different mechanisms: AA protects betacyanins from oxidation, and EDTA binds heavy metal ions. We were interested in finding out whether this combination of AA and EDTA can work synergistically to effectively protect betacyanins during storage. Both AA and EDTA showed significant differences ( $p$ -value $<0.05$ ) compared to Gallic acid and catechin.

The synergistic effect of AA and citric acid on betacyanins stability has been studied, but not with EDTA involved (E. L. Attoe and Von Elbe, 1985). In this study, different combinations of AA (0, 50, 100, 200, and 300 ppm) and EDTA (0, 2.5, 5, 10, and 25 ppm) were chosen to study the synergistic effect on red color stability. In total, 25 samples were stored in daylight for 15 days. Color changes were measured and betacyanins remaining (%) were calculated for day-6 and day-15. Fig. 5a presents betacyanins retention of Bt-Ext with or without the combination of different concentrations of AA and EDTA in daylight for 6 days. Among them, Bt-Ext with a combination of 100 ppm AA and 5 ppm EDTA showed the highest betacyanins remaining (%) ( $80.53 \pm 3.10\%$ ), which is 40% higher than Bt-Ext without any antioxidants. The statistical analyses confirmed that betacyanins remaining (%) of Bt-Ext with a combination of 100 ppm AA and 5 ppm EDTA is significantly different from other binary concentrations ( $p$ -value $<0.05$ ).

For the 15-day study, the highest betacyanins remaining (%) ( $54.05 \pm 5.73\%$ ) was obtained for the Bt-Ext sample with a combination of 200 ppm AA and 10 ppm EDTA on day 15 (Fig. 5b); however, there is no significant ( $p$ -value $>0.05$ ) difference between the “200-25” and “100-25” solutions. Other combinations, e.g., 100 ppm AA and 25 ppm EDTA, 200 ppm AA and 25 ppm EDTA, 100 ppm AA and 5 ppm EDTA, 100 ppm AA and 10 ppm EDTA showed a significantly higher ( $p$ -value $<0.05$ ) betacyanins remaining (%) compared with Bt-Ext ( $25.93 \pm 0.12\%$ ). The use of EDTA is allowed by FDA (FDA, 2020) but the dosage of EDTA for color protection in acidic beverages must be less than 100 ppm, while no restriction is reported for AA dosage (Bauernfeind, 1982).

Images of Bt-Ext with or without a combination of different concentrations of AA and EDTA on days 0, 6, and 15 were recorded (Fig. 5c). Bt-Ext with combinations of AA (100, 200, and 300 ppm) and EDTA (5, 10, and 25 ppm) still preserve their pink colors on day 15, while the red color disappeared for other samples, which supports the data presented above. In conclusion, taking into consideration the fact that lower concentrations of EDTA are more desirable in food systems (Faiman et al., 1983), a combination of 200 ppm AA and 10 ppm EDTA is recommended for use in any further experiments.

### 3.3. Optimization of Bt-Ext with polysaccharides and antioxidants in acidic solutions

As showed above, 0.25% (w/w) polysaccharides (Alg and LMP) and a combination of antioxidants (200 ppm AA and 10 ppm EDTA) were proved to increase the color stability of Bt-Ext, separately. To further

evaluate the effect of a combination of polysaccharides and antioxidants on Bt-Ext at pH 3.2. Four different samples (Bt-Ext, Bt-Ext with AA and EDTA, Bt-Ext with AA, EDTA, and Alg, Bt-Ext with AA, EDTA, and LMP) were prepared and stored under natural daylight for up to 20 days. The color change was measured with a colorimeter. Fig. 6a and b indicate the percentage of red color retained and  $\Delta E^*_{ab}$  with daylight exposure for the four samples in 5 days. Bt-Ext solution without antioxidant and polysaccharides has been preserved the lowest red color ( $49.57 \pm 7.64\%$ ) compared with the other three samples; also, the highest  $\Delta E^*_{ab}$  value of Bt-Ext suggests the significant ( $p$ -value $<0.05$ ) total color change, while AA/EDTA/polysaccharides improved color stability. Similar trends are also observed on day 10 (Fig. 6c & d).

However, according to Fig. 6e, a significant difference ( $p$ -value $<0.05$ ) in red color retained between 4 samples appeared on 20 days. The order in red color retention is Bt-Ext + AA + EDTA + LMP > Bt-Ext + AA + EDTA + Alg > Bt-Ext + AA + EDTA > Bt-Ext. The obtained differences are statistically different ( $p$ -value $<0.05$ ).  $\Delta E^*_{ab}$  value decreased (Fig. 6f) due to an increase of obtained  $b^*$  value of four samples. We also noticed the obvious decrease of red hue and increase of yellow hue for 20 days storage from images showing in Fig. 6g. In short, the combination of 0.25% (w/w) LMP, 200 ppm AA, and 10 ppm EDTA proved to be the most effective stabilization agents for Bt-Ext in acidic solutions (pH 3.2).

### 3.4. QCM-D measurements

To detect the real-time interaction between LMP and Bt-Ext, both frequency and dissipation were recorded as a marker of molecule-molecule interaction, and consequently, the mass was added over the sensor. Fig. 7a exhibits simultaneous recorded frequencies shift ( $f_3$ ,  $f_5$ ,  $f_7$ ,  $f_9$ ,  $f_{11}$ , and  $f_{13}$ ) and corresponding dissipations (D3, D5, D7, D9, D11, and D13) vs. time at each injection period. A buffer wash step was repeated before and after each injection to prime the sensor and remove loose bound layers. LMP solution (0.1% w/w) and Bt-Ext (0.2%) were prepared as previously stated for these tests. A significant frequency drop was observed upon injection of LMP, confirming its interaction with the surface of the gold sensor. Electrostatic interaction could be contributed for such an interaction, as the pKa of LMP is close to 3.5 (Ström et al., 2014) and the isoelectric point of gold sensor is 5.2 (Cuddy et al., 2013), which operating at pH 3.2, means the surface of the sensor could be positively charged and LMP might have negatively charged patches of  $\text{COO}^-$ . This strong interaction was confirmed even after the buffer wash step, as LMP was formed a stable layer.

Afterward, the Bt-Ext solution was injected for a sufficient length of time (approx. 25 min) to ensure that the recorded signals were stable. The data show 7% mass of Bt-Ext as adsorbed initially over LMP layer; however, at the final step, upon rinsing with buffer, we observed 7.7% of mass removal, Fig. 7b. This could render physical interaction of Bt-Ext molecules in a formed layer of LMP, or weak surface interaction because of negative charges of betacyanins at pH 3.0—isoelectric point of betacyanins is around 1–2. Also, the final buffer wash step confirmed the weak interaction as the adsorbed Bt-Ext was completely washed out. Therefore, as there is no direct binding between LMP and betalains at pH 3.2, LMP could lower the water activity of the solution and protects Bt-Ext indirectly (Girhammar and Nair, 1992; Serris and Biliaderis, 2001).

### 3.5. ATR-FTIR analysis on the samples before and after storage

Three different samples (Bt-Ext, Bt-Ext + AA + EDTA, Bt-Ext + AA + EDTA + LMP) were prepared, stored for 10 days, and freeze-dried for ATR-FTIR analysis. Fig. 8a indicates ATR-FTIR results of Bt-Ext in 0 and 10 days. A characteristic group of bands attributed to carbohydrates is exhibited in the 1750–900  $\text{cm}^{-1}$  region (Biswas et al., 2013). The degradation products of betalains include yellow chemical compounds such as betalamic acid, neobetanin, cyclo-Dopa-5-O-b-glucoside, and isobetalamic acid may also be released (Herbach et al., 2006). Different

functional groups were generated in the 10 days sample compared with 0 day, indicating degradation of betacyanins. Major changes happened in the 1250–1100  $\text{cm}^{-1}$ , 1450–1300  $\text{cm}^{-1}$ , and 1750–1675  $\text{cm}^{-1}$  regions. However, smaller changes were observed for Bt-Ext with 200 ppm AA and 10 ppm EDTA at 0 and 10 days (Fig. 8b), which proves that the combination of AA and EDTA protects red color of Bt-Ext. In addition, no obvious difference has been observed on Bt-Ext with 200 ppm AA, 10 ppm EDTA and 0.25% (w/w) LMP in 0 and 10 days (Fig. 8c).

### 3.6. Long-term storage study in a sports beverage model

Bt-Ext with or without 0.25% (w/w) LMP and a combination of 100 ppm AA and 5 ppm EDTA are incorporated in a sports beverage (pH 3.2). Fig. 9 shows images of color changes of different samples in 45 days. The red color of the Bt-Ext sample completely disappeared in 20 days. Bt-Ext with 0.25% (w/w) LMP and a combination of 100 ppm AA and 5 ppm EDTA samples present slightly better red color protection compared with Bt-Ext with a combination of 100 ppm AA and 5 ppm EDTA from 20 days. Compared with the commercial red colored sports beverage, our samples with stabilizers exhibit acceptable red color in 45 days. The results showed that the synergistic effects of AA, EDTA, and LMP work in actual acidified recipes.

In this study, we have discovered and shown that the synergistic effects of different polysaccharides and antioxidants can potentially protect the red color of Bt-Ext in acidic solutions (pH 3.2). The screening studies showed that Alg and LMP help increase the color stability of Bt-Ext in acidic solution (pH 3.2) during thermal treatment. Further, Bt-Ext with a combination of 200 ppm AA and 10 ppm EDTA has ideal betacyanins remaining ( $54.05 \pm 5.73\%$ ) in light stability studies at 15 days (compared to just AA or EDTA binary solution). In addition, the combination of AA, EDTA, and LMP helps stable Bt-Ext in acidic solution (pH 3.2) in storage studies. Although QCM-D results did not indicate any chemical binding between LMP and Bt-Ext, the ATR-FTIR results confirmed the effectiveness of the selected method on Bt-Ext stabilization. The red color stabilized by AA, EDTA, and LMP was stable for 45 days in a model sports beverage recipe. In conclusion, combination of 200 ppm AA, 10 ppm EDTA, and 0.25% (w/w) LMP exhibits synergistic effect on Bt-Ext stabilization. This study proposed a new way to further improve betacyanins stability in acidic solutions. The synergistic effect of AA, EDTA, and LMP provides references for other similar studies. In the future, chemical interaction mechanisms of betacyanins stability should be recommended for further studies.

### CRediT authorship contribution statement

**Qi Guo:** Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft. **Zhong Zhang:** Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft. **Younas Dadmohammadi:** Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft. **Ying Li:** Methodology, Investigation, Data curation. **Alireza Abbaspourrad:** Supervision, Conceptualization, Project administration, Funding acquisition.

### Declaration of competing interest

The authors have no conflicts of interest to declare.

### Acknowledgment

The authors would like to thank Nanoscience Instruments and Biolin Scientific for providing the QCM-D equipment, the QSense Analyzer, and for their generous technical support.

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