COVER SHEET

TITLE: Ultrasound Alters Pigmentation in Aqueous Solution

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ABSTRACT

Ultrasound Alters Pigmentation in Aqueous Solution

The use of ultrasound technology has been gaining popularity in many industries as a relatively inexpensive, simple and energy saving technique. This study investigated the efficacy of ultrasound technology in reducing pigment intensity of the annatto pigment as an alternative to current chemical methods.

Annatto extract was added to distilled water. The solution was pumped through a closed ultrasound system, and samples were taken at designated intervals. Spectrophotometric methods were used to determine the degree of color intensity. The effectiveness of ultrasound was compared to chemical means of bleaching. Anthocyanin and β-carotene pigments were also exposed to the same treatments.

When exposed to ultrasound, the annatto pigment intensity was significantly diminished. Ultrasound with and without the addition of oxidizing agents may have utility as a means of reducing pigmentation of aqueous systems with the potential of reducing the usage of chemical-only treatments.

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Ultrasound Alters Pigmentation in Aqueous Solution

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ABSTRACT

The use of ultrasound technology has been gaining popularity in many industries as a relatively inexpensive, simple and energy saving technique. Ultrasound induced cavitation through the generation, growth, and collapse of large bubbles liberates high energy. When water is exposed to this high energy, the cavitation phenomenon generates highly reactive hydroxyl and hydrogen ion species. This study investigated the efficacy of ultrasound technology in reducing pigment intensity of annatto, β-carotene, and anthocyanin in aqueous solution. The cavitation phenomenon and resulting generation of reactive water species was hypothesized to alter the chemical structure of the pigment molecule, thus reducing pigment intensity.

The pigment annatto was studied because of its importance in applications such as whey bleaching. Annatto extract was added to 12 L of distilled water. The solution was pumped through a closed ultrasound system, and samples were taken at designated intervals. Spectrophotometric methods were used to determine the degree of color intensity. The effectiveness of ultrasound was compared to chemical means of annatto bleaching. Anthocyanin and β-carotene pigments were also exposed to the same treatments.

When exposed to ultrasound, the annatto pigment intensity was significantly diminished. A logarithmic relationship was found to exist between absorbance and exposure time. The rates of pigmentation reduction were greatest for annatto, followed by the β-carotene and anthocyanin treatments. Ultrasound with and without the addition of oxidizing agents may have utility as a means of reducing pigmentation of aqueous systems with the potential of reducing the usage of chemical-only treatments.

Key Words: ultrasound, pigment, annatto

INTRODUCTION

Annatto

Annatto is the carotenoid-based orange pigment that is commonly used as a colorant in many dairy foods, most notably in cheddar cheese. The orange and yellow color in annatto dye is produced from the contained bixin and norbixin molecules (Kang, 2010). These oil soluble bixin molecules are found primarily in the cis-form, but trans bixin can also be present. A saponification reaction using alkali salts yields the water soluble norbixin molecule (Technical Resources International, 1997). This allows water-soluble liquid annatto to be used as the major colorant in cheese processes.

Annatto Removal

Though highly desired in cheese product itself, this orange color produced by the norbixin molecule is often eventually removed in order to make a whiter whey powder product. The drained off whey from cheese production can be further processed for use as whey protein concentrate (WPC) or whey protein isolate (WPI). Both WPC and WPI have become increasingly popular among food manufacturers in recent years because of their functional and nutritional properties (Kang et al., 2012). However, fifteen percent of the annatto added to the cheese milk remains in the separated whey after cheese processing (McDonough et al., 1968). This annatto colored cheese whey must be decolorized before being processed into powder.

Bleaching

The unsaturated structure of the norbixin molecule makes it highly susceptible to inactivation through bleaching. Unfortunately, however, current methods of pigment removal used in industry have been known to alter flavor profiles and have potential health and environmental impacts (Kang et al, 2010). The chromophore is the portion of the molecule that emits color and once destroyed, the molecule becomes colorless. Bleaching is known to occur through oxidative, reductive, and biochemical

reactions (Kang et al, 2010).

Oxidation of the double bonds within the chromophore eliminates the color characteristic of the bixin molecule (Kang et al., 2010). The application of certain oxidizing agents, namely benzoyl peroxide and hydrogen peroxide, has been utilized as an industrial method for decolorizing annatto for many years. The specific mechanism of the chemical degradation for these two bleaching agents varies slightly. Hydrogen peroxide attacks the annatto pigment while also attacking other conjugated double bonds of whey compounds, while benzoyl peroxide has only been associated with the annatto molecule (Jervis et al.,2012).

Reduction of the double bonds within a chromophore eliminates the color characteristic of annatto by hindering its ability to absorb visible light (Kang et al, 2010). This form of bleaching is most commonly exhibited upon exposure to UV light, when high-energy photons disrupt the chromophore double bonds. The application of the reducing agents lemon juice and sulfur dioxide has been used in food production as a method of decolorization (Kang et al, 2010).

The third type, biochemical bleaching, is the result of enzymes such as lipoxygenase. There are a few of these lipoxygenases that have been successful at bleaching dairy products including milk, cheese, cream, and whey (Kang et al, 2010). These lipoxygenases react with linoleic acid, producing free radicals which oxidize the double bonds of the annatto molecule. The use of fungal peroxidase has also been investigated for use in place of oxidizing bleach (Kang et al, 2010).

These chemical bleaching agents, however, have been associated with off-flavors. Recent research has been conducted to find alternative bleaching methods that have less of an impact on flavor profile,

including acid-activated bentonite, ultraviolet, and ozone (Kang et al., 2012). Little work, however, has been done in the field of ultrasonic alternatives.

Investigation of Additional Pigments

Anthocyanin and β-carotene are two other natural pigments of significance used in the food industry today. There are over 635 different anthocyanins found in nature, all imparting a scarlet, magenta, or purple color (Glover, et al, 2012). These pigments are used primarily in the beverage industry, appealing to consumers looking for natural ingredients. Acylated anthocyanins are most commonly used due to their increased stability over nonacylated anthocyanins (He, et al, 2010). Currently, sulfur dioxide is the most common way to bleach these molecules by converting them into their colorless flavan structures (Jurd, 1962). β-carotene is the most common carotenoid used as a food coloring as yellow, orange, and red shades. While this compound is abundantly present in a number of sources including algae, carrot, and palm oil, most of the β-carotene used in industry is chemically synthesized. Similar to anthocyanins, β-carotene pigments are commonly used in the beverage industry. B-carotene is highly susceptible to oxidation, leading to degradation (Chapman, S., 2011). Investigation of these pigments will allow for a better understanding of the mechanism of pigment bleaching.

Ultrasound Technology

Ultrasound is a well-known technology with numerous applications in various industries. Because it is a relatively inexpensive, simple and energy saving technique, it is an emerging technology for modifying various processes in food products. Currently, ultrasonic processes in the food industry include emulsification, homogenization, extraction, crystallization, pasteurization, and various other physical and functional property changes.

A high power ultrasound is composed of sound waves with frequency beyond the limit of human hearing that can be tuned for specific application. The main mechanism of higher intensity, lower frequency ultrasound is the induced cavitation due to generation, growth, and collapse of large bubbles, causing the liberation of high energy and thus, the induction of chemical and physical changes in food systems (Awad et al., 2012). Therefore, the chemical effects of ultrasound do not occur from a direct interaction with the molecules, rather the release of high amounts of localized energy formed by cavitation. This cavitation phenomenon is responsible for the breakdown of water molecules into highly reactive hydroxide radicals and hydrogen atoms. The dissociation of water molecules in aqueous solutions intensifies chemical reactions, can induce crosslinking of molecules, and enhances the rate of mass transport reactions due to acoustic streaming (Dubrovic et al., 2011).

We hypothesize that, similar to chemical oxidizing agents, the reactive water species generated by ultrasound can attack the π - bonding systems of the chromophores, thereby reducing color pigment intensity.

MATERIALS AND METHODS

Experimental Design

Three different pigments were tested: annatto, anthocyanin, and β-carotene. For each, two experiments were conducted. Experiment 1 was conducted using ultrasound as the only form of bleaching power. Experiment 2 compared the strength of a chemical method alone and also in combination with ultrasound exposure.

Materials

Commercial American cheese color, containing extract of annatto, water, and potassium hydroxide, was acquired from Cargill. Colorfruit Violet pigment, containing anthocyanins, was acquired from Chr-

Hansen, Inc. 10% Cold Water Soluble β-carotene powder from Roche Vitamins and Fine Chemicals was used. Samples were treated using an ultrasonic mixer supplied by Aurizon Ultrasonics and analyzed with a UV-Vis spectrophotometer.

Sample Preparation

Sample preparation varied for the different pigments analyzed. For annatto, 1 mL of the water soluble liquid was diluted in 12L of distilled water. For anthocyanin, 5 mL of the water soluble liquid were diluted in 12L of distilled water. For β-carotene, 1 g of 10% cold water soluble powder was first mixed into 15 g of water. 3 mL of this solution was then diluted in 12L of distilled water. After the addition of the pigment, the color treated water was first passed through the system for 5 minutes with no ultrasound exposure to promote uniform dispersion of the dye in the water. After this initial mixing time, the treatment (none (Control), ultrasound (US), sodium hypochlorite (Cl), or the combination of the two (US + Cl)), was applied.

Ultrasound Treatment

For all samples and experiments, the power output on the ultrasound was held at approximately 1400 Watts. Flow rate was held at a constant 12 gal/min, to maintain equal residence time in the ultrasonic horn holding tank. After 5 minutes of mixing to evenly distribute the pigment, an initial reading was taken (0 min) and the ultrasound device was turned on. Readings were then collected every 10 minutes for 60 minutes. As a control, pigment treated water was run through the system with no ultrasound exposure, and the samples were analyzed following the same protocol.

Chemical Treatment

Sodium hypochlorite, a common industrial bleaching agent, was added at 200 ppm to the color treated water after the first 5 minutes of mixing. The first sample was taken immediately after the addition of the chemical, due to its rapidly induced effects. Samples were collected in shorter, one minute, intervals for 15 minutes, due to the increased bleaching power. The strength of the chemical bleaching power

was tested alone and also in combination with ultrasound. When used in combination, the chemical was added simultaneously to the ultrasound being turned on and samples were taken at the same shortened interval.

Spectrophotometry

The absorbance of the water color treated with annatto was measured at the maximum absorbance wavelength of the bixin molecule, 475nm, using a UV-vis spectrophotometer. For anthocyanin and β-carotene, 520nm and 457nm were used, respectively. Readings were taken immediately after removal from ultrasound exposure.

Statistical Analysis

Statistical analysis was done using JMP software. Analysis of variance (ANOVA) was used to determine significant differences in treatment rates ($p < 0.05$). Rates were log transformed for statistical analysis, to best fit the test.

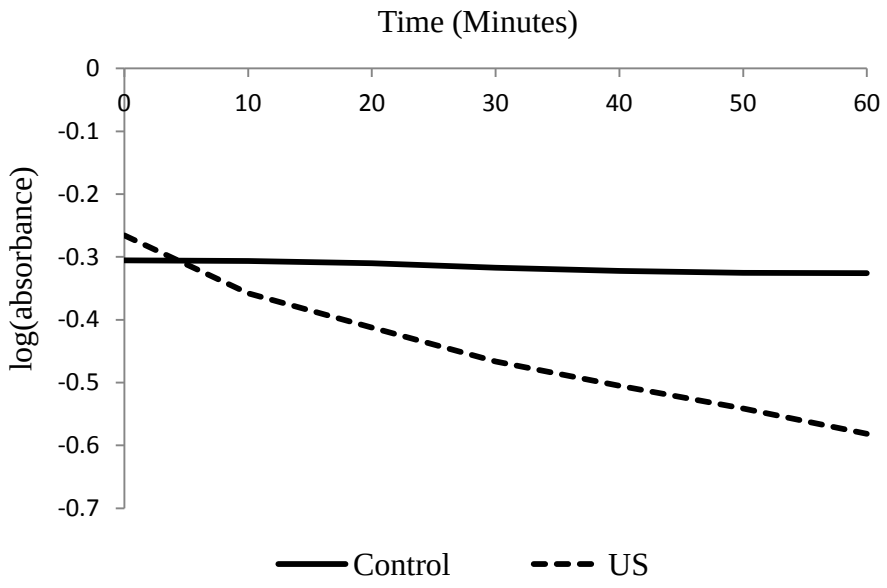
RESULTS

Effect of Ultrasound on Annatto

Ultrasonic exposure was found to significantly decrease annatto pigment intensity when compared to the control. After 60 minutes of exposure time, samples showed an average degree of bleaching (DOB) of 53% (Table 1). This means that at the end of 60 minutes, the absorbance values were reduced by 53% of those at the initial time of 0 minutes. After 60 minutes of simply mixing with no ultrasonic exposure, the control sample showed only a 6% degree of bleaching. This suggests that the high intensity ultrasound was responsible for approximately 47% of the bleaching seen. In addition to the bleaching capability of the ultrasound, the rate of bleaching was also increased significantly upon exposure. Rates of bleaching were determined by taking the slope of the log transformed lines (Figure 1). In order to calculate the most accurate rate, the slope was only taken for segments of the line exhibiting a clear

decline. Data collected prior to and after this period of absorbance decline were excluded because they did not represent bleaching. The rates given in Table 1 are the change in absorbance value per minute. For the control, the rate was found to be 0.0003 minute^{-1}, while the pigment exposed to ultrasound had a significantly increased rate of bleaching of 0.0034 minute^{-1}. While these values allow for a basic understanding of the strength of ultrasound on reducing pigment intensity, their accuracy can be questioned. It is possible that, over time, the control pigment will never truly lose all of its color, and the pigment may not be as responsive to the ultrasound treatment infinitely.

Figure 1. Effect of ultrasound exposure on annatto pigment intensity.



Effect of Chemical Exposure on Annatto

Chemical exposure significantly decreased the pigment intensity when applied both alone and in combination with sonication. It should be noted that the time scale of these treatments was significantly reduced compared to previous treatments (Figure 2). After 15 minutes, these treatments were terminated, as the maximum reduction was recorded. This maximum reduction endpoint was

determined when absorbance values maintained the same level for 3 consecutive readings. Both rate and degree of bleaching were increased significantly upon the addition of the sodium hypochlorite. When the chemical alone was added, an average 98% degree of bleaching at a rate of 0.1421 minute^{-1} was recorded. When added in combination with ultrasound, the average DOB was 99% at a rate of 0.2607 minute^{-1}. Absorbance values recorded in the range of 4-9 minutes were used to calculate the rate of bleaching for the Cl + US treatment. These results suggest that while both treatments are viable options for pigment reduction, the combination of ultrasound and chemical exposure produced the fastest and most notable reduction.

Figure 2. Effect of chemical exposure on annatto pigment intensity.

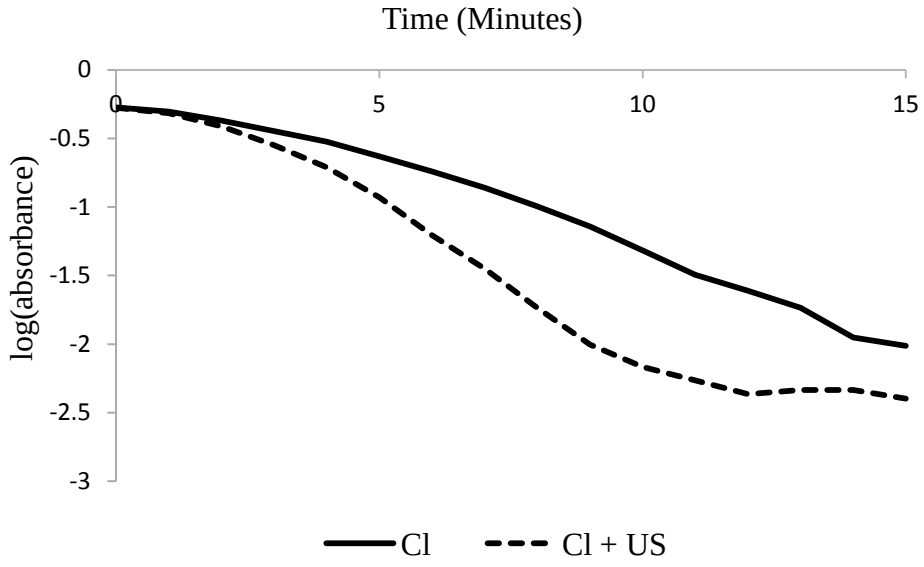


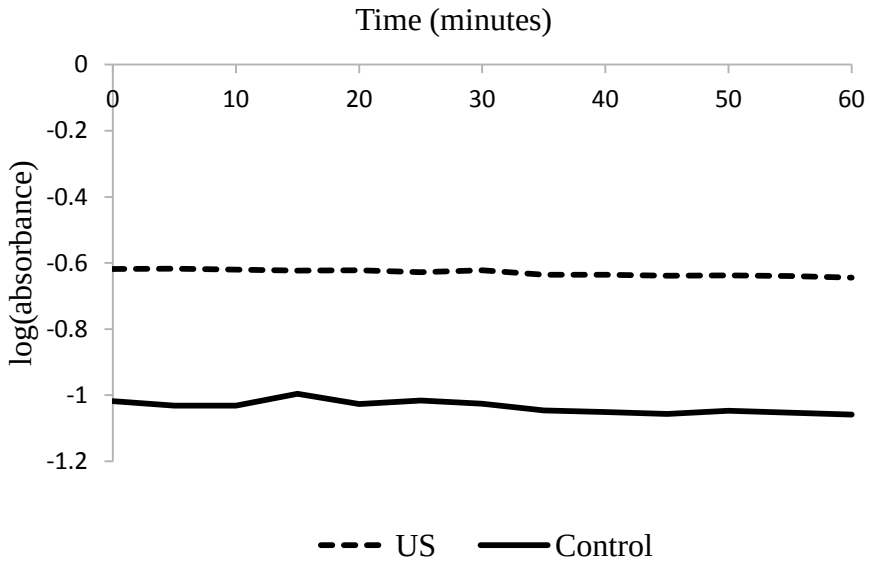
Table 1. Rate and degree of bleaching for various annatto treatments. Significantly different rates are noted by different letter superscripts ($p < 0.05$).

Treatment	Rate of Bleaching (minute^{-1})	Degree of Bleaching (%)
Control	0.0003 ^{a}	6
US	0.0034 ^{b}	53
Cl	0.1241 ^{c}	98
Cl + US	0.2607 ^{d}	99

Effect of Ultrasound on Anthocyanin

Ultrasound exposure was found to have no significant effect on the anthocyanin pigment intensity. Both control and US treatments resulted in statistically similar rates and degrees of bleaching (Table 2). These rates and degrees of bleaching were very similar to the control treatment of annatto. During data collection, starting absorbance values for these two treatments differed slightly (Figure 3). This was not considered particularly problematic because the values of interest, the rates of bleaching, were independent of the starting values. Differences between the molecular structures of this pigment and annatto render it nonreactive to the water species generated by the ultrasound.

Figure 3. Effect of ultrasound exposure on anthocyanin pigment intensity.



Effect of Chemical Exposure on Anthocyanin

Chemical exposure was found to have a significant effect on the anthocyanin pigment intensity. There was no significant difference, however, between the Cl alone and the Cl + US treatments (Figure 4). Since the combination of the two exposures did not result in improved pigment reduction, the bleaching effect seen in the combination treatment can be attributed to the Cl itself. These results further support the previous statements that the structure of the anthocyanin prevents reactivity with sonicated water

molecules. The Cl treatment was far less effective for the anthocyanin pigment than the annatto, attaining only about a 25% DOB. Furthermore, all the bleaching occurred in the first minute of exposure. Because of this, these rates were calculated using data collected in the first minute. This instantaneous response indicates that the mechanism of color loss for anthocyanin is different than for annatto, which took 11 minutes to reach its lowest absorbance.

Figure 4. Effect of chemical exposure on anthocyanin pigment intensity.

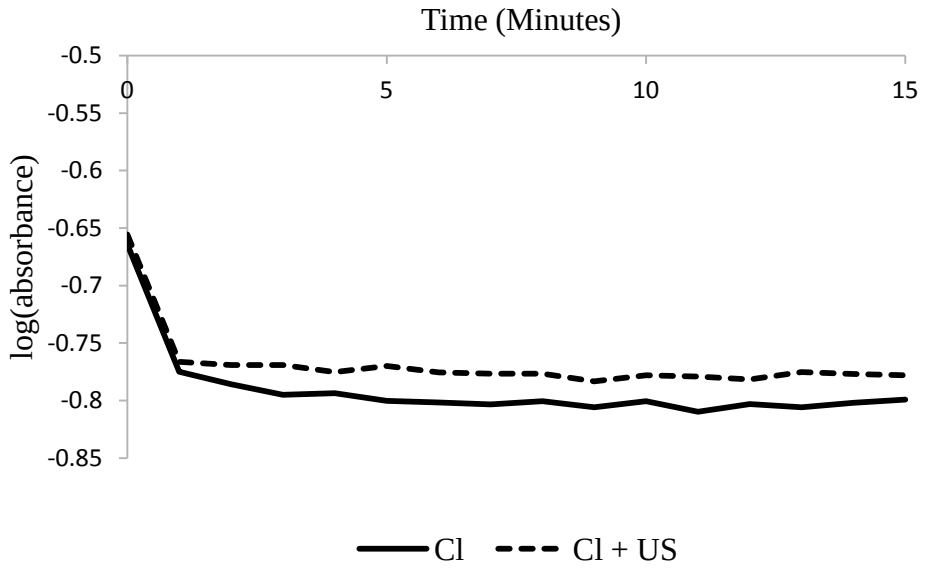


Table 2. Rate and degree of bleaching for various anthocyanin treatments. Significantly different rates are noted by different letter superscripts ($p < 0.05$).

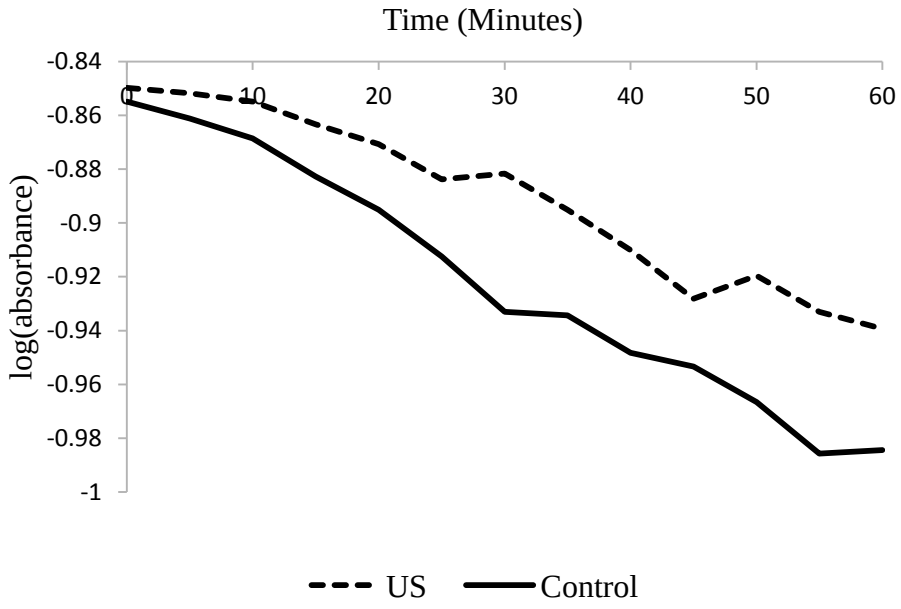
Treatment	Rate of Bleaching	Degree of Bleaching
Control	0.0007 [a]	8
US	0.0005 [a]	6
Cl	0.1107 [b]	27
Cl + US	0.1106 [b]	25

Effect of Ultrasound on β-Carotene

β-Carotene responded to ultrasound exposure differently than both annatto and anthocyanin. Though both US and the control treatments followed similar trends, the control actually resulted in more

bleaching with a faster rate than when exposed to ultrasound (Figure 5). The control treatment showed the highest DOB of all four treatments, at 26%, with a rate of 0.0024 minute^{-1}(Table 3). This rate was significantly faster than the US treatment rate of 0.0016 minute^{-1}, suggesting that US imparts the opposite effect on β-carotene than on the annatto and anthocyanin pigments. Instead of diminishing the pigment strength, it is possible that ultrasound preserves it.

Figure 5. Effect of ultrasound exposure on β-carotene pigment intensity.



Effect of Chemical Exposure on β-Carotene

β-carotene had a similar response to chemical exposure as the anthocyanin pigment. The Cl treatment was found to significantly reduce the pigment intensity, at a rate of 0.0084 minute^{-1} (Figure 6). At the end of 15 minutes, however, the Cl treatment only produced a 21% DOB, lower than the control. Like with anthocyanin, the combined chemical and ultrasound exposure did not further reduce pigment intensity. Instead, out of all the treatments, the combination of US + Cl resulted in the lowest DOB, 11%.

Figure 6. Effect of chemical exposure on β-carotene pigment intensity.

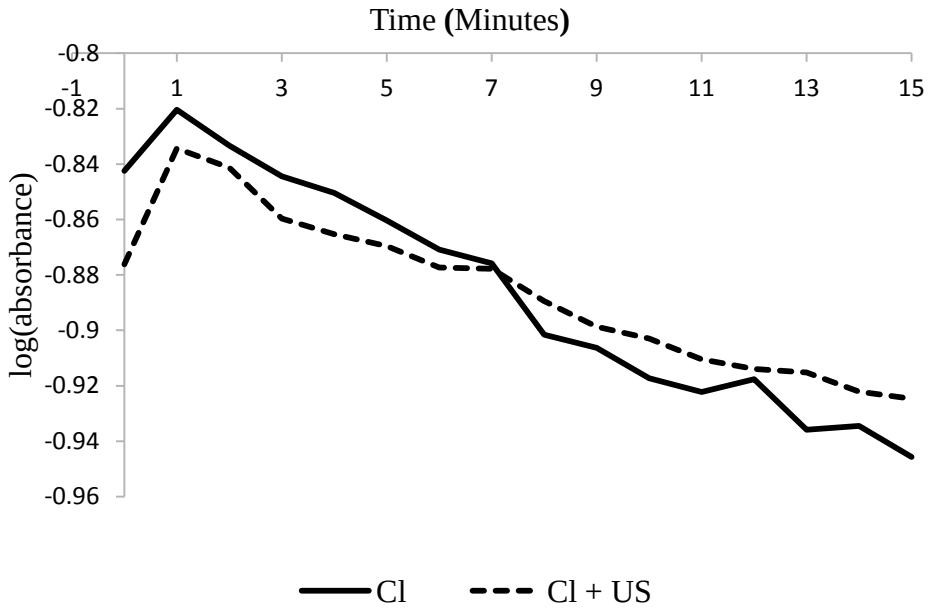


Table 3. Rate and degree of bleaching for various β-carotene treatments. Significantly different rates are noted by different letter superscripts ($p < 0.05$).

Treatment	Rate of Bleaching	Degree of Bleaching
Control	0.0024 [a]	26
US	0.0016 [b]	18
Cl	0.0084 [c]	21
Cl + US	0.0055 [c]	11

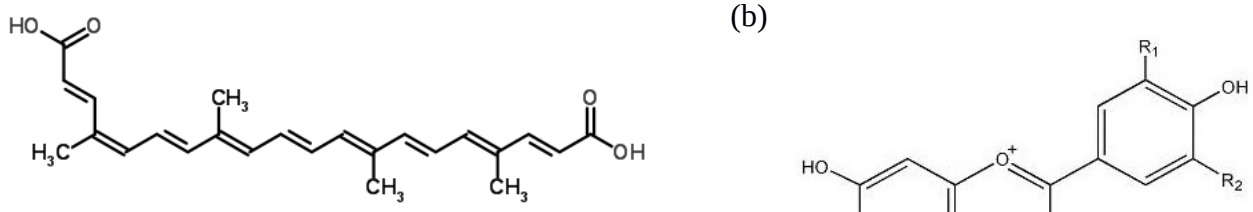
DISCUSSION

The results from this investigation support the hypothesis that the π-bonding systems of the chromophores are affected by exposure to the ultrasound. The molecular structures of the three pigments studied share a similar unsaturated hydrocarbon moiety (Figure 7). These unsaturated hydrocarbons are highly reactive due to their double π-bonds, which are weaker than single σ-bonds. This is due to orbital deformation and strain, leading to easier breakage. Overall stability depends on

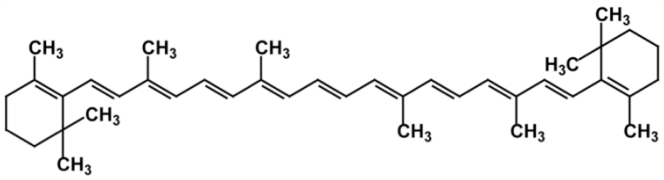
the functional groups attached to these hydrocarbon chains.

Figure 7. Molecular structures of the three pigments. (a) annatto, (b) anthocyanin, (c) β-carotene.

(a) (b)



(c)



(ChemSpider, 2014)

The chromophore of the annatto molecule is made up of repeating isoprene units, flan ked by two carboxyl groups. The chromophore of the β-carotene molecule is also made up of repeating isoprene units, in a slightly different conformation, and with two β-ionone rings in the place of the carboxyl groups. This slight variation in polymer structure, coupled with the unique functional groups, can be responsible for the different responses to ultrasound exposure. The β-carotene molecule is more stable, and less susceptible to oxidation by the reactive water species than the annatto. The chromophore of anthocyanin, while still an unsaturated hydrocarbon, is an aromatic structure. Aromatics are generally relatively stable due to their resonance ability. The anthocyanin structure is further stabilized by the high amount of β-ring hydroxylation. The stability of this molecule explains the lack of effect that ultrasound exposure had. In order to better understand the exact mechanism responsible for this pigment intensity reduction, future work should focus on taking a closer look at molecular structure of

pigments both before and after ultrasound exposure.

CONCLUSION

The results of this study suggest that ultrasound may be considered as a viable option for the reduction of annatto pigment, but not as much for β-carotene and anthocyanin. Ultrasound exposure alone would probably not be feasible in an industrial setting due to the large amount of exposure time necessary to achieve bleaching. The combination of ultrasound and chemical exposure would probably be the best applied treatment, as it would allow for reduced levels of chemical addition while maintaining a rapid rate of bleaching. Lowering the amounts of chemical oxidizing agents would lead to decreased amounts of flavor alteration and deleterious health and environmental effects.

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