MECHANISTIC INSIGHTS INTO COLOR STABILITY IMPARTED TO AQUEOUS BETACYANINS BY THE PRESENCE OF FOOD GRADE ANIONIC POLYSACCHARIDES

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 Meghan Marchuk

May 2018

ABSTRACT

The growing consumer concern surrounding synthetic dyes in food products has driven manufacturers to seek more natural color inclusion alternatives. Betalains, specifically red betacyanins, from beet powder are a promising natural solution for red color in neutral or low acid food products. The current barrier for the widespread use of betalains is their inherent instability to standard heat processing and storage conditions. As shown in this paper, certain food grade anionic polysaccharides (gum arabic, beet pectin, xanthan gum, and sodium alginate) are able to improve storage and thermal stability of red betalains to different degrees under modestly acidic (pH 5) and more acidic (pH 3.2) conditions. The study herein prevents a comprehensive look at the mechanism in which these gums impart stability on betalains by coupling shelf stability data with zeta potential, particle size, auto-fluorescence, and pigment-carbohydrate binding studies via Quartz Crystal Microbalance with Dissipation Monitoring (QCM-D). The results from our accelerated shelf life study with beet extract at 40˚C showed that, qualitatively, sodium alginate and xanthan gum were able to best preserve the red color at both pH values. Beet pectin was able to provide strong stabilization at pH 5 while gum arabic provided modest stability at pH 3.2. However, at 55˚C, xanthan gum lost its ability to impart stability under both conditions while alginate was the most effective stabilizer at pH 3.2. Beet pectin at pH 5 as well as xanthan gum and sodium alginate under both pH conditions showed the most negative zeta potential values. This finding may suggest that the negative charges on these gum substituents, and their ability to form electrostatic interactions, may play a larger role

in their stabilization mechanism. The particle size data revealed that sodium alginate might be the only polysaccharide to form a true soluble complex with beet extract in solution, which may result in its superior stabilization ability overall. The QCM-D binding studies showed that the betacyanins are able to bind strongly and irreversibly to both the xanthan gum and sodium alginate gums. On the other hand, beet pectin and gum arabic, promoted weak and reversible associations between the gums and pigment molecules. The differences seen in the binding may be attributed to the high degrees of linearity, in the case of xanthan gum and sodium alginate, or branching, as seen in both beet pectin and gum arabic. Although beet pectin exhibited weak binding interactions via QCM-D, intrinsic fluorescence studies showed that beet pectin at pH 5 was the only polysaccharide to cause a shift in the beet extract spectra, suggesting an alternative mechanism for it's ability to impart stabilization. It is our intent that the insights garnered from this study will advance the understanding of betalain stabilization and thus improve upon the applications of beet powder in food products.

BIOGRAPHICAL SKETCH

Meghan Marchuk is from London, Ontario, Canada. She received a Bachelor of Science in Chemistry from Queen's University in Kingston, Ontario in 2015. Her previous undergraduate thesis was in the field of Organic and Materials Chemistry, with a focus on creating new catalytic platforms for heterogeneous catalysis.

She is currently completing her Masters of Professional Studies degree in Food Science at Cornell University under the supervision of Dr. Alireza Abbaspourrad. This past year she has been focusing on color-related projects with her lab group as they work towards replacing synthetic food dyes with colors derived from more natural sources. Aside from her research and classes, Meghan spends her free time focusing on her product development team, IFTSA & Mars, and running a Food Science social media platform called Nonfiction Foods. She is looking forward to her next steps as she pursues a fulfilling career in the food industry.

I would like to dedicate this paper to my incredible parents, Stephen and Brenda. Mum you are the smartest woman I know, and dad, I'll never know as much as you've forgotten. Thank you for everything. I couldn't have done this without you.

ACKNOWLEDGMENTS

I would like to express my deepest appreciation to my advisor Dr. Alireza Abbaspourrad, who consistently supported my research efforts and challenged me to be a better scientist.

I would also like to thank my mentor, Michael Joseph Selig. Your unwavering patience and dedication to my development and our project is something I will remember for the rest of my career.

I want to express sincere gratitude to the members of my lab group for helping me through various technical challenges with this project, particularly Giovana Celli. I also want to acknowledge my collaborators Peter Lawrence in the Abbaspourrad group and Dr. Detlef Smilgies at the Cornell High Energy Synchrotron for making this project possible.

Finally, I would like to express my profound gratitude to the Food Science department and staff for their incredible support throughout my time at Cornell.

Thank you, everyone, for such a memorable experience.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

Background

In recent years, negative consumer perception surrounding the use of synthetic colorants in food products has driven the food industry to look for more natural alternatives. In particular, natural pigments such as anthocyanins and betalains have been used as replacements for synthetic red color in food products, such as maraschino cherries, yogurt, and jam (Giusti & Wrolstad, 1996; Wallace & Giusti, 2008; Falcão et al., 2009). Although these natural pigments offer improved consumer acceptability, their inherent instability under both processing and long-term storage conditions is a significant problem for food manufacturers. Since the color of food products is an important predictor of quality and consumer palatability, reducing degradatptonion and improving the stability of these color sources is a priority for companies looking to maintain the appeal and integrity of their products as they transition towards natural ingredient usage (Francis, 1995).

Betalains are a class of nitrogenous heterocyclic pigments that include both yellow betaxanthins and red betacyanins (**Figure 1**). Betacyanins have recently gained popularity as natural red colorants due to their high tinctorial strength and solubility in water (Stintzing & Carle, 2007). Unlike their anthocyanin counterparts, betacyanins exhibit high stability in both neutral and low-acid food products (Stintzing & Carle, 2004). Although betacyanins offer an improved alternative under certain pH conditions, they are innately sensitive to low pH (< 4.0), light,

oxygen, metallic ions, water activity, and temperature (Herbach, Stintzing, & Carle, 2006). The susceptibility of betacyanins to degradation throughout both processing and extended storage conditions has a created a large barrier to use in the food industry. There are a handful of betacyanin sources available to manufacturers although beet powder is currently the only one approved by the US Food and Drug Administration (see: https://www.fda.gov/ForIndustry/ColorAdditives/ColorAdditiveInventories/ucm106626.htm). Consequentially, the degradation mechanisms of betanin, the major source of betacyanin in beets, have been widely studied under different processing and storage conditions. In all cases, a specific color change from red to yellow is an indicator that betacyanin degradation has occurred.

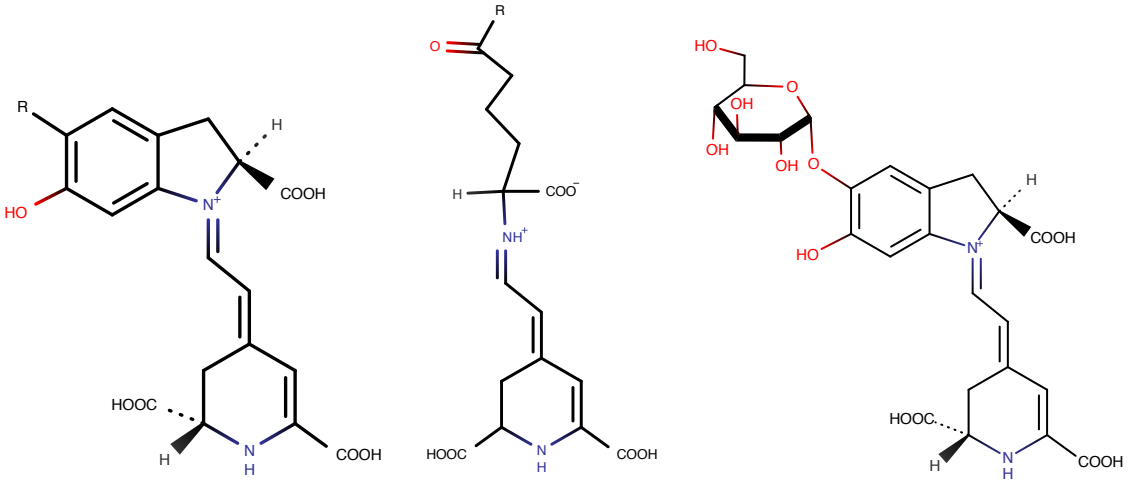


Figure 1. Betalain pigment components: red betacyanin structure (left), yellow betaxanthin structure (middle), and betanin, the major betacyanin in beet pigment (right).

Degradation

Under heat processing, betanin degrades into betalamic acid and cyclo-Dopa 5-O-glucoside to produce an unfavorable yellow color (Herbach, Stintzing, & Carle, 2006). According to Shwartz

and von Elbe (1983) this is due to hydrolytic cleavage of the aldimine bond. The development of betalamic acid, and thus degradation, can easily be monitored by evaluating peak changes in the visible spectra of betanin at 535 nm and betalamic acid at 430 nm (Saguy, Kopelman & Mizrahi, 1978). Other types of degradation pathways observed for betanin include decarboxylation, deglycosylation, and dehydrogenation. Betanin can also isomerize into iso-betanin, which follows similar degradation pathways and can break down into yellow neobetanin upon aerobic thermal treatment (Herbach, Stintzing & Carle, 2004, 2006).

Previous Stabilization Efforts

Storage methods aimed at the stabilization of betalains have focused on minimizing exposure to heat, light, oxygen, and water. Studies have shown that betacyanin stability can be maintained longer under vacuum or if blanched and then kept under dark conditions (Jackman & Smith, 1996; Ravichandran et al., 2013). While these methods are simple and effective, they are ultimately impractical solutions for food manufacturers that utilize clear, multi-use packaging or have specific thermal processing requirements. Inclusion of food additives, such as antioxidants and chelating agents, appear to be a more realistic solution for many food products. As an example, previous studies have shown the addition of ascorbic acid or small amounts of EDTA could preserve the tinctorial strength of red beet pigments (Reynoso et al., 1997; Woo et al., 2011). Furthermore, the encapsulation of betalains with certain polysaccharides has shown stabilizing effects on betalains attributed to lower water activity levels within the carbohydrate complex (Savolainen & Kuusi, 1978). Recently, our own work has shown that high pressure processing (HPP) increases the stability of beetroot-carbohydrate complexes under acidic conditions (Selig et al, 2018). While progress has been made to improve the stability of beet

pigments using common hydrocolloids, research uncovering the mechanisms that impart stability is warranted.

Research Intent

The present study attempts to provide such insights into the stabilization mechanisms imparted by anionic carbohydrates on betacyanins, as noted previously. Herein, the mild improvements to shelf stability and thermal stability of beetroot combined with beet pectin, xanthan gum, gum arabic and sodium alginate are highlighted. Visual assessment of the ability of said carbohydrates to retain beet pigments is made and then association/binding studies between purified betacyanin and the above polysaccharides are pursued through use of quartz crystal microbalance with dissipation (QCM-D) techniques. The interactions reported in this latter context are informative and may lead researchers interested in betalain stabilization to new and improved strategies.

CHAPTER 2

MATERIALS & METHODS

Pigment Source & Polysaccharides

Beetroot powder was purchased from Bulk Supplements (Henderson, NV). Beet pectin (BP) was acquired as Genu® BETA Pectin provided by CP Kelco. TIC Prested® Gum Arabic Spray Dry Powder (GA) was provided as a sample by TIC Gums (White Marsh, MD). The xanthan gum (XG) used in this study (type C) was provided as a sample by Colony Gums (Monroe, NC). Sodium alginate (Manugel GHB; Alg) was provided by FMC Biopolymer (Philadelphia, PA). Food grade citric acid was used for the preparation of all buffered solutions and all other chemicals utilized were reagent grade.

Pigment-Polysaccharide Complex Stability Studies

The shelf stability of four betalain-carbohydrate complexes in water was observed over a 2-week period in 24-well polystyrene microplates at room temperature (20^{o}C). Accelerated shelf-stability testing was also performed in a similar fashion in 10 mM sodium citrate buffeer at pH 3.2 and 5. 1% aqueous solutions of beet root powder were well mixed to allow complete hydration, centrifuged at 3000 rpm for ten minutes, and then the pigment containing solution was decanted and used for testing. Five stock solutions were made for evaluation, including a control sample of the beetroot powder solution. Single carbohydrate components (BP, XG, GA, Alg) were then added at a loading of 1% (w/w) and then all solutions, including a no-polysaccharide control, were diluted 1 in 2 in 0.01 M sodium citrate buffer at either pH 3.2 or pH 5. The change

in absorbance of the solutions were compared over time using a SpectraMax iD3 Multi-Mode Microplate Reader. Photographs were used to qualitatively show the mild color stability imparted to the beet solutions over time.

Thermal stability of the beetroot-carbohydrate complexes was compared to the control using a SpectraMax iD3 Multi-Mode microplate reader from Molecular Devices (San Jose, CA). Identical solutions (pH 3.2 and 5) to those prepared above where prepared into triplicate wells (200 uL/well) of a 96-well polystyrene microplate. The temperature of the plate reader was set at 55°C and absorbance was measured every 5 minutes from 400 to 700 nm in increments of 5 nm for 6 hours. Data from these tests were presented as average percentages of the initial absorbance at 530 nm minus the absorbance at 650 nm (internal blank).

Colorimetry

The 1976 CIE L*, a*, and b* color space method was used to assess color and color changes in the extract solutions during our accelerated shelf stability studies (Hunter et al., 1948). L*, a*, and b* values were collected using a Konica Minolta Chroma Meter CR-400/410 (Osaka, Japan) portable colorimeter and scanning the beet extract solutions directly over the wells of the 24-well microplates used for the study. Color changes (ΔE_{ab}) were assessed from these values before and after the storage period and calculated according to the following equation:

$$\textbf{(1)} \qquad \Delta E_{ab} = \sqrt{(L_2 - L_1)^2 + (a_2 - a_1)^2 + (b_2 - b_1)^2}$$

All color data presented represent averages from scans of triplicate color stability tests at each condition.

Size and Zeta-Potential Measurements

1% (w/w) aqueous beet root powder extract and polysaccharide solutions as prepared above were diluted 1 in 10 into 0.01 M sodium citrate at either pH 3.2 or pH 5. The surface charge and size of the complexes were evaluated using a NanoZS90 zeta-sizer (Malvern Instrument Ltd., UK) at 25°C with a fixed scattering angle of 90°. All samples were run in triplicate and the data presented as averages of these replicates.

Polysaccharide Complex Pigment Retention Testing

To visually assess the ability of the anionic polysaccharide to retain the beet root pigments 5% (w/w) slurries of beet root powder were prepared using a T-25 high shear homogenizer (IKA; Wilmington, NC) at 10,000 rpm. The selected anionic polysaccharides (BP, XG, GA, Alg) were then homogenized into solutions at a concentration of 5% (w/w). 2 g of each "gel-like" slurry was then carefully pipetted into the bottom of 20 mL glass scintillation vials filled with 18 mL of 0.01 M sodium citrate buffer adjusted to either pH 3.2 or 5. Digital images and visible light absorbance spectra (as noted above) from 400 to 700 nm were taken on 200 uL samples the upper volume of buffer to monitor diffusion of the pigment from the ploysaccharide matrix.

Purification of Betacyanin from Beet Root Extract

A 10% solution of beetroot powder was created in 0.01 M Sodium Citrate Buffer at both pH 5 and pH 3.2. The solutions were mixed well, centrifuged at 3000 rpm for ten minutes, and decanted. The decanted solutions were each purified 10 ml at a time using an anion-exchange column, Bio Rad BioLogic LP FPLC (Bio-Rad; Hercules, CA) with Q-Sepharose Fast Flow resin (GE Healthcare). A 0.01 Sodium Citrate buffer at pH 5 was employed as mobile phase A.

After an initial red fraction came off the column a 20% gradient of 0.01 M Sodium Citrate Buffer with 1 M NaCl at pH 5 was introduced as mobile phase B. After the desired second red/pink fractions were collected, the fraction of mobile phase B was increased to 100% to collect remaining fractions off the column. The desired red/pink fractions were added to a Bio-Rad BioLogic FPLC size column using Bio-Gel P-2 Gel, 45-90 um (Bio-Rad Laboratories, CA) for salt removal. The eluting mobile phase was 0.01 M Sodium Citrate Buffer at pH 3.2 or pH 5, and the resulting purified betacyanin solutions were adjusted to the desired pH. Fractions were collected using a Bio-Rad Model 2110 Fraction Collector.

Visible Light Spectroscopy

The visible light absorption spectra of the beet root extract pigment and purified forms thereof obtained using the SpectraMax iD3 Multi-Mode plate reader described above. The original soluble beetroot solution and purified fractions from FPLC purifications were first freeze dried using a FreeZone 2L Benchtop Freezedryer (Labconco; Kansas City, MO). 2 mg of each powder was dissolved in 1 ml of water and 200 ul of each solution was pipetted in triplicates into a 96 well plate. Spectra presented within this manuscript are averages of the replicate spectra.

Mass Spectrometry

Vulgaxanthin I, Vulgaxanthin II, and Betanin levels compared to total sample signal levels were determined by comparing the area under the curve of SRM scans for each analyte to the total area of MS1 scans. The samples were analyzed using a Thermo LTQ Linear Ion Trap with and ESI source and with an Aglient 1100 HPLC on the front end. Samples were separated using a Phenomenex Luna 3μm PFP 100 column (100x 4.6mm) at room temperature. The mobile phase

consisted of A) 2 % formic acid and B) Acetonitrile and the following gradient at a constant flow rate of 0.6 ml per minute was used; time 0 A} 95%, time 35 minutes A) 80%, total run time was 35 minutes. All reagents were of HPLC grade from Sigma Aldrich. MS2 scans of Vulgaxanthin I, Vulgaxanthin II, and Betanin were performed to determine the best transition ions to scan for in SRM mode the transition used were 381>279, 382>279, and 550> 389 respectively. All SRM scans were performed using a collision energy of 15.0 and a mass window of 3.0 mass units in positive ion mode. Sample peak areas were calculated using Thermo Excalibur software.

Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy (ATR-FTIR)

FTIR spectra of freeze-dried (as above) pigment extracted from beet root powder and the subsequent fractions associated with the purification process were taken on a IRAffinity-1S spectrometer equipped with a single-reflection ATR accessory (Shimadzu Corporation; Kyoto, Japan). All spectra were averages of 32 scans from 500 to 4000 cm^{-1} with the resolution of 4 cm^{-1}. The presented data are single spectra representative of triplicate readings displayed as the standard normal variate of the original spectra, which were determined according to Barnes and co-workers (1989).

Association Study via Quartz Crystal Microbalance with Dissipation (QCM-D)

Quartz Crystal Microbalance with Dissipation Monitoring (QCM-D) was used to evaluate the association of the purified betacyanin (above) to the studied anionic polysaccharides at pH 3.2 and pH 5. All QCM-D for was performed on a Q-Sense Explorer single-module system from Biolin Scientific (Gothenburg, Sweden) using gold-coated SiO_2 base sensors (QSX 301). Base polysaccharide layers were prepared on the bare gold sensors by flowing citrate buffer (pH 5 and

3.2) 0.1 % (w/w) solutions of either xanthan gum, beet pectin, gum arabic, or sodium alginate over the gold sensors in the QCM-D module at a flow of 100 uL/min. Following the formation or a stable polysaccharide layer and washing with clean buffer, buffered solutions of the purified betacyanin from above was run over the coated sensors at the same flow rate. This analysis was repeated in triplicate at each pH for each type of anionic polysaccharide. Data presented are frequency change and dissipation change data from the 7^{th} harmonic resonant frequency of the base gold-coated sensors and are individual data set representative of the triplicate analyses.

All replicate experimental runs were performed with reproducible results on sensors cleaned according to the following protocol; the sensors sonicated in a water bath for 10 minutes with 50% ethanol at 60°C, wash rinsed with water and then sonicated in 6% hydrogen peroxide for 30 minutes at 60°C. Following, the peroxide solution was discarded and the sensors were rinsed with deionized water and then sonicated in deionized water or 30 minutes at 60°C. The sensors were rinsed with clean de-ionized water thoroughly before re-use.

Intrinsic Fluorescence Testing

Intrinsic fluorescence of the raw and purified beet root extracts alone and in combination with the tested polysaccharides was observed using a RF-6000 spectro-fluoro-photometer (Shimadzu, Kyoto, Japan). Sample solutions were prepared at pH 5.0 in citrate buffer at beet root or polysaccharide concentrations of 0.1% (w/w) and loaded into 1 cm quartz cuvettes. Full excitation-emission specrtra from 250 nm to 700 nm incrementing by 5 nm were collected under all scenarios to fully assess changes in intrinsic fluorescence imparted by the polysaccharide, although only the relevant emission spectra at an excitation wavelength of 290 nm were presented herein.

Statistical Analyses

The QCM-D frequency and dissipation profiles provided within this manuscript are individual data sets representative of triplicate runs at each condition. Data sets are presented as averages of triplicates tests under each scenario and error bars displayed all represent standard deviations determined from triplicate tests. When relevant, the statistical significance between means was determined by one-way analysis of variance (ANOVA) with post-hoc Tukey's test of honest significant difference (HSD) with $p < 0.05$ (Tukey, 1949); indication of relevant differences is noted in the caption of each data figure.

CHAPTER 3

DISCUSSION

Research Intent

The intent of the present study was to gain insights into mechanisms that may enable mild to moderate improvements in the red color shelf-stability and thermal-stability of beet root extracted pigments in the presence of a selection of anionic polysaccharides. The work presented herein highlights the potential for improved storage stability and thermal stability imparted to aqueous sodium citrate buffered beet root extract (Bt-Ext) solutions by the presence the uronic acid containing polysaccharides, gum arabic (GA), beet pectin (BP), xanthan gum (XG), and sodium alginate (Alg). These observations are then related to the zeta-potentials and average particle sizes associated with the pigment-polysaccharide solutions. This data is then compared to QCM-D studies comparing the associative interactions between the selected polysaccharides and betacyanins purified from the beet root extracts studied.

Shelf life and Thermal Stability

Initial qualititative shelf-stability tests of Bt-Ext-carbohydrate complexes at room temperature highlight the potential for polysaccharides to stabilize the red color in aqueous beet extract solutions. The imparted stability was greatest for the sodium alginate tested and for all polysaccharides as ordered, Alg > XG > BP > GA (**See Figure 2**). After two weeks, the soluble beet root powder extract controls degraded from a vibrant red hue to a yellow-brown hue, the addition of GA produced a yellow solution, the inclusion of BP and XG provided moderate preservation of the red betacyanins resulting in red-orange hues, and sodium alginate addition

proved superior in preserving the red hue of the betacyanins present. This is in line with previous studies showing sodium alginate encapsulation of betalains offered improved red color retention through storage (Otalora, et al., 2016; Rodríguez-Sánchez et al., 2017).

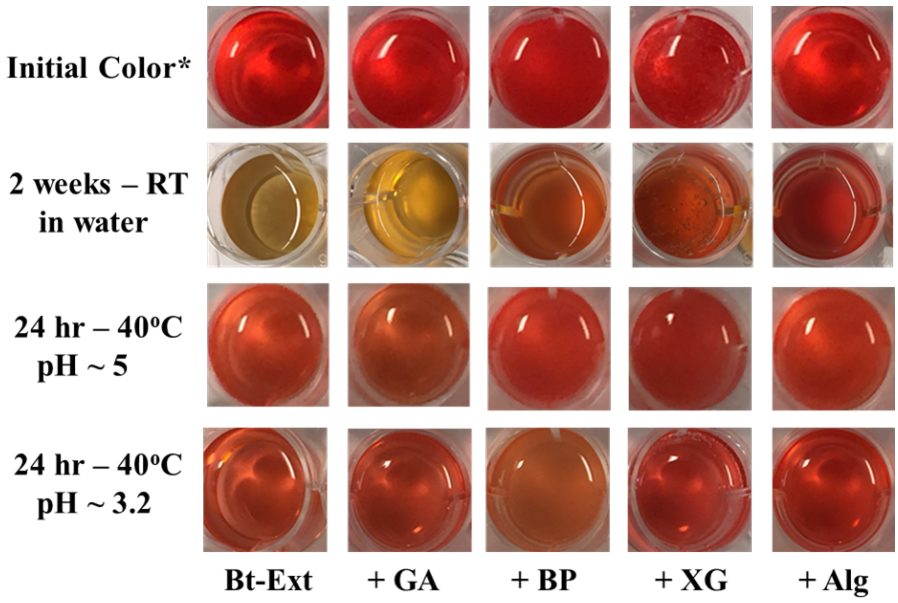


Figure 2. Images depicting the general storage and 10 mM citrate buffered-40°C storage of 1% beet root powder extracts (Bt-Ext) without and with the inclusion of gum arabic (GA), beet pectin (BP), xanthan gum (XG), or sodium alginate (Alg) at 1% (w/w).

Thermal stability testing of the polysaccharide complexed beet extracts was performed at pHs near the lower pKa values for citric acid (3.13, 4.76), pH 3.2 and pH 5.0. Buffering with citric acid is common in many food systems and not only affects the stability of betalains but also modulates the charge density of the polysaccharides by altering the extent to which the uronic acid constituents are deprotonated. Accelerated storage testing at acidic pHs and an elevated temperature of 40˚C showed both Alg and XG were the most stabilizing of the original red color of extract pigment, particularly at pH 3.2, while BP was most effective at pH 5, and Gum arabic showed only slight stability improvement at pH 3.2 (**Figure 2**). Color changes values (ΔE_{ab}) and

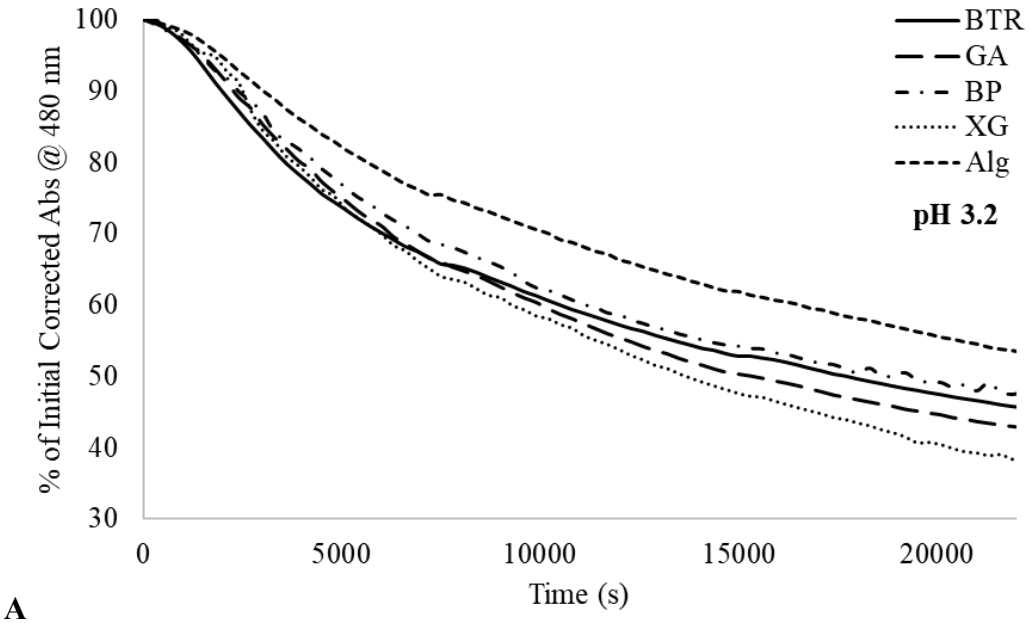
the associated red color changes (Δa*) derived from L*, a*, and b* color space data for these tests are presented in **Table 1**. In terms of red color preservation specifically (a*), the weighted values in Table 1 show that Alg and XG were most stabilizing under both acidic conditions, while BP was also effective at pH 5. Furthermore, a* values for the xanthan gum tests increased somewhat over the storage period and suggest a level of co-pigmentation may occur in this case.

Table 1. ΔE_{ab} values and percentage of red color retained (a*) using L* a* b* color data from 40˚C accelerated shelf stability tests after 24 hours.

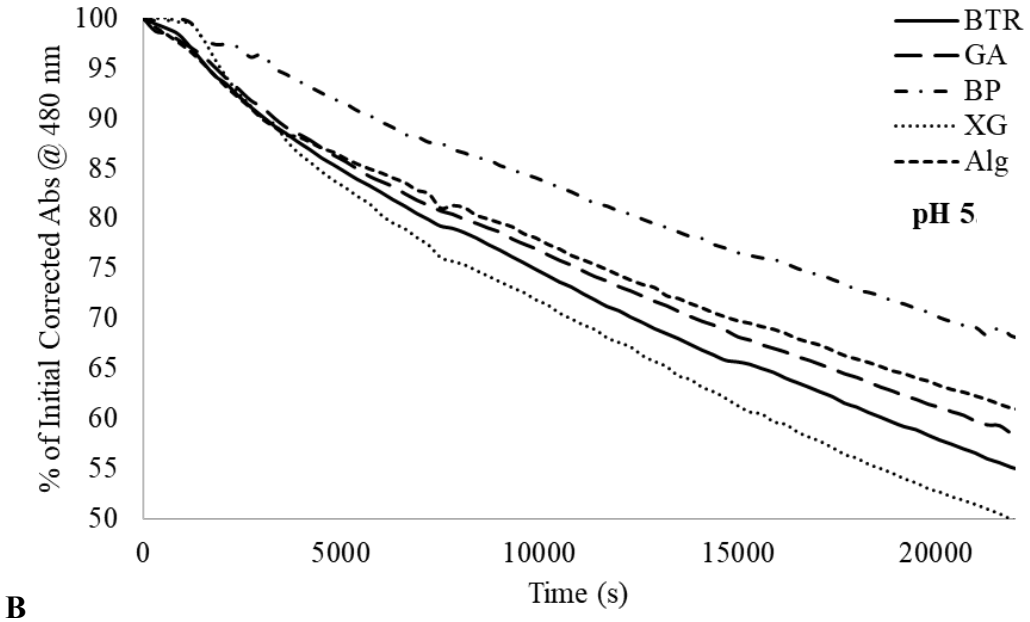
	ΔE_{ab}		% a* retained	
pH	3.2	5	3.2	5
Bt-Ext	11.43 ± 2.27	15.33 ± 3.44	69	64
Bt-Ext + BP	12.21 ± 2.48	7.25 ± 1.98	58	77
Bt-Ext + Alg	8.54 ± 3.53	6.50 ± 0.80	78	77
Bt-Ext + GA	13.07 ± 1.93	14.17 ± 3.99	69	55
Bt-Ext + XG	12.53 ± 1.49	3.03 ± 0.14	132	102

At higher temperatures, 55˚C, the decline in absorbance signals associated with betaxanthins (480 nm) and betacyanins (530 nm) was most reduced by Alg at pH 3.2 and by BP at pH 5 (**Figure 3 A-D**). At this temperature, Alg was also the second most stabilizing at pH 5 and more notably the XG did not impart color stability and may have accelerated the rate of color loss at both pH. The improved thermal stability of beet betalains with inclusion of Alg at the lowest pH is corroborated by our previous high pressure study which indicated that Bt-Ext-Alg complexes mildly improved thermal stability prior to the high pressure treatment (Selig et al., 2018). We

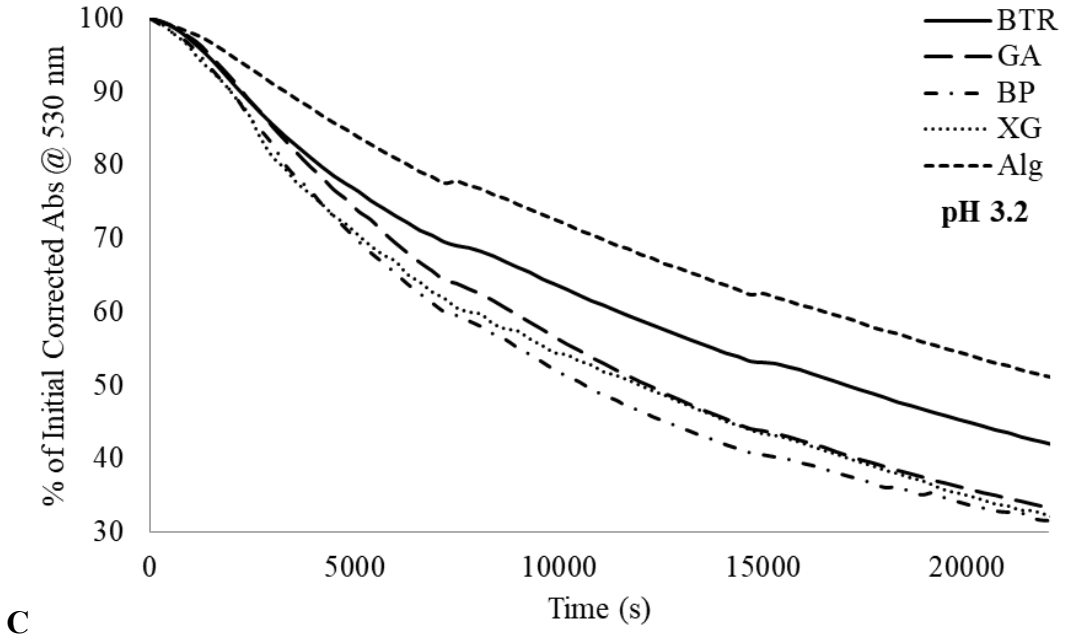
also speculate here, that the lost color stabilizing potential of XG at higher temperatures may be due to conformational changes in the polysaccharide network formed at higher temperatures (Holzwarth, 1976).



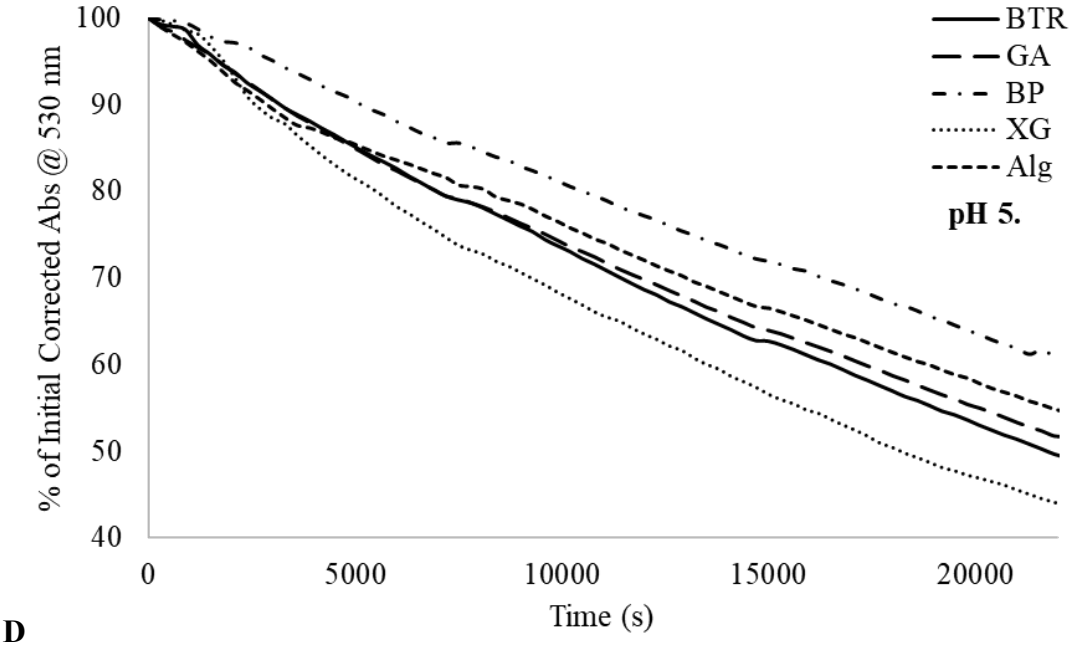
A



B



C



D

Figure 3 – Declining absorbance at 480 nm (betaxanthins; **A & B**) and at 530 nm (betacyanins; **C & D**) corrected for internal background absorbance at 650 nm for 1% (w/w) beet powder extract solutions (Bt-Ext) inclusive of 1% (w/w) gum arabic (GA), beet pectin (BP), xanthan gum (XG), and sodium alginate (Alg) buffered to pH 3.2 or 5 in 10 mM sodium citrate. Data are averages of triplicate in-plate thermal-stability test at 50^{o}C run in 96-well microtiter plates.

Zeta Potential & Particle Size

Zeta potential measurements in **Figure 4** provide some insight into the mechanisms imparting betalain color stability. Most notably, inclusion of Alg or XG at both pH, and BP at pH 5, resulted in the most negative zeta-potentials measured in solution. It is possible that the ability of these polysaccharides to stabilize the betalains is related to the high density of negative charge they impart to a solution. In this context, it is worth noting the drastic increase in negative zeta-potential of BP as the pH is raised from 3.2 to 5, the latter of which BP is an effective color stabilizer. Furthermore, light-scattering measurements to assess the average particle size in the studied solutions (**Figure 5**) indicate that sodium alginate and soluble components (betalains) of the beet root extracts likely form soluble complexes that are larger than the particle sizes measured for the Bt-Ext and Alg by themselves; this is not the case with the other polysaccharides. Since the tested Alg does exhibit the best and broadest color stability to beet root betalains, these data suggest that pigment-polysaccharide association resulting in stable complex formation may be beneficial. A study on the properties of alginate mentions that at low pH, the intramolecular hydrostatic interactions of alginate become weaker and intermolecular bonds as well as entanglement forces start to dominate (Kjonikson, 2005). The tendency to form intermolecular bonds in combination with the notably high surface charge and particle size data may explain why alginate has a superior ability to stabilize betalins at pH 3.2.

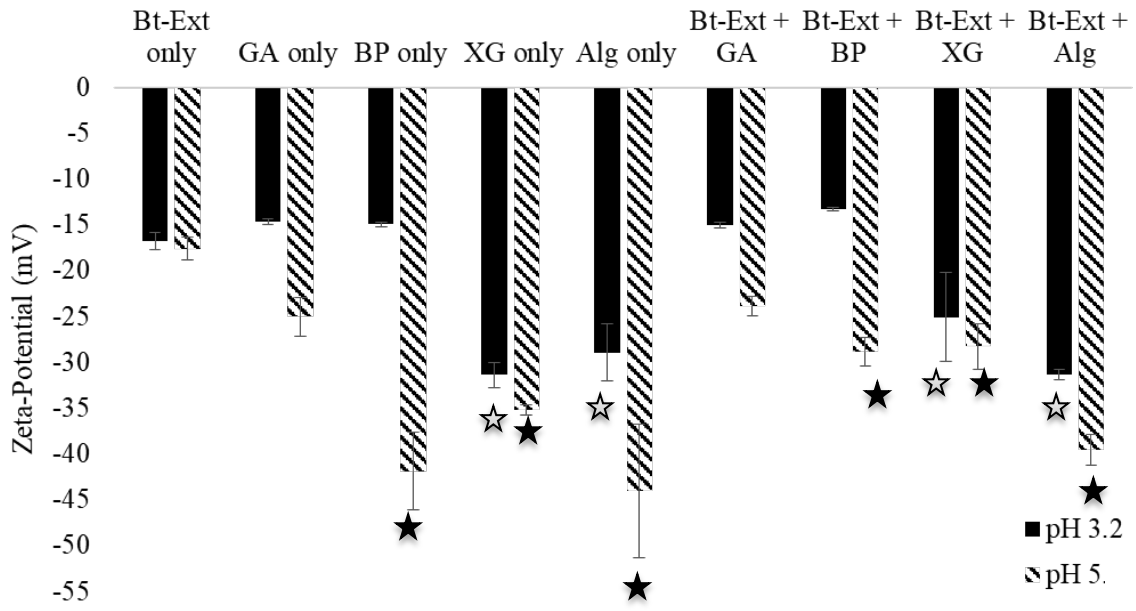


Figure 4. Zeta-potentials of 0.1% (w/w) Solutions of beet root powder extract (Bt-Ext), gum arabic (GA), beet pectin (BP), xanthan gum (XG), and sodium alginate (Alg) and combination of extract plus the polysaccharides buffered in 10 mM sodium citrate at pH 3.0 and pH 5. Values reported represent averages of triplicate analyses at each condition and error bars represent the standard deviations amongst the triplicate measurements. Grey stars denote significant statistical differences to the original beet root extract at pH 3. Black stars denote significant statistical differences to the original beet root extract at pH 5.

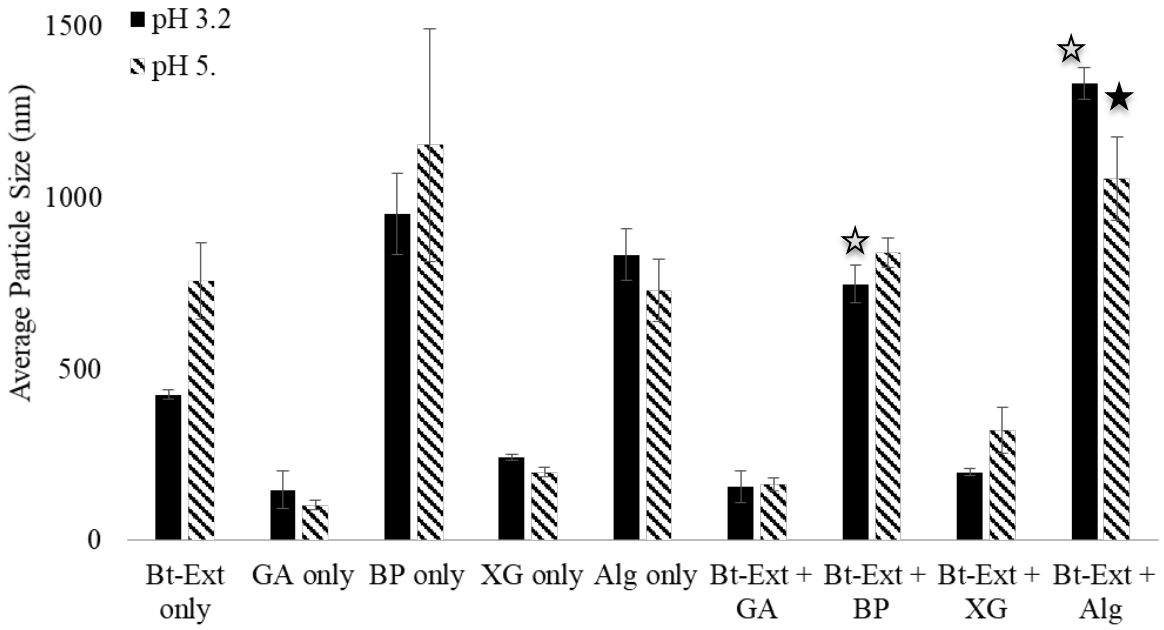


Figure 5. Average particle sizes measured on 0.1% (w/w) Solutions of beet root powder extract (Bt-Ext), gum arabic (GA), beet pectin (BP), xanthan gum (XG), and sodium alginate (Alg) and combination of extract plus the polysaccharides buffered in 10 mM sodium citrate at pH 3.2 and pH 5. Values reported represent averages of triplicate analyses at each condition and error bars represent the standard deviations amongst the triplicate measurements. Grey stars denote significant statistical differences to each gum on its own compared to the complexed version at pH 3. Black stars denote significant statistical differences to each gum on its own compared to the complexed version at pH 5.

Beet pigment purification

To further understand the mechanisms imparting betalain stability by the polysaccharides tested, we investigated the association of purified betacyanins from the Bt-Ext with the polysaccharides via Quartz Crystal Microbalance with Dissipation Monitoring (QCM-D). QCM-D provides real-time data on surface mass interactions by exploiting the piezoelectric properties of quartz crystal

sensors. QCM-D has been historically used to analyze biomolecular interactions comprising of DNA, proteins, fats, and cells (Dixon, 2008), and herein the binding affinity between carbohydrate-coated sensors and the pigment components of beetroot powder is assessed. Although QCM-D is known to be an extremely sensitive system toward mass and dissipation changes at the sensor surface, it is not capable of discriminating between the types of molecules deposited on a surface. Therefore, it was necessary to purify the red/magenta betacyanins from the Bt-Ext to better ensure that the binding events observed were related to the binding of pigment to the polysaccharide coated surfaces rather than other components of the Bt-Ext.

Betacyanin isolates were prepared by a series of anion exchange and size exclusion separations steps and the resulting solution was analyzed using a variety of spectroscopic techniques. **Figure 6** shows the changes in the absorbance spectra throughout each step of the purification. Here, these data show that the original beetroot slurry has equal absorbance at wavelengths indicative of both betaxanthins (480 nm) and betacyanins (530 nm), while the final solution has an increased ratio of betacyanin to betaxanthin. This isolation of betacyanin was further corroborated by LC-MS analysis (not shown) which indicated 50% of the mass associated with the first 5 minutes of the LC elution consisted of ionization products related to betacyanins, while those associated with betaxanthin ionization products comprised less than 1% of the total.

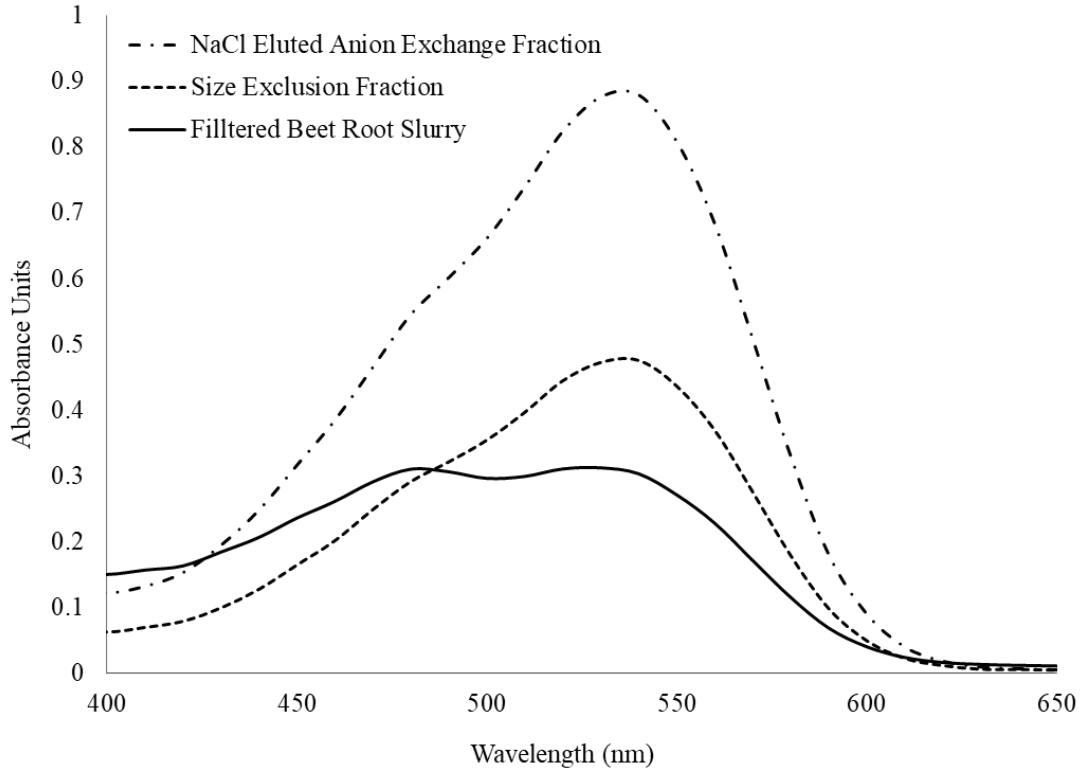


Figure 6. Visible light spectra of isolated beet root extract fractions

FTIR

Furthermore, the FTIR spectra in **Figure 7** show the originally sourced beetroot powder obtained has strong characteristic bands for carbohydrates in the 1500 – 900 cm^{-1} region, and particularly at 980 and 1030 cm^{-1} (Garrigues, 2000). Peak assignments for the ATR-FTIR spectra of the purified sample can be seen on Figure 7 and are based on work by Perez and co-workers (2017). In the final purified powder a large reduction in the carbohydrate peaks is evident. The purified pigment spectra exhibit dominant peaks associated with N-H vibrations and C-N stretches; both present in the betacyanin pigment structures. The aforementioned analyses provided confidence that the pigment purified adequately to move forward with the QCM-D binding studies.

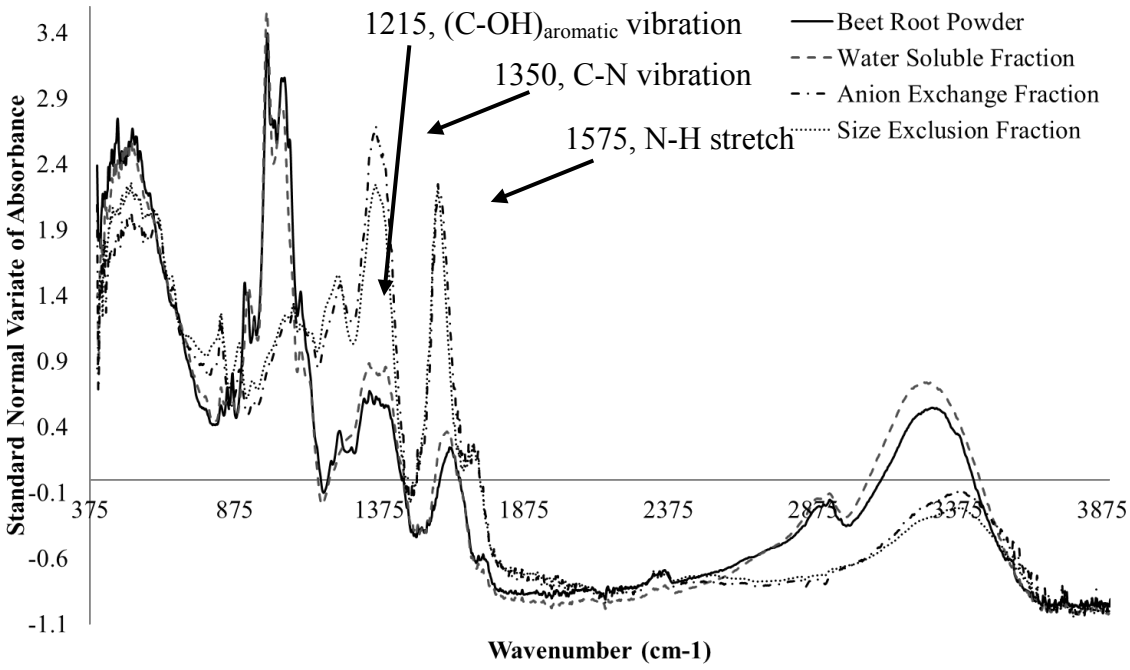
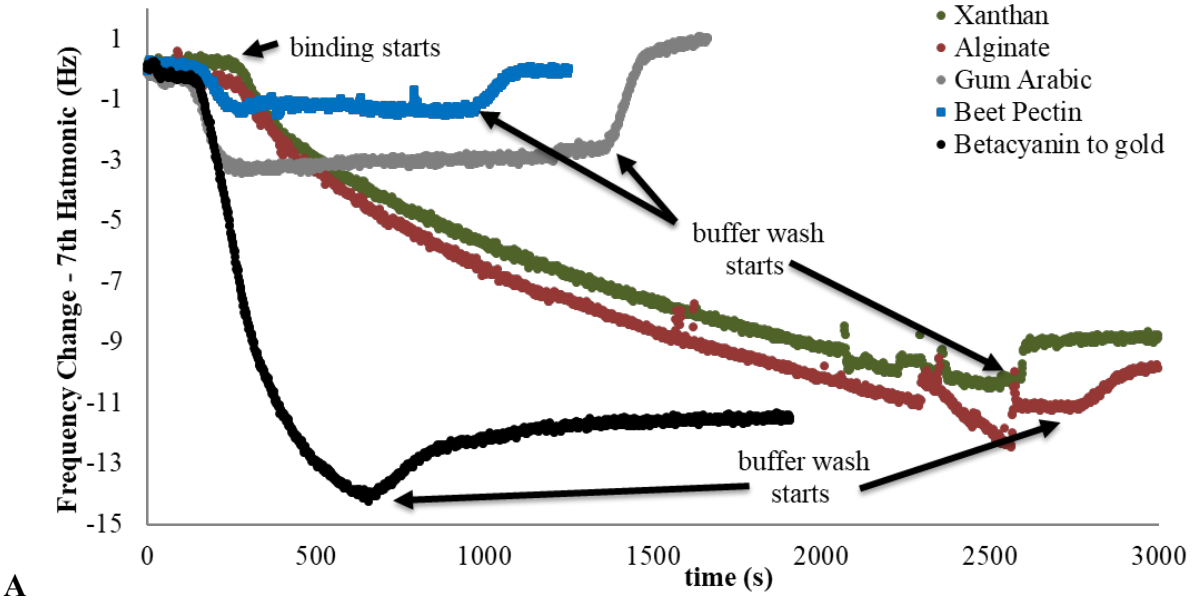


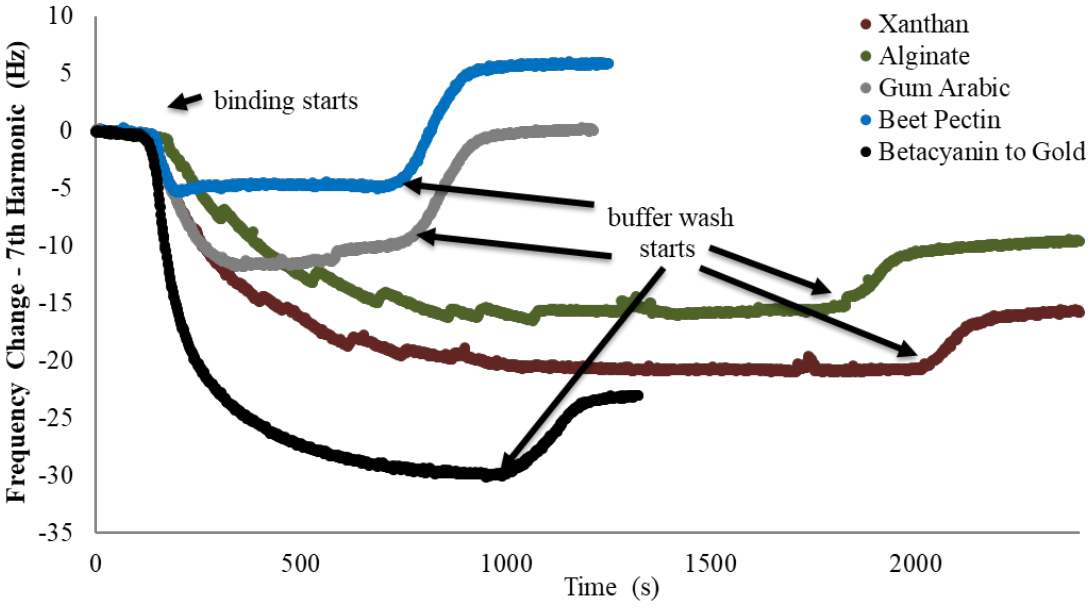
Figure 7. Standard normal variates of ATR-FTIR Spectra of freeze dried samples of beet root powder through the process of betacyanin isolation. Spectra are single data sets representative of triplicate analyses on each sample.

QCM-D Binding Studies

In QCM-D, the application of alternating currents across a quartz sensor results in the oscillations at the sensor resonant frequency; changes in resonant frequency can be related to the addition or removal of mass to the surface where a drop in frequency is indicative of mass deposition. Further, the rate of oscillation dissipation, provides insight into the rigidity of the surface layer with increasing dissipation indicating less rigidity. **Figure 8** shows the QCM-D frequency changes versus time for the association of the purified betacyanins to gold-coated quartz sensors coated with the polysaccharides studied herein at both pH 3.2 and pH 5 at room temperature (21°C).



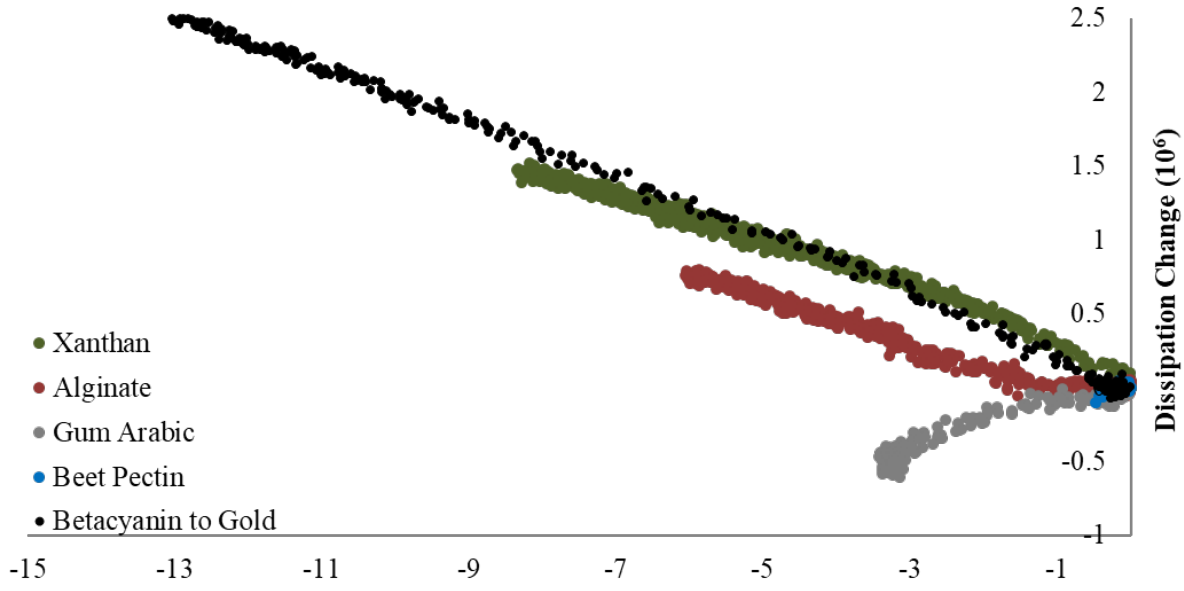
A



B

Figure 8 - QCM-D frequency change data for betacyanin binding events at pH 5.0 (A) and pH 3.2 (B). All data represent single experimental binding and buffer wash events that are representative of triplicate experiments for each condition tested.

Regardless of the environment studied, both alginate and xanthan gum displayed a higher affinity for binding the pigment when compared to the other gums. Not only was greater betacyanin mass deposited on the alginate and xanthan-coated sensor surfaces, but the pigment also appeared to bind irreversibly following a buffer wash. In the case of beet pectin and gum arabic, very little mass was deposited on the surface and all of the mass binding was reversible, and removed following the wash step, indicating that the binding interactions between betacyanin and BP or GA were not very strong at these pH. Furthermore, plots of the dissipation changes vs frequency changes in **Figure 9** for these association events show that at pH 3.0 the slopes of the plots for binding to XG and Alg are lower than those for GA or BP, or even the control, and suggests that the interaction between the pigment and these polysaccharides is likely greater and forms a more rigid layer on the sensor surfaces.



A

B

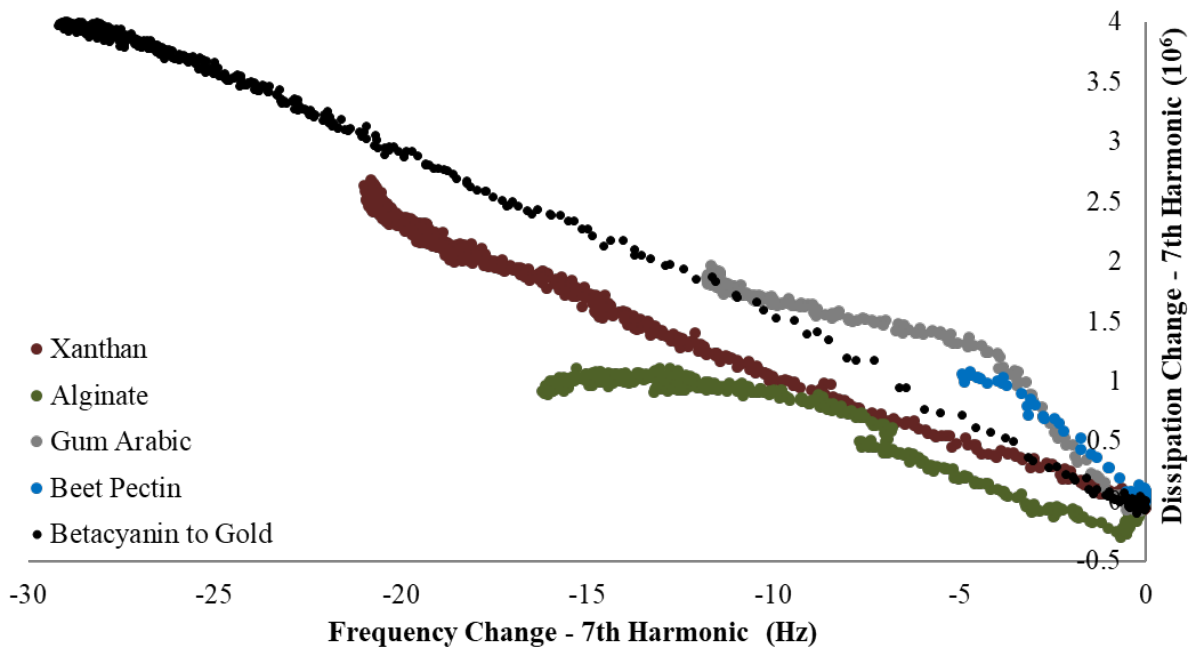


Figure 9 - Frequency vs dissipation plots for all betacyanin binding events observed by QCM-D at pH 5.0 (A) and pH 3.2 (B). All data represent single experimental binding events that are representative of triplicate binding experiment for each condition tested.

The above observations by QCM-D provide added support suggesting that stronger and irreversible pigment-polysaccharide association may play a critical role in the superior color stability imparted by XG and Alg to the Bt-Ext at storage temperatures. It has been proposed that the gums with small amounts of polyvalent cations, like sodium alginate, are able to form bridges between the carboxyl groups and increase complex stability. This same work also suggested that the complexes and pigment may be physically trapped in hydrocolloid matrices such that formed in the self-association of xanthan gum (Navagnana, 1987). Furthermore, the structures of both Alg and XG are relatively linear in nature compared to the structures of GA and BP, which are rather branched. Such linear conformations may present greater opportunities

for stable per-molecule binding interactions, such as hydrophobic associations coupled with intermolecular hydrogen bonding, as they likely create fewer steric hindrances to the pigments than the heavy branching patterns present in BP and GA do. While pigment-polysaccharide association may make an important contribution to the imparted color stability, one must consider that there are other possible mechanisms that may impart pigment stability.

In the case of beet pectin, at high pH (5.0) high charge density and qualitatively good color stability is exhibited but poor binding was observed by QCM-D. This specific result suggests a different stabilizing mechanism exists in this case. Examination of changes to the intrinsic fluorescence of the beet root extracts when coupled with beet pectin may shed light into this. While no significant changes to the fluorescence spectra of beet extract was observed with the inclusion of Alg, XG or GA, significant changes were observed with BP inclusion at pH 5; shown in **Figure 10**. At this pH the inclusion of BP results in significant reduction of fluorescence in the range of 330-370 nm, and 300-330 nm; regions traditionally associated with fluorescence of the indole derived tryptophan and the phenolic tyrosine amino acids, respectively (Goldberg 2012). In beet pectin similar phenolic structures exist as feruloyl ester substitutions throughout the polysaccharide and, like tryptophan, betacyanins are also indole derivatives. So, while it is possible some of the control beet extract fluorescence may be related to proteins present, it is likely the strong fluorescence is related to the ferulate and betacyanin structures themselves, were relevant. Indole fluorescence at 355 nm has been attributed to deprotonated forms, while the fully protonated form does not fluoresce (Bridges, 1968); the suppression of fluorescence at 355 nm with BP inclusion may suggest some interaction occurs that affects betalain fluorescence, which may be related to the observed color stability in this case.

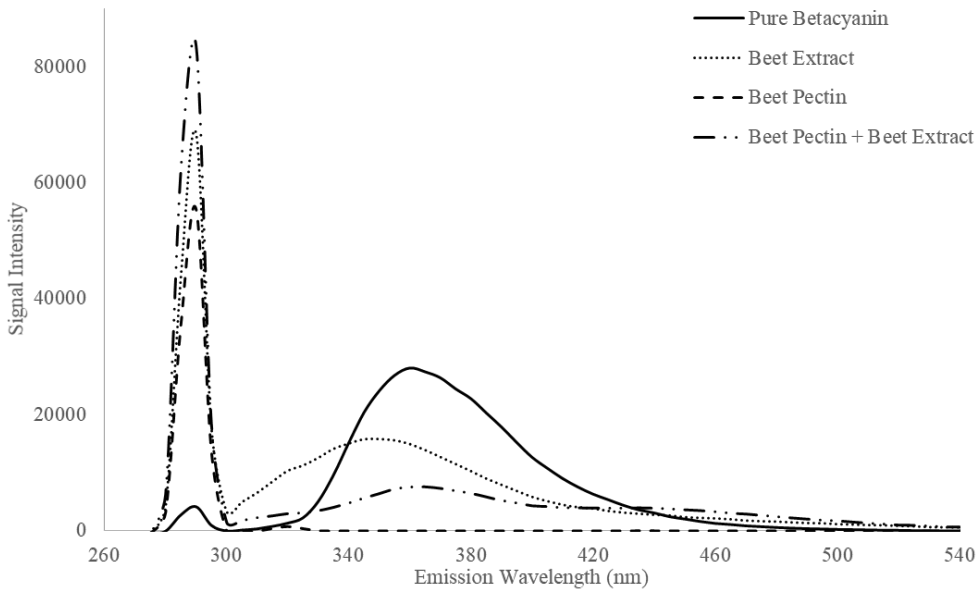


Figure 10. Fluorescent emission spectra for purified betacyanin, beet extract, beet pectin and the combined beet extract and beet pectin following excitation at 290 nm. Spectra presented are signal spectra representative of replicate tests.

CHAPTER 4

CONCLUSION

This study provides insight into the mechanism of stabilization imparted on betacyanin by four anionic polysaccharides (BP, Alg, X, GA) by relating QCM-D binding data to shelf stability studies, zeta potential, and particle size measurements. Initial shelf life studies of beet root extract indicate that Alg and XG offered improved color shelf stability in aqueous environments and superior thermal stability at 40˚C at both pH values when compared to the other tested complexes. BP showed similar effectiveness as Alg and XG to stabilize the red color specifically at 40˚C and pH 5. Alginate in particular demonstrated the best, yet modest, color preservation at 55˚C at pH 3.2. Pigment-polysaccharide complex zeta potential data suggest the ability to form electrostatic interactions may play a significant role in each gum's ability to impart stability on beet pigment. Both xanthan gum and alginate under both pH conditons and beet pectin at pH 5 demonstrated the highest surface charge values and qualitatively the best color stability. The combination of the zeta potential and particle size values indicate that alginate's ability to enhance pigment stability may be based on the formation of stable soluble complexes with the betalains. Purified betacyanins exhibited strong and irreversible binding to Alg and XG-coated sensors, indicating such pigment-polysaccharide binding may be a significant stabilizing mechanism, which does not occur with the less stabilizing BP and GA studied. BP did demonstrate stabilizing potential along with a high surface charge at pH 5, but only weak and reversible binding of betacyanin to BP surfaces was observed by QCM-D studies, although differences in auto-fluorescence displayed by beet extract-BP complexes at this pH suggest some

other mechanism may cause color stabilizing change to the betalains therin. Future work should include a deeper dive into the stabiliation mechanism of the aforementioned gums in addition to the inclusion other types of carbohydrates. The insights gathered from this study, and future work on polsaccharides, will help enhance the general understanding of beet pigment stabilization and thus promote the widespread use of betalains in food products.

REFERENCES

Barnes, R.J., Dhanoa, M.S., & Lister, S.J. (1989). Standard Normal Variate Transformation and De-trending of Near-Infrared Diffuse Reflectance Spectra. *Applied Spectroscopy, 43,* 772-777.

Bridges, J.W. and Williams, R.T., 1968. The fluorescence of indoles and aniline derivatives. *Biochemical Journal, 107(2),* p.225.

Falcão, A., Chaves, E., Falcão, L., Gauche, C., Barreto, P., & Bordignon-Luiz, M. (2009). Rheological behavior and color stability of anthocyanins from Merlot (*Vitis vinifera L.*) and Bordô (*Vitis labrusca L.)* grapes in a jam model system. *Ciência e Tecnologia de Alimentos*, *29(4),* 857-862.

Francis, F. (1995). Quality as influenced by color. *Food Quality and Preference, 6(3),* 49-155.

Goldberg, J.M., Wissner, R.F., Klein, A.M. and Petersson, E.J., 2012. Thioamide quenching of intrinsic protein fluorescence. *Chemical Communications, 48(10)*, pp.1550-1552.

Giusti, M., and Wrolstad, R. (1996). Radish anthocyanin extract as a natural red colorant for maraschino cherries. *Journal of Food Science, 61(4)*, 688-694.

Herbach, K.M., Stintzing, F.C., & Carle R. (2004). Impact of thermal treatment on color and pigment pattern of red beet (*Beta vulgaris L.)* preparations. *Journal of Food Science, 69,* 491–498.

Herbach, K. M., Stintzing, F. C., & Carle, R. (2006). Betalain stability and degradation - Structural and chromatic aspects. *Journal of Food Science, 71*, R41–R50.

Holzwarth, G. (1976). Conformation of the extracellular polysaccharide of *Xanthomonas campestris*. *Biochemistry, 15*, 4333–4339.

Jackman, R.L., & Smith, J.L. (1996). Anthocyanins and betalains. In: Natural food colorants. Hendry, C.F., & Houghton, J.D., editors. *Blackie Academic & Professional, London,* 244–309.

Ravichandran, K., Saw, N. M., Mohdaly, A. A., Gabr, A. M., Kastell, A., Riedel, H., & Smetanska, I. (2013). Impact of processing of red beet on betalain content and antioxidant activity. *Food Research International, 50(2),* 670-675.

Reynoso, R., Garcia, F. A., Morales, D., & Mejia, E. G. (1997). Stability of Betalain Pigments from a Cactacea Fruit. *Journal of Agricultural and Food Chemistry, 45(8),* 2884-2889.

Saguy, I., Kopelman, I. J., & Mizrahi, S. (1978). Thermal kinetic degradation of betanin and betalamic acid. *Journal of Agricultural and Food Chemistry, 26(2),* 360-362.

Savolainen, K., & Kuusi, T. (1978). The stability properties of golden beet and red beet pigments: Influence of pH, temperature, and some stabilizers. *Zeitschrift Lebensmittel-Untersuchung und Forschung, 166(1),* 19-22.

Schwartz SJ, Von Elbe JH. (1983). Identification of betanin degradation products. *Z Lebensm Unters Forsch 176*, 448–453.

Stintzing, F. C., & Carle, R. (2004). Functional properties of anthocyanins and betalains in plants, food, and in human nutrition. *Trends in Food Science & Technology, 15,* 19-38.

Stintzing, F.C., & Carle, R. (2007). Betalains - emerging prospects for food scientists. *Trends in Food Science & Technology, 18,* 514-525.

Wallace, C., & Giusti, M.M. (2008). Determination of color, pigment, and phenolic stability in yogurt systems colored with nonacylated anthocyanins from *Berberis boliviana L.* as compared to other natural/synthetic colorants. *Food Chemistry, 73(4),* 241-248.

Woo, K., Ngou, F., Ngo, L., Soong, W., & Tang, P. (2011). Stability of Betalain Pigment from Red Dragon Fruit (Hylocereus polyrhizus). *American Journal of Food Technology, 6(2),* 140-148.

Rodríguez-Sánchez, J. A., Cuatzo-Lozano, M. I., Pérez-Loredo, M. G., Abarca-Sarro, D. I., & Navarro, Y. G. (2017). Alginate Encapsulation as a Preservation Method of Pitaya Fruit Juice (Stenocereus spp.). *Journal of Food Science and Engineering*, 7(3).

Bu, H., Kjøniksen, A., & Nyström, B. (2005). Effects of pH on dynamics and rheology during association and gelation via the Ugi reaction of aqueous alginate. European Polymer Journal, 41(8), 1708-1717.

Otálora, M. C., Carriazo, J. G., Iturriaga, L., Osorio, C., & Nazareno, M. A. (2016). Encapsulating betalains from Opuntia ficus-indica fruits by ionic gelation: Pigment chemical stability during storage of beads. *Food Chemistry,* 202, 373-382.

Dixon, M. C. (2008). Quartz Crystal Microbalance with Dissipation Monitoring: Enabling Real-Time Characterization of Biological Materials and Their Interactions. *Journal of Biomolecular Techniques*, 19(3), 151–158.

Garrigues, J. (2000). Direct ATR-FTIR determination of sucrose in beet root. *Talanta*, 51(2), 247-255.

Pérez, E., Ibarra, I. A., Guzmán, A., & Lima, E. (2017). Hybrid pigments resulting from several guest dyes onto γ-alumina host: A spectroscopic analysis. Spectrochimica Acta Part A: *Molecular and Biomolecular Spectroscopy*,172, 174-181.

Hettiarachchy, N. S., Muffett, D. J., & Wedral, E. R. US Patent 4,699,664, 1987.