Synergistical Effects of Ascorbic Acid, Low Methoxy Pectin, and EDTA on Stabilizing the Natural Red Colors in Acidified Beverages

A Project Paper

Presented to the Faculty of the Graduate School

of Cornell University

in Partial Fulfillment of the Requirements for the Degree of

Master of Professional Studies in Agriculture and Life Sciences

Field of Food Science

by

Qi Guo[1], Zhong Zhang[1], Younas Dadmohammadi, Ying Li, Alireza Abbaspourrad*

May 2020

[1] Equally contributing author
* Corresponding author

Department of Food Science, College of Agriculture and Life Sciences, Cornell University, Ithaca 14853, NY, USA

Correspondence: Alireza Abbaspourrad*, Department of Food Science, College of Agriculture and Life Sciences, Cornell University, Ithaca 14853, NY, USA. Tel: (607) 255-2923. Email: alireza@cornell.edu

ABSTRACT

Betacyanin (*i.e.,* the red pigment found in beet root) is one of the reddish to violet betalain pigments, and it provides a type of natural red colorant. Unfortunately, betacyanin is unstable under an oxygen atmosphere, greatly limiting its application and commercial potential. In order to improve the betacyanin color instability, we explored and utilized synergistic effects between ascorbic acid (AA), polysaccharides, and Ethylenediaminetetraacetic acid (EDTA) to significantly improve the color stability of betacyanin in acidified conditions. We found that alginate and low methyl pectin (LMP), among the thirteen studied polysaccharides, increased the red color stability of beet root extract in the acidic solution (pH 3.2) during thermal treatment. Further, beet root extract with a combination of 200 ppm AA and 10 ppm EDTA kept 54.05±5.73% of its red color, proving to be much more effective compared to other combinations of ascorbic acid (0 - 300 ppm) and EDTA (0 - 25 ppm) in light stability studies after 15 days. Our results proved that there is a synergistic effect between polysaccharides, AA, and EDTA, for enhancing the betacyanin color stability. Further, the red color in Gatorade recipe protected by the LMP, AA, and EDTA was stable for up to 45 days at room temperature and under natural light. In short, we found that the combination of 200 ppm AA, 10 ppm EDTA, and 0.25% (w/w) LMP could synergistically stabilize betacyanin in acidified beverages, which was confirmed through ATR-FTIR analysis. However, Quartz crystal microbalance with dissipation analysis results showed no binding between LMP and beetroot extracts, indicating the protection of red colors due to the synergistic antioxidating effect rather than hydrophobic interactions or others.

Keywords: Beetroot extracts; Color stabilization; Betacyanin; Low methoxy pectin; Ascorbic acid; EDTA; Betalain

BIOGRAPHICAL SKETCH

Qi Guo was born and grew up in Beijing, China. She completed her Bachelor of Food Science and Engineering in Collage of Biological Sciences and Biotechnology at Beijing Forestry University. She then came to Cornell University to pursue a MPS degree in Dr. Alireza Abbaspourrad's lab, working on the Betacyanin color stability. Qi work hard and has a passion on food and food science.

I would like to dedicate this work to my dear mother and father.

谨以此论文献给我亲爱的父母。

ACKNOWLEDGMENTS

Deepest thanks to my advisor, Dr. Alireza Abbaspourrad and my lab mates for their help and support. Without their help, this project would not have been possible to be done.

First and foremost, I would like to express my deepest appreciation to my advisor Dr. Alireza Abbaspourrad, without whom it would not have been possible for me to realize this project.

Secondly, I would like to thank Zhong Zhang and Younas Dadmohammadi, both are Post Doctor students in Dr. Abbaspourrad's lab, for their patient support and guidance, both academically and psychologically, throughout this project.

Then, I would like to thank Mr. Peter Lawrence, for providing me training on laboratory safety and instruments usage, and efficiently supporting me on lab materials throughout this project.

In addition, I would like to applaud all my lab mates Ying Li for her advice.

Also, I would like to thank my family and friends for all of their encouragement.

Last, I would like to thank Nanoscience and Biolin Scientific for providing the QCM-D equipment, the QSence Analyzer, and for their generous technical support.

TABLE OF CONTENTS

Biographical Sketch v
Dedication vi
Acknowledgements vii
1.Introduction 1
2.Material and Methodology 3
- 2.1.Materials and Chemicals
- 2.2.Extraction of red colorants from beet root
- 2.3.Screening of polysaccharides for betacyanin stabilization
- 2.4.Light stability studies of antioxidants in acidic solution
- 2.5.Short-term storage stability of Bt-Ext: effect of polysaccharides and antioxidants
- 2.6.UV-vis spectrophotometry
- 2.7.Colorimetry
- 2.8.Quartz crystal microbalance with dissipation monitoring (QCM-D)
- 2.9.Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR)
- 2.10.Validation of the method in a sports beverage model
- 2.11.Statistical analysis

3.Results and Discussion 8
- 3.1.Effect of polysaccharides on Bt-Ext stability in acidic solutions
- 3.2.Effect of antioxidants on Bt-Ext stability in acidic solutions during light treatment
- 3.3.Optimization of Bt-Ext with polysaccharides and antioxidants in acidic solutions
- 3.4.QCM-D measurements
- 3.5.ATR-FTIR analysis on the samples before and after storage
- 3.6.Long-term storage study in a sports beverage model

4.Conclusion 15
References 16
Figures 18

1. INTRODUCTION

Food products formulated with natural colorants are increasingly demanded recently [1]. Betacyanin, typically extracted from beet root, is getting more attention as a type of natural red colorant. Betacyanin (reddish to violet) is one major category of betalains, which are a class of red and yellow indole-derived pigments found in plants of the Caryophyllales. Beetroot red has been approved as food/color additives in US [2]. However, betacyanin is unstable under light and oxygen atmosphere, which greatly limits its application and commercial potential. Temperature, water activity, light, pH, and the presence of oxygen and metal cations can all influence the degradation speed of betacyanin [3].

A variety of stabilization methods have been proposed to stabilize the betacyanin [4]. Among them, the encapsulation of betacyanin using various shell materials has been broadly investigated [5]. For example, polysaccharides (mainly Xanthan gum, guar gum, gelatin, and gum Arabic) have been reported to lower the hygroscopicity and increase the stability of betacyanin after encapsulation [6-9]. Although some improvements have been achieved in further stabilizing the natural colorant through encapsulation, this technique has received much criticism because of the employment of energy-intensive processes such as spray drying and freeze-drying [10]. Our previous study showed that some polysaccharides could stabilize betacyanin in aqueous solutions at different pH through complex formation [11]. Nevertheless, the role of polysaccharides to enhance the stability of betacyanin has not been explored considering a wide range of available polysaccharides.

In addition, antioxidants have been reported as able to stabilize betacyanin. Ascorbic acid and iso-ascorbic acid can remove oxygen and protect betacyanin from oxidation [12, 13]. As chelating agents, citric acid, and EDTA also help with betacyanin stabilization by making the heavy metal ions unavailable [14]. In many cases have these agents have been found to stabilize betacyanin in real food systems. For instance, reports emphasize that encapsulated betacyanin can maintain its stability after storage in gummies and chewing gums [15, 16]. To date, however, research lacks any study on synergistic effects of ascorbic acid (AA), Ethylenediaminetetraacetic acid (EDTA), and polysaccharides to further stabilize the betacyanin.

In this study, we first screened up to thirteen types of polysaccharides, such as carboxymethyl cellulose, Lambda-carrageenan, Kappa-carrageenan, Iota-carrageenan, HMP, Gum Arabic, Tara Gum, Xanthan gum, Konjac Gum, Gum Guar, Locust Bean Gum, LMP, and Alg, that could potentially protect betacyanin from degradation on thermal and light. Second, we designed different combinations of polysaccharides and antioxidants in order to explore whether synergistic effects existed. In addition, we also used characterization techniques such as FT-IR and QCMD to confirm the protection of betacyanin through these discovered synergistic effects. Finally, a simulated Gatorade recipe was used to validate such synergistic effects in real solution and storage conditions.

2. MATERIALS AND METHODS

2.1Materials and chemicals

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 (Konjac Gum), TIC Pretested® Gum Guar 8/22 Powder (Gum Guar), and TIC Pretested® Locust Bean Gum PORIA Power (Locust Been Gum) were provided by TIC gum (White Marsh, MD, US). Sodium alginate (Manugel GHB; Alg) (Alg) was purchased from FMC Biopolymer (Philadelphia, PA, US). (Ethylenedinitrilo) tetra-acetic 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), citrate acid (food graded), and other chemicals used for beverage were purchased from Sigma Aldrich (St. Louis, MO, US).

2.2. Extraction of red colorants from beet root

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°C until used.

2.3. Screening of polysaccharides for betacyanin stabilization

Thirteen different polysaccharides such as carboxymethyl cellulose, Lambda-carrageenan, Kappa-carrageenan, Iota-carrageenan, HMP, Gum Arabic, Tara Gum, Xanthan gum, Konjac Gum, Gum Guar, Locust Bean Gum, LMP, and Alg were screened, targeting betacyanin stabilization. An aqueous solution of 0.25% (w/w) of each polysaccharide was prepared and combined with the Bt-Ext at 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°C for 16 hr. 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 hr for accelerated studies. The betacyanin's color intensity was measured every 36 hr 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 betacyanin 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°C, 10 mins), and stored under natural light (Ithaca, NY) for 15 days (September through October). The degradation of betacyanin was measured on day-6 and day-15.

2.6. UV-vis spectrophotometry

Spectroscopy has been used for detecting betacyanin 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). Betacyanin remaining (%) is calculated based on the followed equation.

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

2.7. Colorimetry

To access color changes of prepared solutions, we followed CIE system (Francis & 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). Color changes (ΔE^*_{ab}) and red color remaining (% a* remaining) were assessed according to the following equations.

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

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

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 QCM-D technique. In this study, we used QSence Explorer machine (Biolin Scientific,

Gothenburg, Sweden) equipped with gold sensor (QSX 301). Briefly, we employed this machine to monitor changes due to frequency (Δf) and dissipation (ΔD) of the sensor. This information can be further post-processed through DFind software using the composite Sauerbrey equation to quantify the mass (Sauerbrey, 1959)[11, 17]. 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°C±2) and at the flowrate 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 unbounded LMP) was removed. This assures the formation of uniform and stable LMP layer on the sensor. Then, Bt-Ext were run over the coated sensors at the same flow rate. Finally, 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 were prepared and stored for 10 days in daylight. Samples of solutions at 0 day and 10 days were freeze-dried for the measurements. FTIR spectra of samples were taken on 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 cm^{-1} with a resolution of 2 cm^{-1}.

2.10. Validation of the method in a sports beverage model

The basic sports beverage formulation was derived from Gatorade nutrition facts as follows: sodium citrate dihydrate (1.95 g/1000 ml), monopotassium phosphate (0.44 g/1000ml), citric acid monohydrate (3.70 g/1000 ml), dextrose (57.53 g/1000 ml). The solutions of 0.2% (w/w) Bt-Ext with or without AA, EDTA, and LMP, were incorporated in the beverage model. Final pH was adjusted to 3.2 with citric acid, and the beverages were pasteurized for storage at 95°C (internal solution temperature 90°C, 2.5 min). Final products were bottled in plastic containers, stored under daylight. Photos were taken periodically to monitor color change during storage.

2.11. Statistical analysis

All analyses were performed in triplicate and data reported as mean ± 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.

4. RESULTS AND 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 hr with light exposure and tested by the microplate reader at 529 nm. The betacyanin remaining (%) was calculated and used to indicate the protection effect of these polysaccharides. **Figure 1** shows the betacyanin remaining (%) of Bt-Ext with or without polysaccharides after 16 hr 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±0.95%, 48.50±1.35%, 47.03±0.45% and 48.43±0.55%, respectively. The Bt-Ext with Alg presents the highest betacyanin retention. Therefore, four polysaccharides, *i.e.,* Alg, Tara gum, LMP and Xanthan gum, were selected to move forward with further testing under pH 3.2, 16 hr and 40°C.

Figure 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 acidic show higher red color retention (*a*-value of 75.40±1.05% and 73.23±0.85%, respectively) compared to the Bt-Ext only solution (*a*-value of 68.13±0.74%). Similar trend is confirmed by spectrophotometric readings (**Figure 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 is not as effective as LMP and Alg in protecting the red colors after excluding the effect of polysaccharides on the absorbance. Previous research has shown that pectin and alginate can be used as an effective wall material in the encapsulation of betacyanin [18, 19].

However, it is uncertain why pectin and alginate can help protect the betacyanin in Bt-Ext. It is believed that binding interactions between beet pigment and two of the polysaccharides may contribute to color stability.

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 hr during thermal treatment (12 hr, 40°C). Absorbance decrease of the three samples are indicated in **Figure 3a** with betacyanin degradation rate: Bt-Ext > Bt-Ext with LMP > Bt-Ext with Alg. The red color (*a*-value) retention curves also show similar results (**Figure 3b**). ANOVA analysis showed a significance difference between the mixtures of Bt-Ext/polysaccharides and Bt-Ext, but no significant difference between Bt-Ext with Alg and Bt-Ext with LMP. **Figure 3c, d, and e** show the UV-vis spectra of Bt-Ext, Bt-Ext with Alg, and Bt-Ext with LMP, at 0 hr and 8 hr, which indicates smaller absorbances change when Bt-Ext with Alg and LMP. Considering the above results, in this study we conclude that Alg and LMP help increase the betacyanin stability of Bt-Ext in acidic condition (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 betacyanin during storage. The mixture of Bt-Ext and antioxidants at pH 3.2 were prepared and exposed to UV light for 36 and 72 hr. The absorbances of the samples were evaluated in the microplate reader at 529 nm. **Figure 4a** shows betacyanin remaining (%) of Bt-Ext with or without antioxidants in 36 hr. On the one hand, lower betacyanin remaining (%) was found in Bt-

Ext with gallic acid and catechin (48.90±0.52% and 49.97±2.75%, respectively) compared with Bt-Ext itself (57.33±2.55%), which is consistent with previous studies [20, 21]. On the contrary, Bt-Ext with AA and EDTA exhibit much higher betacyanin remaining (%) (72.50±0.87% and 63.67±0.76%, respectively, $P<0.05$). In fact, previous studies have proved that AA helps stabilize betacyanin in a real food system [21-24]. EDTA has also been reported as a stabilizer of betanin [14]. A similar trend was found in the 72 hr (**Figure 4b**). Interestingly, EDTA preserves nearly the same content of betacyanin as AA in 72 hr, which suggests that both EDTA and AA are essential in preservation of betacyanin in long term storage [12, 14]. However, the AA and EDTA protects betacyanin through different mechanisms: AA protect betacyanin 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 betacyanin during storage.

The synergistic effect of AA and citric acid on betacyanin stability has been studied, but not with EDTA involved [20]. 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 for testing. The 25 samples were stored in daylight for 15 days. Color changes were measured and betacyanin remaining (%) were calculated for day-6 and day-15. **Figure 5a** presents betacyanin retention of Bt-Ext with or without combination of different concentration of AA and EDTA in day light for 6 days. Among them, Bt-Ext with combination of 100 ppm AA and 5 ppm EDTA exhibit highest betacyanin remaining (%) (80.53±3.10%), which is 40 % higher than Bt-Ext without any antioxidants. Highest betacyanin remaining (%) (54.05±5.73%) is shown for the Bt-Ext sample with combination of 200 ppm AA

and 10 ppm EDTA on day 15 (**Figure 5b**). While several 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) still have a relative higher betacyanin remaining (%) compared with Bt-Ext (25.93±0.12%) or Bt-Ext with combination of 100 ppm AA and 5 ppm EDTA (48.07±3.17%). The use of EDTA is allowed by FDA. Dosage of EDTA for color protection in acidic beverage must be less than 100 ppm, and no restriction is reported for AA [25].

Images of Bt-Ext with or without combination of different concentration of AA and EDTA on days 0, 6 and 15 were recorded (**Figure 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 [26], a combination of 200 ppm AA and 10 ppm EDTA are 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 color stability of Bt-Ext, separately. To further evaluate the effect of 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. Color change was measured with a colorimeter. **Figure 6a and 6b**

indicates the percentage of red color retained and ΔE^*_{ab} with daylight exposure for the four samples in 5 days. Bt-Ext solution without antioxidant and polysaccharides has been preserved lowest red color (49.57±7.64%) compared with other three samples, also, highest ΔE^*_{ab} value of Bt-Ext suggests biggest total color change, while AA/EDTA/polysaccharides improve color stability. Similar trends are also observed on day 10 (**Figure 6c** & **6d**).

However, according to **Figure 6e**, significant difference ($P<0.05$) in red color retained between 4 samples appeared on 20 days. The order in red color retention is Bt-Ext with AA, EDTA, and LMP > Bt-Ext with AA, EDTA, and Alg > Bt-Ext with AA and EDTA > Bt-Ext. ΔE^*_{ab} value decreased (**Figure 6f**) due to an increase of 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 **Figure 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, mass added over the sensor. **Figure 7a** exhibits simultaneous recorded frequencies shift (f3, f5, f7, f9, f11, and f13) and corresponding dissipations (D3, D5, D7, D9, D11, and D13) *vs.* time at each of 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 frequencies 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 [27] and the isoelectric point of gold sensor is 5.2 [28], which at operating pH 3.2, means the surface of the sensor could be positively charged and LMP might have negatively charged patches of 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-Ex as adsorbed initially over LMP layer; however, at final step, upon rinsing with buffer, we observed 7.7% of mass removal, **Figure 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 betacyanin at pH 3.0—isoelectric point of betacyanin is around 1-2. Also, the final buffer wash step confirmed the week 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 water activity of the solution and protects Bt-Ext indirectly [29, 30].

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

Three different samples (Bt-Ext, Bt-Ext with AA and EDTA, Bt-Ext with AA, EDTA, and LMP) were prepared, stored for 10 days, and freeze dried for FTIR analysis. **Figure 8a** indicates ATR-FTIR results of Bt-Ext in 0 and 10 days. Characteristic group of carbohydrates are exhibited in 1750–900 cm^{-1} region [31]. The degradation products of betalains includes yellow chemical compounds such as betalamic acid, neobetanin, cyclo-Dopa-5-O-b-glucoside, and isobetalamic

acid may also be released [22]. Different functional groups generated in the 10 days sample compared with 0 day, indicating degradation of betacyanin. Major changes happened in the 1250–1100 cm^{-1}, 1450–1300 cm^{-1}, and 1750–1675 cm^{-1} regions. However, smaller changes were observed for Bt-Ext with 200 ppm AA and 10 ppm EDTA at 0 and 10 days (**Figure 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 (**Figure 8c)**. Thus, the effectiveness of using AA/EDTA/LMP on stabilizing the Bt-Ext has been confirmed by ATR-FTIR results.

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

Bt-Ext with or without 0.25% (w/w) LMP and combination of 100 ppm AA and 5 ppm EDTA are incorporated in a sports beverage (pH 3.2). Gatorade (watermelon flavor) were purchased from local market as a reference sample. **Figure 9** shows images of color changes of different samples in 45 days. The red color of Bt-Ext sample completely disappeared in 20 days. Bt-Ext with 0.25% (w/w) LMP and combination of 100 ppm AA and 5 ppm EDTA samples presents slightly better red color protection compared with Bt-Ext with combination of 100 ppm AA and 5 ppm EDTA from 20 days. Compared with commercial Gatorade 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 real acidified recipes.

5. CONCLUSION

In this study, we have discovered and shown that the synergistic effects of different polysaccharides and antioxidants can significantly protect red color of Bt-Ext in acidic solutions (pH 3.2). The screening studies showed that Alg and LMP helps increase 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 betacyanin remaining (54.05±5.73%) in light stability studies at 15 days (compared to AA only results and EDTA only results). 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 effectiveness on selected method on Bt-Ext stabilization. The red color stabilized by AA, EDTA, and LMP was stable for 45 days in a Gatorade 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 significantly improve betacyanin stability in acidic solutions. Synergistic effect of AA, EDTA, and LMP provides references for other similar researches. In the future, chemical mechanisms of betacyanin stability should be recommended for further studies.

REFERENCES

1. Tuli, H. S.; Chaudhary, P.; Beniwal, V.; Sharma, A. K., Microbial pigments as natural color sources: current trends and future perspectives. *Journal of food science and technology* **2015,** *52* (8), 4669-4678.

2. Esatbeyoglu, T.; Wagner, A. E.; Schini-Kerth, V. B.; Rimbach, G., Betanin—A food colorant with biological activity. *Molecular nutrition & food research* **2015,** *59* (1), 36-47.

3. Herbach, K. M.; Stintzing, F. C.; Carle, R., Betalain stability and degradation—structural and chromatic aspects. *Journal of food science* **2006,** *71* (4), R41-R50.

4. Khan, M. I., Stabilization of betalains: A review. *Food Chemistry* **2016,** *197*, 1280-1285.

5. Chronakis, I. S., On the molecular characteristics, compositional properties, and structural-functional mechanisms of maltodextrins: a review. *Critical reviews in food science and nutrition* **1998,** *38* (7), 599-637.

6. Barbosa, M.; Borsarelli, C. D.; Mercadante, A. Z., Light stability of spray-dried bixin encapsulated with different edible polysaccharide preparations. *Food Research International* **2005,** *38* (8-9), 989-994.

7. Fang, Z.; Bhandari, B., Encapsulation of polyphenols–a review. *Trends in Food Science & Technology* **2010,** *21* (10), 510-523.

8. Ravichandran, K.; Palaniraj, R.; Saw, N. M. M. T.; Gabr, A. M. M.; Ahmed, A. R.; Knorr, D.; Smetanska, I., Effects of different encapsulation agents and drying process on stability of betalains extract. *Journal of Food Science and Technology* **2014,** *51* (9), 2216-2221.

9. Castro-Muñoz, R.; Barragán-Huerta, B. E.; Yáñez-Fernández, J., Use of gelatin-maltodextrin composite as an encapsulation support for clarified juice from purple cactus pear (Opuntia stricta). *LWT-Food Science and technology* **2015,** *62* (1), 242-248.

10. Nath, S.; Satpathy, G. R., A SYSTEMATIC APPROACH FOR INVESTIGATION OF SPRAY DRYING PROCESSES. *Drying Technology* **1998,** *16* (6), 1173-1193.

11. Marchuk, M.; Selig, M. J.; Celli, G. B.; Lawrence, P.; Smilgies, D.-M.; Abbaspourrad, A., Mechanistic investigation via QCM-D into the color stability imparted to betacyanins by the presence of food grade anionic polysaccharides. *Food Hydrocolloids* **2019,** *93*, 226-234.

12. Attoe, E. L.; Von Elbe, J. H., Degradation kinetics of betanin in solutions as influenced by oxygen. *Journal of Agricultural and Food Chemistry* **1982,** *30* (4), 708-712.

13. Strack, D.; Vogt, T.; Schliemann, W., Recent advances in betalain research. *Phytochemistry* **2003,** *62* (3), 247-269.

14. Pasch, J. H.; Von Elbe, J. H., Betanine stability in buffered solutions containing organic acids, metal cations, antioxidants, or sequestrants. *Journal of food Science* **1979,** *44* (1), 72-75.

15. Chranioti, C.; Nikoloudaki, A.; Tzia, C., Saffron and beetroot extracts encapsulated in maltodextrin, gum Arabic, modified starch and chitosan: Incorporation in a chewing gum system. *Carbohydrate polymers* **2015,** *127*, 252-263.

16. Otálora, M. C.; de Jesús Barbosa, H.; Perilla, J. E.; Osorio, C.; Nazareno, M. A., Encapsulated betalains (Opuntia ficus-indica) as natural colorants. Case study: Gummy candies. *LWT* **2019,** *103*, 222-227.

17. Rodahl, M.; Höök, F.; Fredriksson, C.; Keller, C. A.; Krozer, A.; Brzezinski, P.; Voinova, M.; Kasemo, B., Simultaneous frequency and dissipation factor QCM measurements of biomolecular adsorption and cell adhesion. *Faraday discussions* **1997,** *107*, 229-246.

18. Rodríguez-Sánchez, J. A.; Cuatzo-Lozano, M. I.; Pérez-Loredo, M. G.; Abarca-Sarro, D. I.; Navarro, Y. G.,

Alginate encapsulation as a preservation method of pitaya fruit juice (Stenocereus spp.). *Journal of Food Science and Engineering* **2017,** *7* (3).

19. Lejeune, B.; Pouget, M. P.; Pourrat, A., Le rouge de betterave. Essais de stabilisation et utilisation dans la formulation des gels. *Problematic Technol* **1983,** *31*, 638-643.

20. Attoe, E. L.; Von Elbe, J. H., Oxygen involvement in betanine degradation: effect of antioxidants. *Journal of Food Science* **1985,** *50* (1), 106-110.

21. Khan, M. I.; Giridhar, P., Enhanced chemical stability, chromatic properties and regeneration of betalains in Rivina humilis L. berry juice. *LWT-Food Science and technology* **2014,** *58* (2), 649-657.

22. Herbach, K. M.; Rohe, M.; Stintzing, F. C.; Carle, R., Structural and chromatic stability of purple pitaya (Hylocereus polyrhizus [Weber] Britton & Rose) betacyanins as affected by the juice matrix and selected additives. *Food Research International* **2006,** *39* (6), 667-677.

23. Moßhammer, M. R.; Rohe, M.; Stintzing, F. C.; Carle, R., Stability of yellow-orange cactus pear (Opuntia ficus-indica [L.] Mill. cv.'Gialla') betalains as affected by the juice matrix and selected food additives. *European Food Research and Technology* **2007,** *225* (1), 21-32.

24. Leong, H. Y.; Ooi, C. W.; Law, C. L.; Julkifle, A. L.; Pan, G.-T.; Show, P. L., Investigation of betacyanins stability from peel and flesh of red-purple pitaya with food additives supplementation and pH treatments. *LWT* **2018,** *98*, 546-558.

25. Bauernfeind, J. C., Ascorbic Acid Technology in Agricultural, Pharmaceutical, Food, and Industrial Applications. In *Ascorbic Acid: Chemistry, Metabolism, and Uses*, AMERICAN CHEMICAL SOCIETY: 1982; Vol. 200, pp 395-497.

26. Faiman, M. D.; Artrnan, L.; Maziasz, T., Diethyldithiocarbamic acid-methyl ester distribution, elimination, and LD50 in the rat after intraperitoneal administration. *Alcoholism: Clinical and Experimental Research* **1983,** *7* (3), 307-311.

27. Ström, A.; Schuster, E.; Goh, S. M., Rheological characterization of acid pectin samples in the absence and presence of monovalent ions. *Carbohydrate polymers* **2014,** *113*, 336-343.

28. Cuddy, M. F.; Poda, A. R.; Brantley, L. N., Determination of isoelectric points and the role of pH for common quartz crystal microbalance sensors. *ACS applied materials & interfaces* **2013,** *5* (9), 3514-3518.

29. Girhammar, U.; Nair, B. M., Certain physical properties of water soluble non-starch polysaccharides from wheat, rye, triticale, barley and oats. *Food Hydrocolloids* **1992,** *6* (4), 329-343.

30. Serris, G. S.; Biliaderis, C. G., Degradation kinetics of beetroot pigment encapsulated in polymeric matrices. *Journal of the Science of Food and Agriculture* **2001,** *81* (8), 691-700.

31. Biswas, M.; Dey, S.; Sen, R., Betalains from Amaranthus tricolor L. *Journal of Pharmacognosy and Phytochemistry* **2013,** *1* (5).

Fig1 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) Carboxymethyl cellulose (CMC), Lambda-carrageenan (L-C), Kappa-carrageenan (K-C), Iota carrageenan (I-C), High methoxy pectin (HMP), Gum Arabic (GA), Alg, Tara gum (TG), LMP, and Konjac Gum (KG), Xanthan gum (XG), Gum Guar (GG) and Locust Been Gum (LG) in pH3.2.

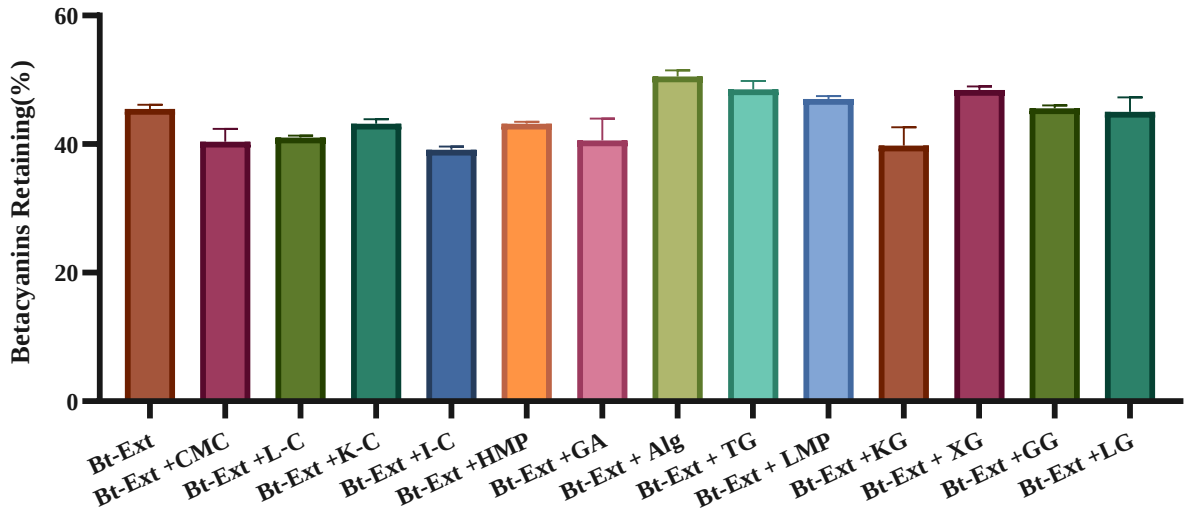


Fig2 40 °C accelerated shelf stability tests after 16 h for 0.2% (w/w) Bt-Ext with or without 0.25% (w/w) Alg, Tara gum (TG), xanthan gum (XG), and LMP in pH3.2: (a) Percentage of red color retained (% a* retained) using CIE L* a* b* colorimeter; (b) betacyanin remaining(%) using Spectra-Max iD3 Multi-Mode Microplate Reader

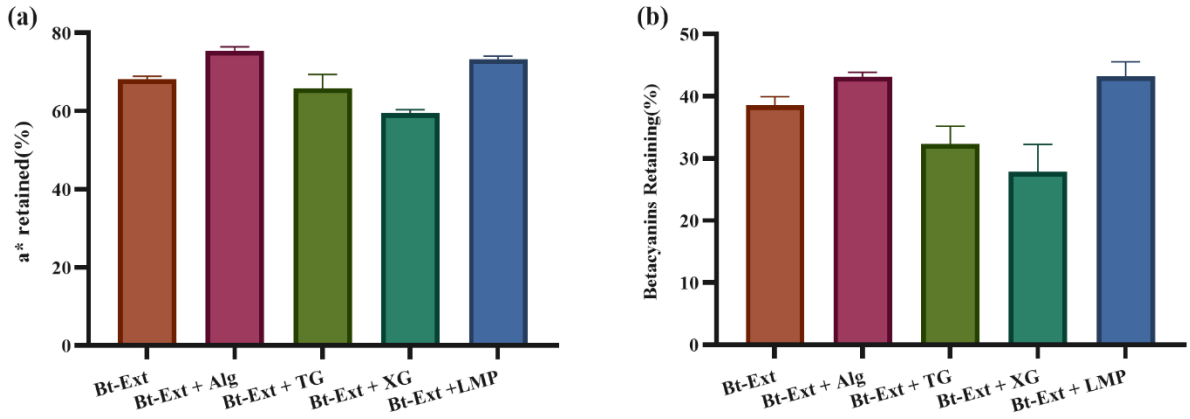


Fig3 40 °C accelerated shelf stability tests for 0.2% (w/w) Bt-Ext with or without 0.25% (w/w) Alg and LMP in pH3.2: **(a)**Declining absorbance at 529nm every 2 hours; **(b)** Declining red color retained (%a* retained) using CIE L* a* b* colorimeter; **(c, d and e)** Absorption cure of Bt-Ext, Bt-Ext with Alg and Bt-Ext with LMP from 40 °C accelerated shelf stability tests before and after 8h.

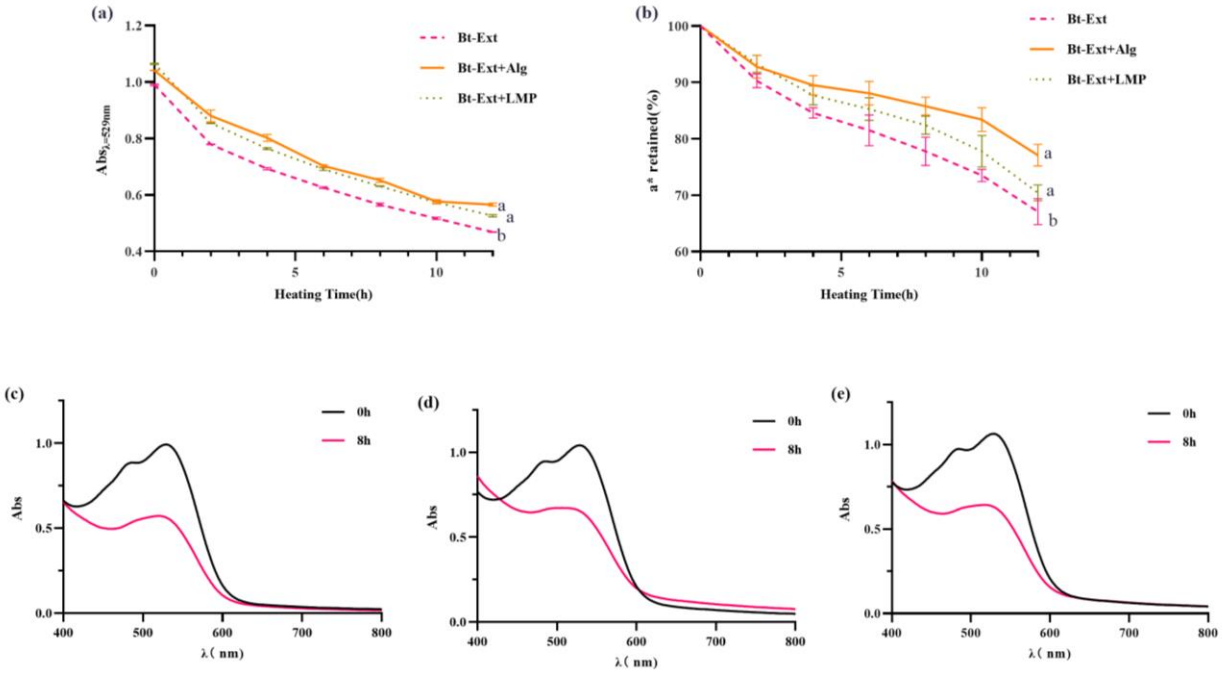


Fig4 Betacyanin remaining(%) with UV light exposure for 0.2% (w/w) Bt-Ext with or without 100ppm AA, EDTA, Gallic acid and Catechin: **(a)** 36 hours **(b)** 72 hours.

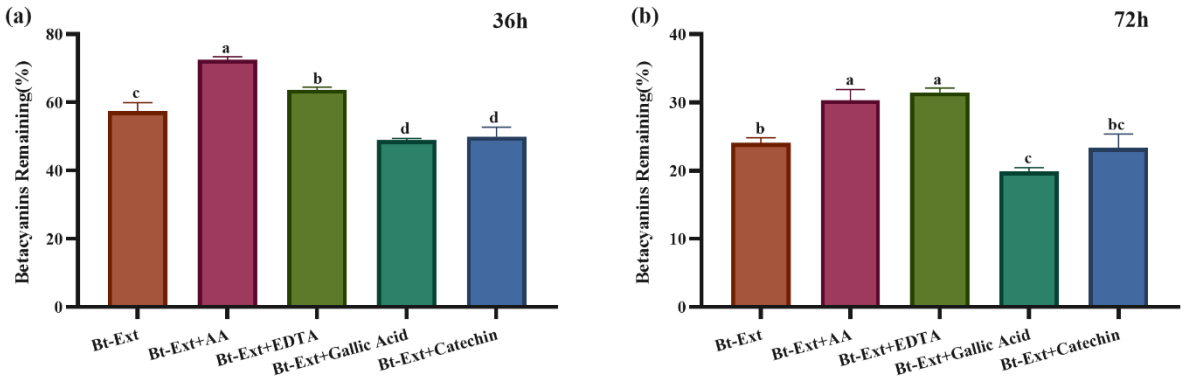
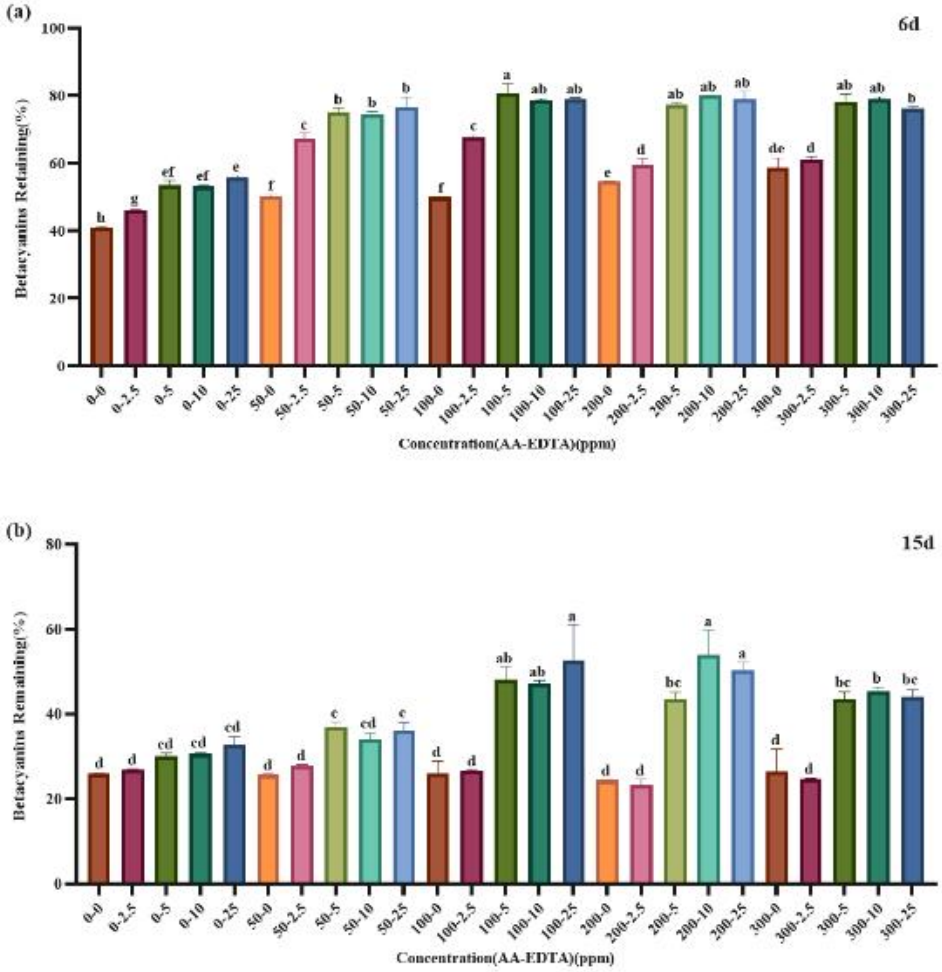


Fig5 Betacyanin remaining (%) with day light exposure for 0.2 % (w/w) Bt-Ext with or without different concentration of AA and EDTA: **(a)** 6 days **(b)** 15 days **(c)** images



(C)

day	0					6					15				
AA / EDTA	0	50	100	200	300	0	50	100	200	300	0	50	100	200	300
0															
2.5															
5															
10															
25															

Figure6 Percentage of red color retained (% a* retained) and ΔE*ab with day light exposure for 0.2 % (w/w) Bt-Ext with or without combination of AA-EDTA, Alg and LMP: **(a, b)** 5 days **(c, d)** 10 days **(e, f)** 20 days

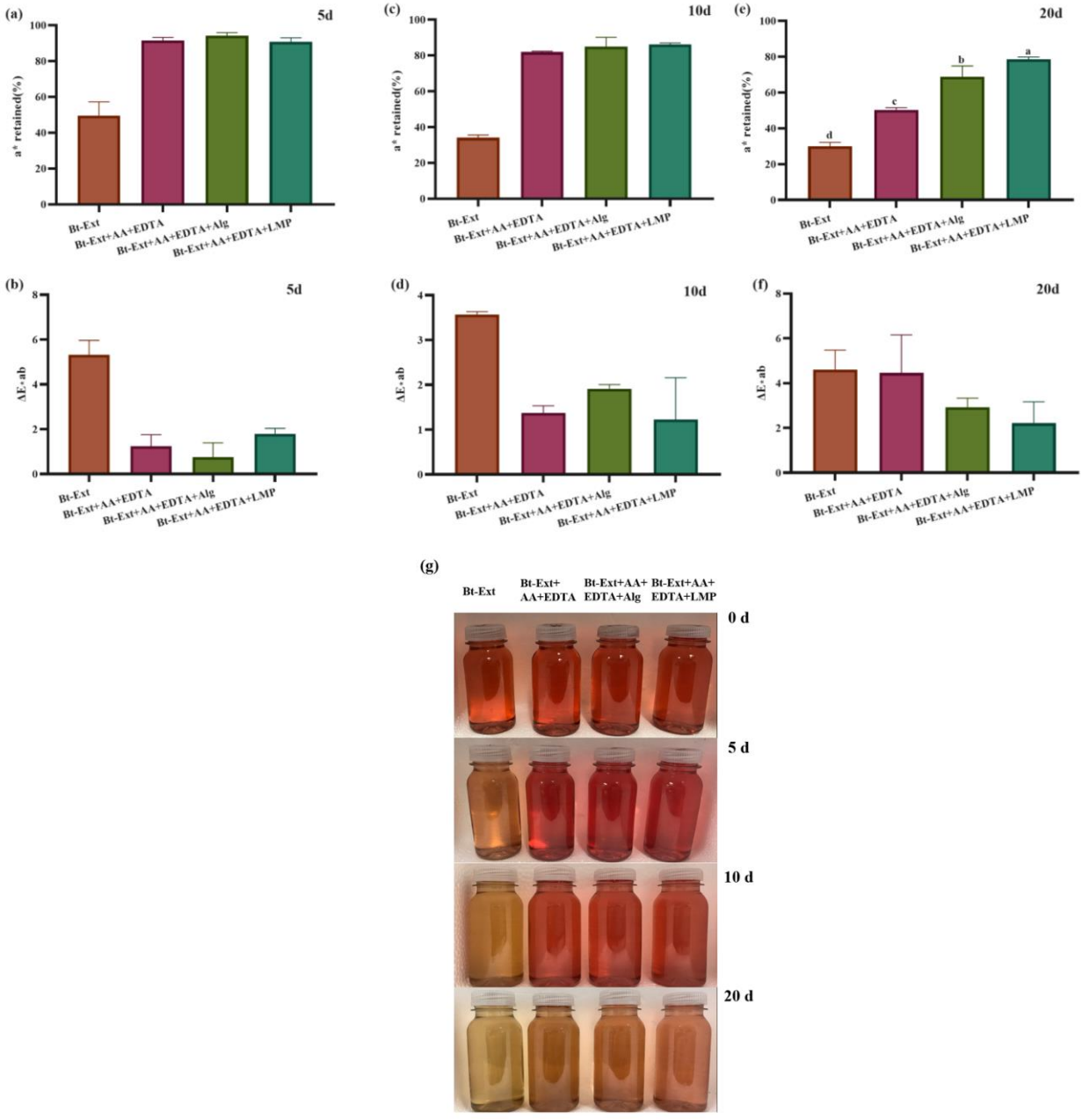


Figure7 a QCM-D frequency change data for Bt-Ext binding events at pH3.2. Data represent single experimental binding and buffer wash events that are representative of triplicate experiments for each condition tested, **b** Mass addition of Bt-Ext

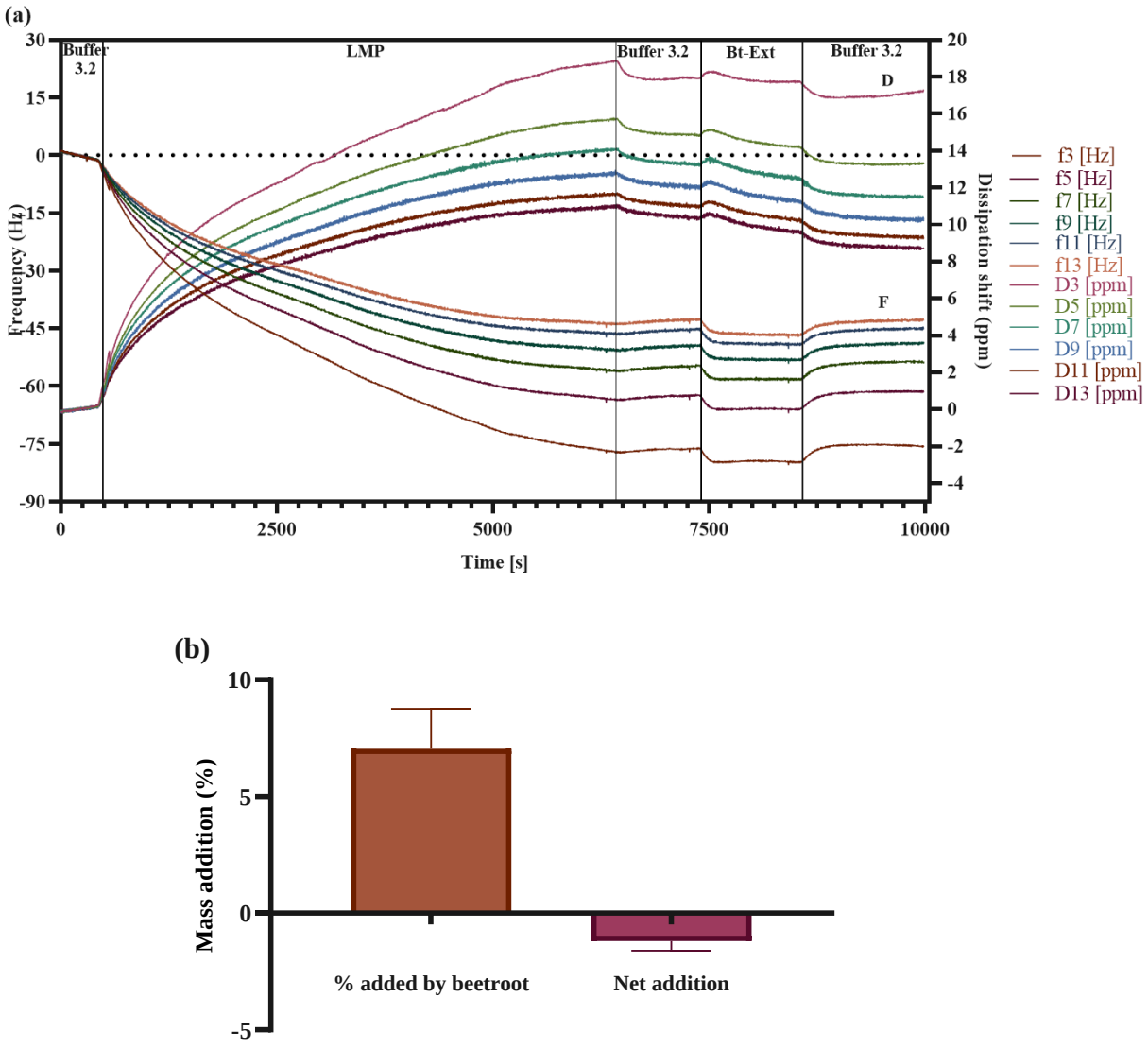


Figure8 ATR-FTIR spectra of freeze-dried samples in 0 day and 10 days: **(a)** Bt-Ext, **(b)** Bt-Ext with AA and EDTA, **(c)** Bt-Ext with AA, EDTA and LMP.

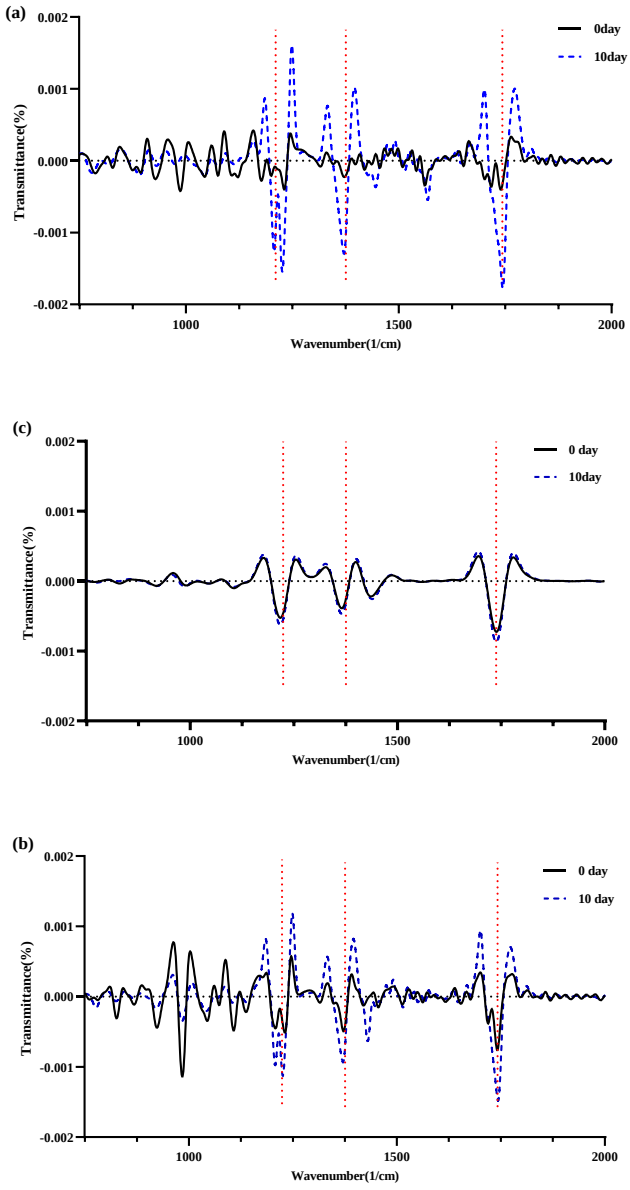


Figure9 images of storage studies conducted in a Gatorade recipe at pH 3.2: the recipe and red color was formulated with different antioxidant combinations.