A Study on the Photo-Stability of Spray Dried Bixin That is Encapsulated with Carbohydrates.

Master's Thesis in Food Technology

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Acknowledgement

The Master's degree project was carried out entirely in Lund University, Lund, Sweden during January 15th, 2017 till August 18th, 2017. The experimental work was executed in the labs of food technology department as well as the solar lab in the architecture department. The solar lab provided the source of light and space for all the samples under examination. The project involved investigation of the stability of color encapsulated in different carbohydrates towards light using two approaches.

I would like to thank many people who have made this project possible as well as have constantly supported me through thick and thin. Firstly, I would like to thank my principle supervisor Björn Bergenståhl, who has given me the opportunity to pursue this project. The project literally started from nothing, the way it has shaped to be is solely due to his presence of mind and his way of thinking. I have never met someone who knows as many things as he does, not only in chemistry. I am very much inspired by his vast knowledge and hopefully one day I would reach the same stage at least in one field of interest. I have always learnt something new and interesting from all our meetings so far. Secondly, thanks to my assistant supervisor Cecilia Curi, who has helped me more than I could have ever asked for! This project is part of her PhD work and therefore she knows a lot of things which I had the privilege to learn from her. She always had time to explain things to me very patiently even if I asked her twice. She played a major role in my project and her encouragement was energizing. Thirdly, a special thanks to Niko Gentile, without whom this project would not have been possible. He is from the architecture department, he has helped me immensely with the solar lab, the equipment there and the measurements related to light. He is a kind soul who has always offered to help in all the ways possible and remained available whenever needed, his enthusiasm is contagious. I also had the best group of classmates I could imagine, throughout the thesis stage we stuck together and were there for each other. We worked hard and played hard together. Last but not least, a huge thank you to my family for all their support and encouragement through these two years of masters, without whom I would not have had the chance to come here or be who I am now.

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Abstract

Bixin is a carotenoid which is extracted from annatto (Bixa orellana). Its main application is as a coloring agent. It consists of conjugating double bonds with a carboxylic end, making it prone to degradation by oxygen, light and heat. This study investigates the light stability of bixin encapsulated with maltodextrin (MD), sucrose and whey by spray drying. MD was added with additives such as carboxymethylcellulose (CMC), gum arabic (GA) and pectin to examine their interaction effects. Two sets of samples were used; one with pure bixin and carbohydrates, and the other, with non-filtered bixin encapsulated with the same set of carbohydrates and a carbohydrate naturally present in the seeds named botanical tissue. The samples were illuminated using a plasma sulfur lamp for a period of 30 days. UV-Visible spectrometer was used to keep track of the degradation. Statistic tests such as Shapiro-Wilk, ANOVA and t-test were used to confirm that the degradation was linear and that they follow first order decay. Quantum yields of photo-bleaching were calculated and compared between samples. Lower the quantum yield, higher the light stability. The presence of carbohydrates significantly improved the stability of bixin in comparison with non-encapsulated samples. The non-encapsulated samples lost their color completely by day 9 while the others lasted till 30 days. Sucrose showed the highest degradation rate in the absence of botanical tissue. Although sucrose and whey showed highest stability towards light in the presence of botanical tissue, the mix of MD and CMC worked better without botanical tissue. Botanical tissue seems to play a complex role in protecting bixin as the results varied with different carbohydrates.

Key words: Bixin; carbohydrates; botanical tissue; decay constant; quantum yield of photo-bleaching

Table of Contents

1. Introduction

1.1 Bixin as a carotenoid

Carotenoids consist of more than 600 naturally existing plant pigments that have essential biological benefits. (Rodriduez et al., 2006) Carotenoid pigments comprise a characteristic polyenoic chain which accounts for their chemical and physical properties. These properties include coloring and anti-oxidant activities as well as their biological functions. The conjugating double bonds present in the molecule lead to degradation through a chain of oxidation reactions caused by reactive species thus acting as an anti-oxidant. (Rascón et al., 2011) Carotenoids are quite unstable due to their sensitivity to oxygen, light and heat. They are insoluble in water and very slightly soluble in oil at room temperature in their natural form. For this reason, the pure pigments are not used for food coloring and this increases the demand for water dispersible natural colorants. (Rodriduez et al., 2006)

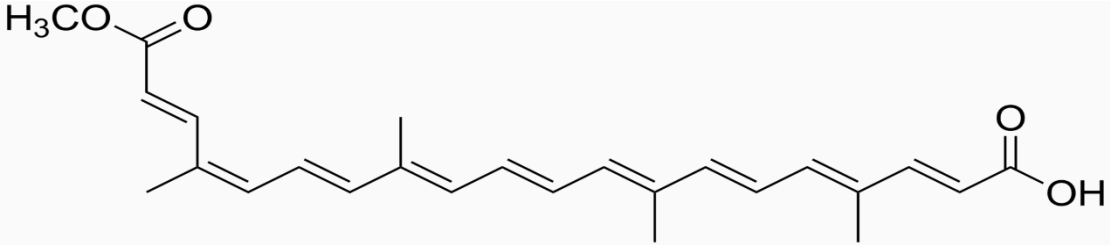


Figure 1.1: The structure of cis-bixin. (En.wikipedia.org, 2017)

Bixin belongs to the group of carotenoids and therefore has a structure of conjugating double bonds, along with carboxylic ends (Figure 1.1). (Lobato et al., 2013) It is part of annatto (Bixa orellana) and is extracted from the seeds of annatto's fruit. (Zhang and Zhong, 2013) Annatto is a tree found usually in South and Central America, cultivated in African and Asian countries as well these days. The fruits of annatto are covered in flexible thorns and are produced in the form of clusters. The red seeds inside the fruits are used to extract the color pigments. (Barbosa, Borsarelli and Mercadante, 2005) Bixin contributes to about 80% of annatto along with trace amounts of other carotenoids. Numerous methods are available for the extraction of bixin from annatto such as the use of oil, hot water or even solvents. (Shahid-ul-Islam et. al, 2016) Cis-bixin is the main form of bixin present in annatto, which is insoluble in water but soluble in most organic solvents. The saponified product of bixin is norbixin, which is water soluble and is stored as potassium or sodium salts in alkali solutions. Bixin extracted from annatto is used in a wide range of food applications including dairy products, fish, bakery products, soft drinks, snack foods, meat products and dry powdered mixes. (Barbosa, Borsarelli and Mercadante, 2005) Similar to other carotenoids, factors such as pH, temperature, oxygen and light also promote the degradation of bixin in both forms, resulting in color instability. (Zhang and Zhong, 2013) The presence of

conjugating double bonds gives the characteristic red color for bixin but it is also responsible for its poor stability during processing and storage conditions. (Barbosa, Borsarelli and Mercadante, 2005) Due to their extreme hydrophobicity, carotenoids are found in the membrane core or other hydrophobic regions, where they are believed to act as antioxidants and accordingly have important roles in the protection of plant tissues from possible damages caused by oxygen and light. (Boon *et al.*, 2010)

1.2 Spray drying

Since carotenoids are sensitive to oxygen, light and heat, it is difficult to preserve their color during storage. The degradation rate of color pigments is usually determined by keeping track of the change in their concentration over time, with the use of spectrophotometer. (Rascón et al., 2011) However, photo-degradation has not been focused upon to the same extent as the other factors. This kind of degradation cannot be completely eliminated but can be reduced to an extent as it is influenced by the accessibility to oxygen and light. One way to deal with color loss during storage is to encapsulate the color pigments, thus minimizing oxidation. Spray drying is a very efficient way to encapsulate food colors, giving them a better protection from degradation by improving their stability. (Ranveer *et al.*, 2015)

Figure 1.2: The setup of a spray drier

Spray drying is used to fabricate powders owing to its capability to synthesize a product of quality specifications at a continuous rate with precision. It is a widely used procedure to convert suspensions or liquid foods into powders in one go. (Krishnaiah, Nithyanandam and Sarbatly, 2014) It involves three principle steps: (i) atomization of the liquid, (ii) drying of the formed droplets, and (iii) transport of the dried powder (Shabde and Hoo, 2008)The principle of spray drying is the removal of moisture from the liquid feed by disintegrating it into minute droplets

with the aid of hot air to give a dry powder. In this process, the slurry is charged through the atomizing system into a drying chamber. Within the chamber, the droplets are trapped by a steam of hot gas and then carried towards the product recovery system. The heated gas causes evaporation of the relatively cool droplets when in contact. (Asheh et al., 2003)The time taken for drying is less compared to that of other drying processes. Process conditions such as temperature, pump rate, flow rate and aspiration rate have an impact on the drying time (Roustapour *et al.*, 2009)(Figure 1.2).

The main aim of spray drying to encapsulate colors by entrapping the pigments within a wall material that would isolate the pigment from the environment. The wall material acts as a barrier and provides protection against environmental factors to an extent. Generally, the wall material is made out of network forming compounds such as starch, gelatin and polymers. (Krishnaiah, Nithyanandam and Sarbatly, 2014) The encapsulation yield and encapsulation efficiency indicate how effectively the substance of interest has been encapsulated within the wall material and how much of it was lost in the process. (Ranveer *et al.*, 2015) The most frequently used wall materials in the food industry include gums, proteins, maltodextrins of distinct dextrose equivalent (DE) values and their mixtures. The choice of wall material has a strong impact on the solubility and stability of the core material. (Barbosa, Borsarelli and Mercadante, 2005)

1.3 Encapsulation using carbohydrates

When it comes to the use of carbohydrates as encapsulating agents; starches, maltodextrins and corn syrup solids have been used commonly. They are thought to be good at encapsulating due to their high solubility and low viscosities with high solid contents, but a lot of them do not provide high microencapsulation efficiency as they lack the interfacial properties necessary for that. Hence, they are usually combined with other wall materials like gums or proteins. Polysaccharides also exhibit gelling properties that aid in stabilizing emulsions towards coalescence and flocculation. Chemical modifications of common carbohydrates used as wall materials has been in practice in order to improve their encapsulating properties. (Krishnaiah, Nithyanandam and Sarbatly, 2014)

Studies have shown that the use of maltodextrin (MD) as a wall material results in higher retention of the spray dried powder compared to that without maltodextrin. (Krishnaiah, Nithyanandam and Sarbatly, 2014) MD with dextrose equivalent values between 10 and 20 could be dispersed in water without forming haze and result in very high retention of flavor, making them fit for use as wall material. (Gharsallaoui *et al.*, 2007) In one study, the powders spray dried without MD were quite sticky which mainly deposited on the wall of the spray drying chamber and cyclone, resulting in very low yield. It was also found that increasing the concentration of MD has a positive effect on the overall yield as well as the quality of the powders. MD alters the surface stickiness, facilitating drying and reduced stickiness of the product. (Krishnaiah, Nithyanandam and Sarbatly, 2014)

Another study involving encapsulation of ß-carotene has shown that MD has a low emulsifying property leading to reduced stability of the emulsion due to harder dispersion of lipid constituents. A blend of starch, gum arabic (GA) and MD were used in the study. An increased content of MD in the mixture gave a higher density to the spray-dried powder. In contrast, MD gave a lower density to hydrolyzed proteins and this was explained by the author. It was said that MD is low in density, and has the ability to surround the surface of the droplets with an impermeable film during evaporation. Consequently, air bubbles are trapped within the particles, increasing the internal porosity and giving a lower density. Whatsoever, a mixture of GA and MD produced powders with the highest retention and increased half-life. This was linked to the higher density of the particles, leading to an efficient protection of the core material against external factors. GA is quite malleable and defiant to deformation and cracking of the microcapsule decreasing the loss of the core material. In addition to that, MD has complimented well with GA, boosting its protective properties. (Przybysz *et al.*, 2016) It will be interesting to look at how MD interacts with different compounds and changes the properties of bixin when encapsulated.

Materials encapsulated with sucrose have a lower encapsulation efficiency and a higher solubility in water. (Barbosa, Borsarelli and Mercadante, 2005) Adding sucrose as a wall material gives a smooth outer surface to the microcapsules. The reason for this is that the sucrose molecules retain water molecules with their structure, occupying the hollow space of the microparticles, hydrating them from inside and preventing depressions on the outer surface, smoothening it uniformly. (Shu *et al.*, 2006) But it is difficult to work with, as it has caramelization properties, it adheres to the surface of the drying chamber and has a heterogenous nature which causes the clogging of the spray nozzle. (Gharsallaoui *et al.*, 2007)

Whey protein isolates have been used widely for encapsulation of milk fats with a low lactose concentration. In general, hydrophilic compounds with glass temperatures higher than ambient conditions like hydrocolloids or sugars are suitable as fillers. Lactose can act as a filler for microencapsulated fat powders, but owing to it being hygroscopic, there is a high chance of it recrystallizing in humid conditions causing fat globule coalescence. Increased lactose concentration improves the barrier function of protein wall of the fat against solvent. It also limits the aggregation of fat globules during drying, producing powder with lower free fat. (Keogh and O'Kennedy, 1999)

Pectin is another suitable material for encapsulation, as it gives stable emulsions at very low concentrations. It is a polymer with protein residues within its chain and a high content of acetyl groups, making it a great emulsifier. As low as 1-2% of pectin is adequate to prepare a stable emulsion for encapsulation by spray drying. The spray drying conditions have no major effect on the functional properties of pectin. (Leroux et al., 2003)

1.4 Light stability

Color degradation can occur through one of many mechanisms, but the main focus in this study will be on photo-degradation and auto-oxidation. Photo-degradation occurs when light induces electronically excited states of the carotenoid or oxygen, called the singlet states. Three ways of photo-degradation were described by Jørgensen and Skibsted, out of which two involve just carotenoids and oxygen; (i) reaction of ground state oxygen with singlet states of carotenoids produced by direct absorbance of light which are vibronically unrelaxed and (ii) reaction of singlet oxygen with carotenoid in ground state. The possible explanation for the reactivity of the carotenoids and oxygen while in singlet states, which requires high energy is that a fraction of the energy is provided by the photons from light in turn lowering the activation energy.(Jørgensen and Skibsted, 1990) Auto-oxidation of carotenoids was observed to take place quite easily, especially when purified and added in organic solvents. It is proposed that the auto-oxidation is initiated by isomer formation in the solution. The molecule gets twisted during the isomerization process which leads to an unpaired spin state, making it reactive towards oxygen. This is then followed by a chain of oxidative reactions leading to degradation products. (Boon *et al.*, 2010)

Photo-degradation of bixin can be controlled to some extent by encapsulating it with carbohydrates using spray drying. The encapsulated bixin has much greater stability against light in comparison to non-encapsulated bixin. Carotenoid photo-bleaching requires an energy barrier of about 20kcal/mol, therefore it can happen with relative ease under illuminated conditions. Encapsulated bixin anyway has a better tolerance against light compared to non-encapsulated bixin, but it was seen that it has 10 times higher stability compared to non-encapsulated bixin even under dark conditions. (Barbosa, Borsarelli and Mercadante, 2005)

Although, encapsulation can protect bixin better, there is always a catch depending on the type of wall material used. For instance, MD is very effective in protecting carotenoids, but GA makes bixin more stable towards light. This could be due to the structure of GA which is highly branched, making it better at film-forming and at emulsifying non-polar substances. (Gharsallaoui *et al.*, 2007)

1.5 Degradation kinetics and Quantum yield of photo-bleaching

Multiple methods can be used to determine the rate of color loss when it comes to degradation kinetics. The most common method employed to check for degradation is UV-Vis spectroscopy. The spray dried samples are usually exposed to any form of light over a period of time, and the absorbance is recorded during regular intervals. This absorbance is then correlated to the color pigments concentration, giving the result in retention percentage. The retention of the encapsulated samples would be higher than the non-encapsulated samples in any case. (Wang *et al.*, 2012)(Barbosa, Borsarelli and Mercadante, 2005)

Such an approach is very general to apply for color pigments, as the results are not quantitative enough. An intriguing way to determine the photo degradation is to find the quantum yield of photo-bleaching. Jørgensen and Skibsted indicated that carotenoids have a significant wavelength dependence when they are photo-bleached by calculating their quantum yield of photo-bleaching at four different wavelengths. (Jørgensen and Skibsted, 1990)

Photo-oxidation was found to be higher in the UV region than in the visible region. Carotenoids are efficient physical quenchers of singlet oxygen which are nothing but molecular oxygen in a higher energy state. There is a proportionality between the quantum yield of photo-bleaching and the square root of partial pressure of oxygen, pointing towards an intricate photo-oxidation pathway in carotenoids. More than one molecule of oxygen is usually needed for each carotenoid molecule to get bleached. The dependence on the oxygen partial pressure displays a complex chain reaction. This could involve an initiation reaction following light absorption, wherein two reactive radicals emerge from one oxygen molecule, which later on react with the carotenoids.(Jørgensen and Skibsted, 1990)

The objective of this study is to evaluate the stability of bixin in different encapsulation systems towards light. These encapsulation systems are made of mainly carbohydrates along with some additives. The carbohydrates used include maltodextrin (MD), sucrose and whey. The additives that were added with MD include gum arabic (GA), carboxymethylcellulose (CMC) and pectin. The light stability of bixin will be evaluated in terms of the degradation rate and the quantum yield of photo-bleaching. The samples used in this study were prepared and provided by Ms. Cecilia Curi, as this study is part of her PhD work.

2. Materials and Methods

2.1 Materials required

Six different encapsulation systems were considered for the study, where two sets of samples were used. One set consisted of pure bixin encapsulated with carbohydrates, while the other set consisted of non-filtered bixin encapsulated with the same carbohydrates as the other set. Non-filtered (NF) bixin contains an additional carbohydrate naturally presents in the seeds it is extracted from, named botanical tissue (BT). Non-filtered bixin means that the BT was extracted along with bixin during the solvent extraction procedure. In addition to these systems, reference samples were used for comparison purposes. These reference samples included pure bixin, non-filtered bixin and encapsulated non-filtered bixin. It can be noticed that the reference samples used are in absence of external carbohydrates. The samples with their composition and code names are listed in table 2.1. The exact ratios of components in the encapsulated systems are given in Appendix I

Table 2.1: List of samples with their compositions and code names.

	Samples	
Bixin+Carbohydrates	MD	Ao
	MD+GA	Bo
	MD+CMC	Co
	MD+Pectin	Do
	Whey	Eo
	Sucrose	Fo
NF Bixin+ Carbohydrates	MD	Ho
	MD+GA	Ae
	MD+CMC	Be
	MD+Pectin	Ce
	Whey	De
	Sucrose	Ee
Without Carbohydrates	Pure Bixin	Fe
	Bixin+BT	Ge
	Bixin+BT(enc)	Go

Plasma sulfur lamp (Philips) was used for illumination of samples, of which the illuminance was measured by Hagner EC-1 luxmeter. Data logger was used to keep a check on the environmental conditions such as temperature and humidity in and around samples. The chemicals used for the sampling and further analysis included Acetonitrile and Methanol, both of HPLC grade from VWR. UV-Visible spectrophotometer (Varian Cary 50 Bio) was used for the absorbance measurements. IBM® SPSS® Statistics 24.0 was used for statistical tests.

2.2 Methods

2.2.1 Method formulation

Initial trials were carried out to establish a protocol for the sampling and further analysis. The samples can be dispersed easily using distilled water. This was however crossed out of the options due to the noise produced in the absorbance curve. Later, a mixture of glycerol and water was used for a 22-day trial. This had given a smooth absorbance curve but the bixin was not completely dissolved in glycerol, resulting in a low absorbance value. This way, different options were examined until finally, a mix of distilled water, acetonitrile and methanol was chosen for the sampling.

2.2.2 Sample preparation

The spray-dried powders were weighed and added onto 96-well microplates in duplicates. Each sample was added into a separate assigned well. The encapsulated samples with pure bixin were weighed to be 3mg while the samples with non-filtered bixin were weighed to be 4mg, except the one with CMC (Be), was weighed to be 2mg. The encapsulated reference sample was weighed to be 1.2mg while the other reference samples; pure bixin was 1.5mg and the non-filtered bixin was 3.914mg. The arrangement of samples in the microplate is shown in the figure below (Figure 2.1). From table 2.1, the samples marked "o" represent those in the odd columns of the microplate while those marked with "e" represent those in the even columns of the microplate.

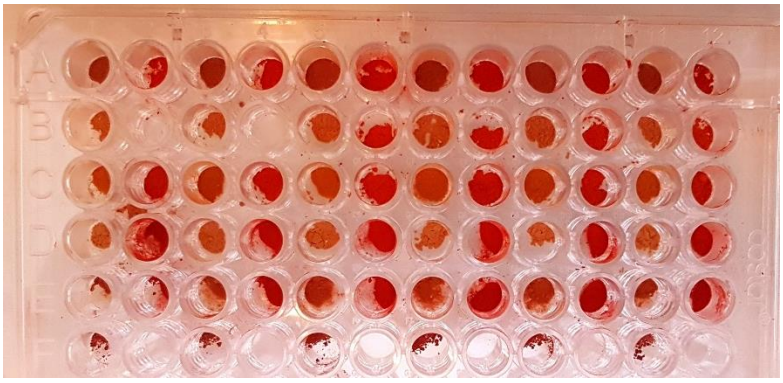


Figure 2.1: Sample arrangement in a 96-well microplate.

2.2.3 Light stability trial

The 96-well microplates with the samples were placed on a white glossy desk having a measured reflectance of 96%. The desk was approximately 5 meters below the plasma sulfur lamp (used for illumination). Plasma sulfur lamp provides a lighting of continuous spectral emission within the visible region of the electromagnetic spectrum, similar to the daylight. (Florentine *et al.*, 1997; Turner *et al.*, 1997) Although it imitates the daylight in the visible region, the lamp light has a very low emission in the UV and the infrared region of the spectra unlike daylight.

In order to measure the spectral power distribution (SPD) of the lamp in the 340-840 nm spectral range, an Avantes Avaspec ULS-2048 was used. Figure 2.2 shows a comparison between the measured relative SPD of the standard daylight illuminant D65 with the SPD of the lamp as defined by CIE (International Organization for Standardization, 2007).

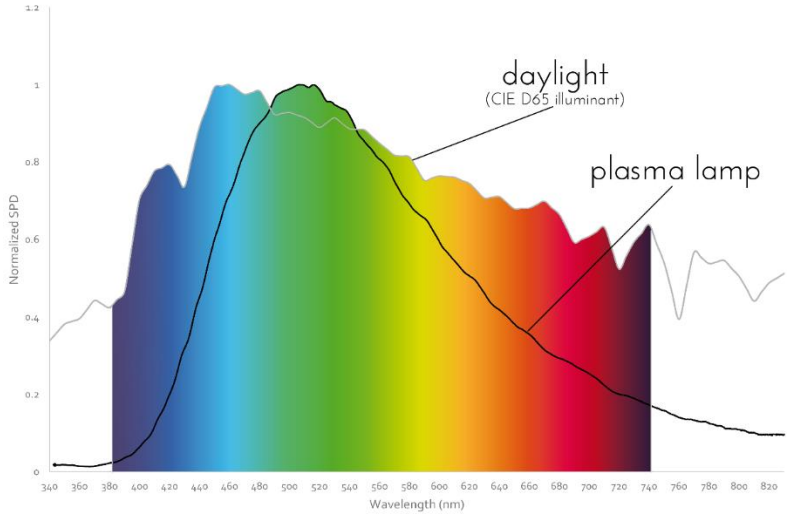


Figure 2.2: Measured relative SPD for the plasma sulfur lamp in comparison to standard CIE daylight D65 illuminant.

All SPD measurements of the plasma sulfur light were taken at the sample height, in the center of the desk. The experimental setup is shown in Figure 2.3. Two wires were inserted into the wells to measure the internal temperature as well as a probe to measure the surrounding temperature and a probe to measure the humidity (important due to the nature of the powder).

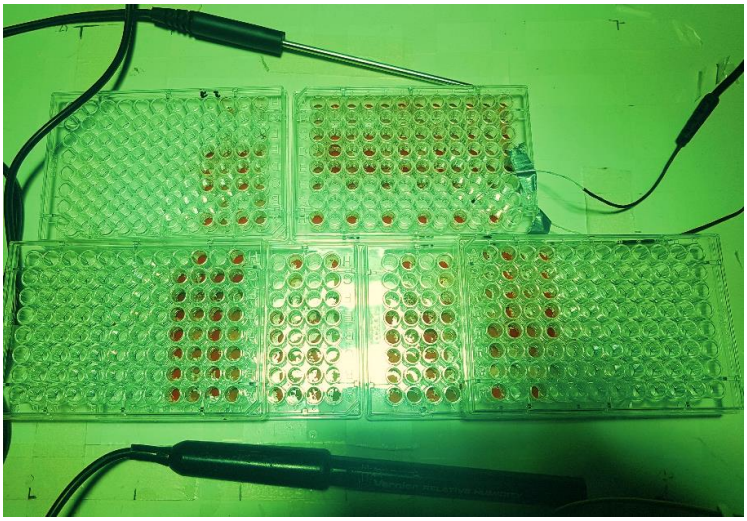


Figure 2.3: Experimental setup in the solar lab under the plasma lamp.

A Hagner EC-1 luxmeter was used to measure the illuminance (E) on the desk. The area of the desk where the samples were placed showed an illuminance in the range of 116000 and 186000lux, with a uniformity ratio (U_0) of 0.76. The uniformity ratio U_0 is defined as the ratio between the minimum E_{min} and the average E_{avg} illuminance on a surface (CEN, 2011)

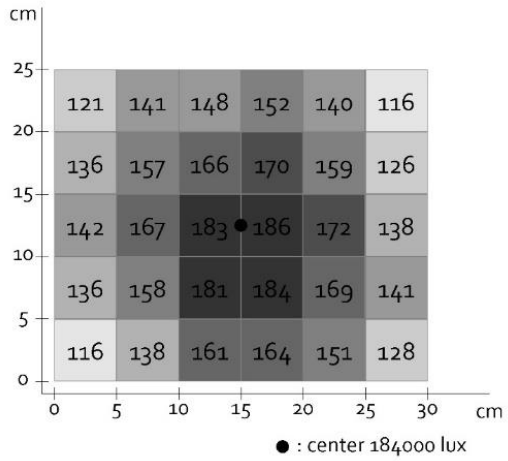


Figure 2.4: Illuminance distribution on the samples area. Values are shown in Klux

The maximum irradiance on the desk was 747 W/m^2 (in terms of energy flux) and the irradiance distribution was similar to that of the illuminance. In this case, a relatively low irradiance corresponds to a very high illuminance as the plasma sulfur light emits almost completely in the visible spectrum. The samples were placed in the center most region (the boxes shaded darkest) of the illuminated area, but each sample was exposed to a different light intensity, which was taken into consideration for further calculations (Figure 2.4).

The experimental setup was located in a big solar laboratory with some access to daylight. Moreover, the laboratory contains a solar simulator powered by fluorescent lamps. Despite the precautions taken, there was a chance that the two additional light sources might have contributed to the delivered SPD to the samples. Therefore, additional SPD and illuminance measurements were executed for the plasma sulfur light combined with the solar simulator on, the daylight, and a combination of both. The resulting measurements of the different combinations were almost identical to that for the plasma sulfur lamp alone. This is due to the fact that the plasma sulfur lamp delivered an illuminance on the desk with two orders of magnitude higher than the other light sources.

It should however be noted that, because of other ongoing research activities, the solar simulator was sometimes running during this experiment. This had a very little effect on the delivered radiation but it caused an increase in the ambient temperature. The samples were left under the light for a total period of 30 days, during which the temperature and humidity were measured throughout. Samples were taken in intervals of 3 days starting from day 0, with additional samplings after 12 hours and day 1. Unlike the normal samples, the reference samples were taken every hour for 12 hours each and then they followed the pattern of the other samples until they showed complete degradation/color loss.

2.2.4 Analysis of samples

In order to remove the samples from the wells, they were diluted with distilled water and taken into volumetric flasks of 50ml. They were further diluted with a mixture of Acetonitrile and Methanol, resulting in a solution comprising of water, Acetonitrile and Methanol (1:2:2). This procedure was applied to all the samples, and then the reference samples (Fe and Ge) were diluted 10 times more to give a final concentration of 3ppm. UV-spectrophotometer was used to measure the absorbance of the samples, the spectra of each sample was recorded.

2.2.5 Calculations

Jørgensen and Skibsted had formulated an equation to calculate the number of photons absorbed by carotenoid irradiated with monochromatic light while in a photolysis solution (Equation 1). This solution was either saturated with a mixture of oxygen (3%) and nitrogen (97%) 30 min prior to photolysis or saturated with air and kept in contact with air during photolysis. An Osram HBO 100/2 high pressure mercury lamp was used to produce the monochromatic light setup in an optical train with other accessories, different filters were used to vary the wavelength of irradiation. The number of photons absorbed by the color pigment in the photolysis solution was determined over finite intervals of time, starting from time 0 (t_0) till time t_i (in s). (Jørgensen and Skibsted, 1990)

Quantum Yield equations taken over time intervals:

$$Q(t_i) = \frac{\sum_i (I'_o / V \cdot N_A)(1 - 10^{-A_{irr}})(t_i - t_{i-1})}{C_o} \quad (1)$$

Where $Q(t_i)$ is the no. of photons absorbed (mol. Einstein^{-1}), I'_o is the light intensity (quanta.s^{-1}) A_{irr} is the absorbance of the sample at $½ (t_i - t_{i-1})$ for a specific wavelength, V is the Volume of the photolysis solution (l^{-1}), N_A is the Avogadro's number and C_o is the initial concentration of carotenoids in the photolysis solution. (Jørgensen and Skibsted, 1990)

Whereas the quantum yield of photo-bleaching ($Ø^{irr}$) takes the relative absorbance over time into consideration and is defined as: (Equation 2)

$$\emptyset^{irr} = \frac{(A(t_o) - A(t_i))/A(t_o)}{Q(t_i)} \quad (2)$$

These quantum yield equations take into account the change in absorbance of photons over time intervals for specific wavelengths. However, in this case, it is required to consider an entire spectrum with a wide range of wavelengths as well as the time (in s). Therefore, the modified versions of the equations are given below (Equation 3 & Equation 4):

$$Q(t_i) = \frac{a_w \sum_t \sum_\lambda \left(I_o \left(1 - 10^{-A_{(\lambda t)} \delta} \right) \right) t}{m \cdot C_o \cdot N_A} \quad (3)$$

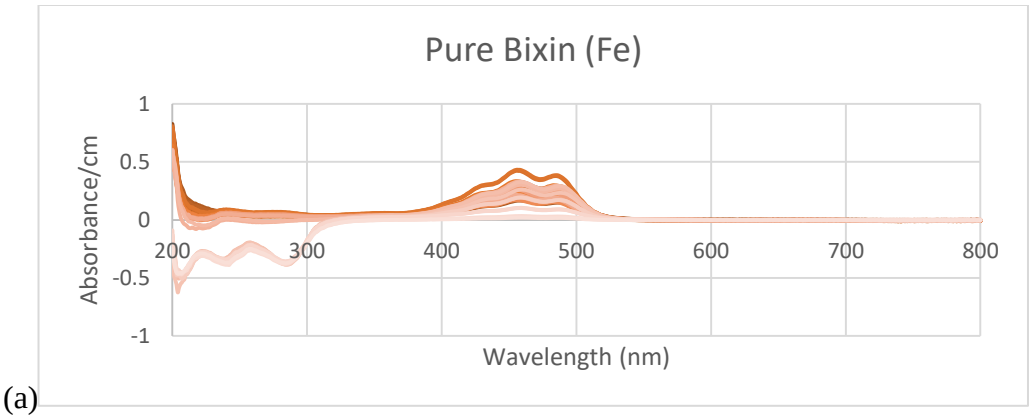
$$\emptyset^{irr} = \frac{m \cdot C_o \cdot \left(A(t_o) - A(t_i) \right) / A(t_o)}{Q(t_i)} \quad (4)$$

Where a_w is the area of the well in which the sample is added (m^2), I_o is the intensity of light (quanta. $m^{-2}.s^{-1}$), $A_{(\lambda t)}$ is the calculated real absorbance of bixin in the powder at a specific wavelength and time (cm^{-1}), δ is the thickness of the sample powder in the well (cm), t is the time of sampling (in s), m is the mass of the sample (g), C_o is the concentration of bixin present in the sample (mol. g^{-1}) and N_A is the Avogadro's number. The formulas required to calculate $A_{(\lambda t)}$ and δ are given in Appendix II.

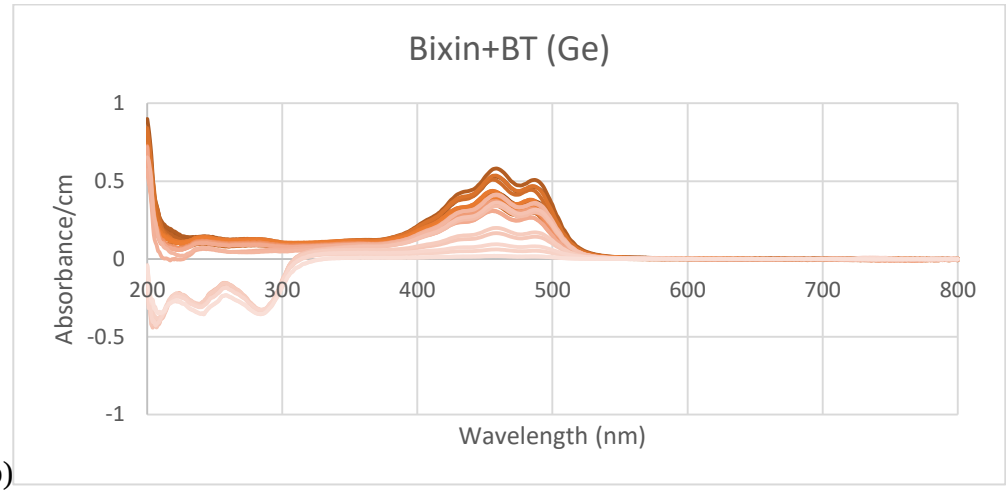
3. Results

3.1 UV-Vis spectroscopy

The absorbance spectra was taken for all the samples at regular intervals of 3 days in a period of 30 days using UV-Vis spectrometer except the initial stages, where the intervals were shorter (Day 0, 12 hours and day 1). The main absorbance peaks can be observed between the wavelength 350-550nm of the absorption spectra. There are three main absorption peaks that occur with all the samples in their spectra. It is seen from the absorption curves that the main peaks reduce over time in the visible region of the spectra, while the absorbance increases in the ultraviolet region of the spectra over time. The peaks in the visible region and the ultraviolet region seem to be inversely proportional. Overall, the absorbance of the reference samples have been the lowest of all (Figure 3.1).



(a)



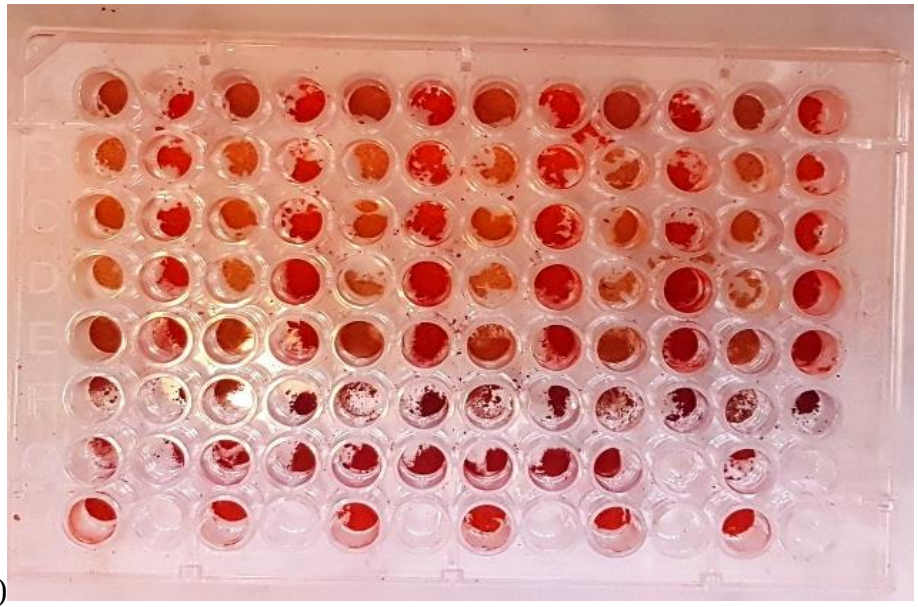
(b)

Figure 3.1: (a) The absorption spectra of pure bixin over 9 days. (b) The absorption spectra of non-filtered bixin with the same concentration of bixin as Fe but has a higher absorbance in the beginning.

3.2 Degradation

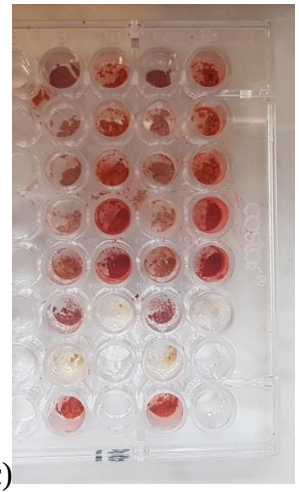
There is a clear visual difference in the color of the samples along the period of 30 days. From the appearance, it can be seen that the samples with botanical tissue retain their color to a greater extent compared to those with just bixin and carbohydrates (Figure 3.2). While this is the case for encapsulated samples, the reference samples (Go, Fe and Ge) lost their color completely by day 9, which is in accordance with the absorption spectra (Figure 3.1).

(a)



(b)

(c)



(d)

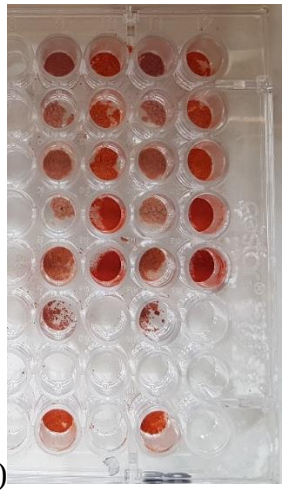


Figure 3.2: Samples at (a) Day 0; (b) Day 9; (c) Day 18 and (d) Day 30.

In order to determine the degradation rate constant (K-constant), a graph of change in bixin concentration in the sample against time and irradiance was to be plotted. The change in bixin concentration in the well was calculated theoretically from the absorbance measured in UV-Vis spectrometer. The amount of bixin present in the well reduced over time, this was proportional to the absorbance. Thus, the absorbance values at 458nm were used to calculate the amount of bixin present in the well and this was then divided by the area of the well to give concentration in g/m^2. The calibration of bixin absorbance was done, from which the absorptivity value was calculated and used in the calculations. The logarithm of the concentration was taken and normalized over the highest concentration, repeated for all the samples (x-axis). For the y-axis, the time for which the sample was exposed was multiplied with the irradiance it faced to give a value in $W/m^2.s$. The slope of the graph plotted gave the K-constant.

When the change in concentration of bixin in the samples was plotted against time and irradiance at 458nm (the highest peak of absorbance), a common trend was noticed in all the samples. The degradation of bixin followed first order reaction and an example of this in one of the samples is shown in figure 3.3.

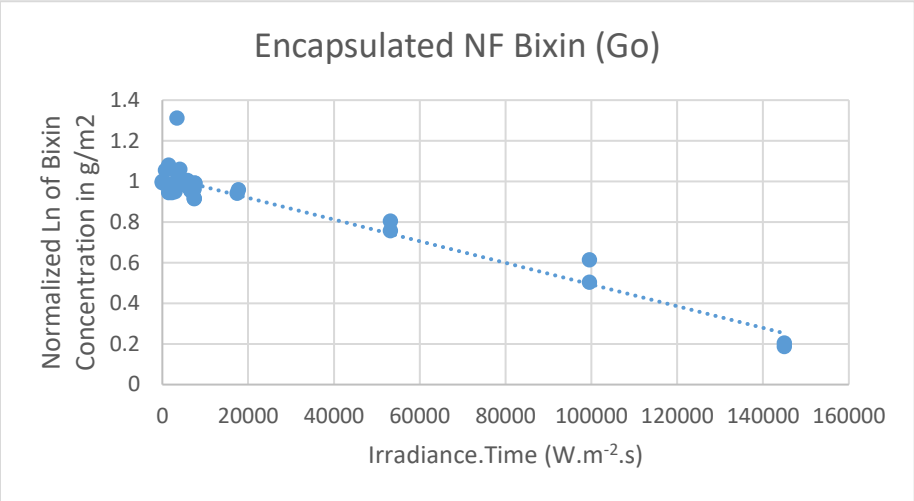
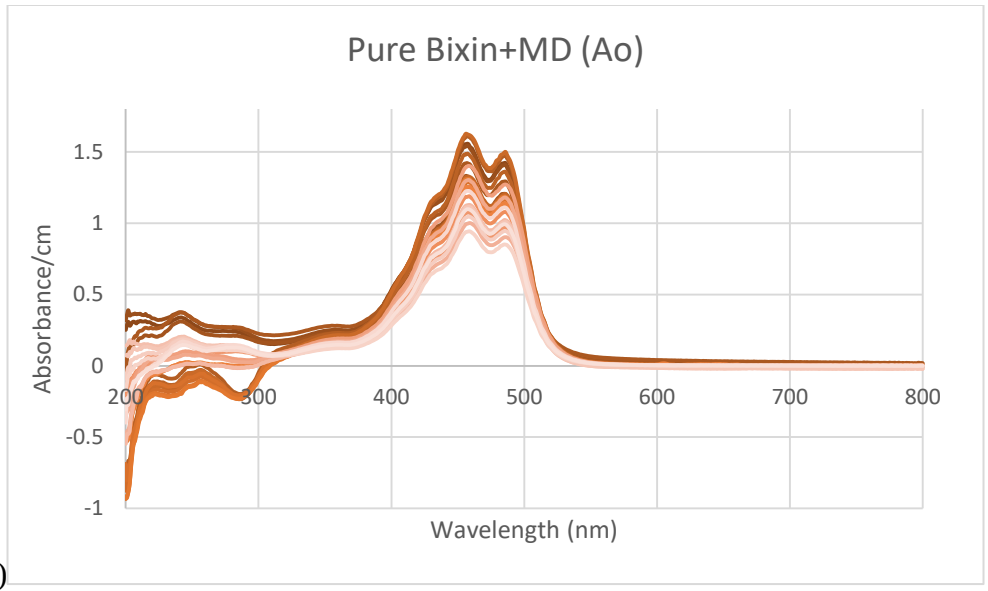
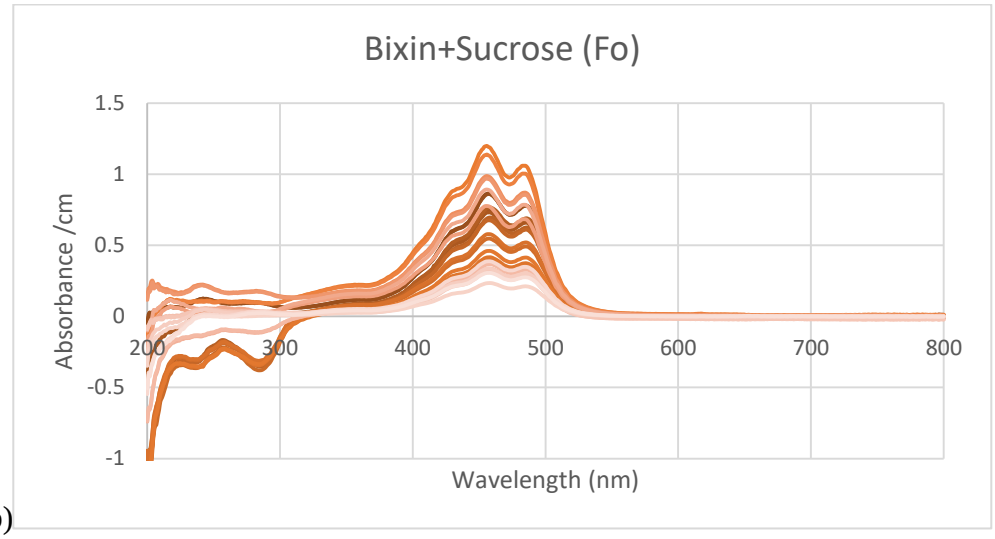


Figure 3.3: An example of the first order decay of bixin.

The overall degradation was low for all the encapsulated samples, but it did vary amongst the samples. A summary of the K-constants is given in table 3.1. The degradation rate constants are approximately in the same range for all the samples except Fo (Bixin + Sucrose) and the reference samples. These have a higher rate constant compared to the other samples. The difference in the degradation is also visible in the absorption spectra when compared to those with a lower k constant (Figure 3.4). The rest of the absorption spectra are included in Appendix IV.



(a)



(b)

Figure 3.4: (a) The absorption spectra of bixin maltodextrin which shows a slower degradation than (b) sucrose with bixin although with similar concentrations.

Statistical tests were carried out on the degradation of bixin in all the samples. Shapiro-Wilk test showed that all the samples followed the normal distribution. F-test and t-test were also performed, and they showed that there is no significant difference at a confidence level of 95% so the degradation occurs in a linear manner. This indicates that the rate of degradation of the samples does follow first order reaction. A summary of the statistic tests is given in Appendix III.

3.3 Quantum Yield

The quantum yield of photo-bleaching was calculated for all the samples, integrated over 30 days and the wavelength of 340-560nm. The list of the quantum yields for all the samples are shown in table 4.1. Out of all the samples, the reference samples have the highest yields of photo-bleaching. However, the non-filtered bixin with botanical tissue (Ge) shows a lower yield compared to the other reference samples. Among the other samples, Co, De and Ee have lower yields of photo-bleaching. The presence of botanical tissue in De and Ee resulted in a better protection of bixin against light than their counterparts without botanical tissue (Eo and Fo). Meanwhile, this does not seem the case for Co (Bixin+ MD+ CMC), the presence of botanical tissue increased the quantum yield of photo-bleaching. The lower quantum yields mean that those systems are more stable to light compared to the others.

4. Discussion

4.1 UV-Vis spectroscopy

Dissolving the samples in a mixture of acetonitrile, methanol and water helped in producing clear solutions which in turn aided in getting smooth absorbance curves. Encapsulation aided in making the samples dispersible in water which was not possible with pure bixin. The solvent mixture had dissolved the carbohydrates completely, resulting in maximum dispersal of bixin. The absorbance of bixin in the samples is directly proportional to their concentration. The information obtained from the absorbance spectra can be used to calculate the bixin concentration by means of its absorptivity value. The absorptivity value can be calculated by calibrating the absorbance of the color pigment at different concentrations. The calibration of bixin was done prior to the main experiment. It was seen that during degradation, the main absorption peaks of bixin in the visible region decreased proportionally and the peaks increased in the UV region of the spectrum. This is the result of disruption of the conjugating double bonds giving rise to degradation products. The degradation products are responsible for the shift in the absorption peaks. (Jørgensen and Skibsted, 1990)

The energy obtained from the absorption of photons during the light stability trials was utilized in the production of degradation products. The photon energy gives rise to excited states of the carotenoid or the oxygen around it shifting them to a singlet state. These singlet states are very reactive and thus react with the other in ground state resulting in oxidative reaction. (Jørgensen and Skibsted, 1990) A chain of these oxidation reactions initially produce epoxides, carbonyl compounds, further followed by secondary short chain carbonyl compounds, carboxylic acids and carbon dioxide. These are basically the degradation products of carotenoids. (Boon *et al.*, 2010)

The presence of degradation products has a huge impact on the absorption spectra, as it displays the absorbance of all the compounds present in the sample. The use of HPLC is an efficient way to determine the oxidation products produced due to degradation. (Boon *et al.*, 2010) Different degradation products absorb at a different wavelength and have different retention times due to the difference in molecular size. This property is of particular advantage for HPLC, making their identification possible. It also produces the spectra of pure bixin in the samples unlike UV-Vis spectrometer which is generic. However, the same diluted samples were also analyzed using High Performance Liquid Chromatography (HPLC) to look for possible errors in measurements. The correlation shown between the results of UV spectrometry and HPLC is shown in figure 4.1. This correlation is an indication of the consistency in the measurements as well as a confirmation that the absorbance from UV-Vis measurements reflect the bixin concentration.

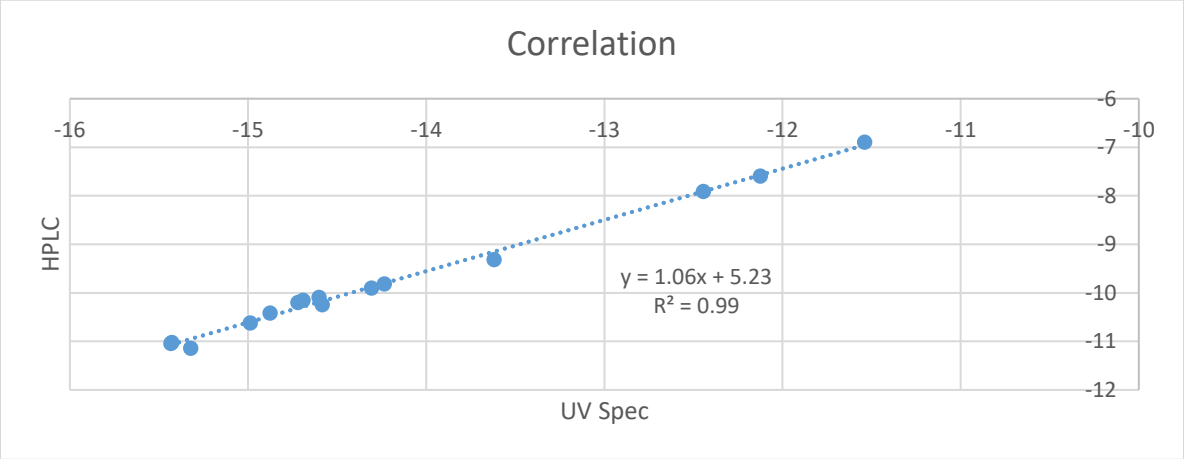


Figure 4.1: The correlation between the decay constants calculated from UV-Vis spectroscopy and HPLC.

4.2 Degradation

The powders with carbohydrates showed better color retention and stability towards light compared to the reference samples. This means that the encapsulation with carbohydrates has a beneficial effect on bixin. Although encapsulation of the color pigment is supposed to improve its stability, the encapsulated reference sample did not have any superior stability compared to the non-encapsulated reference samples. The presence of botanical tissue has however resulted in a more appealing color of the samples. Above a certain concentration, MD can change the characteristic color of the spray dried product. (Krishnaiah, Nithyanandam and Sarbatly, 2014), thus the difference in color. Botanical tissue does not seem to play any role in the maintenance of bixin concentration according to the absorption spectra. But it has preserved the color of the samples encapsulated with carbohydrates well compared to those without botanical tissue at the end of 30 days (Figure 3.2). The other samples lost their attractive color by day 9 itself.

When the k-constants were evaluated, the degradation followed a first order reaction as displayed in figure 3.3, the rest of the samples followed a similar trend of degradation. However, a study shows that encapsulated bixin follows two consecutive first-order decays under light. The explanation for the kinetic behavior of encapsulated bixin under light is divided into two parts. The first part involves the rapid decay of one type of bixin molecules independent of the initial bixin concentration or the wall material used. This decay resembled that of non-encapsulated bixin with a similar lifetime. This means that the first decay of bixin is of the pigment which did not get encapsulated and is present on the surface of the capsules, being fully exposed to the light. The second part has a slower decay signifying the incorporation of that set of bixin molecules inside the microcapsules, protecting them better from oxidative and photo-degradation. (Barbosa, Borsarelli and Mercadante, 2005) This pattern was not observed in this study, possibly due to the time limitation and better encapsulation efficiency. The encapsulated systems degraded at a very low rate overall.

There was not much difference between the degradation rates of all the encapsulated samples except Fo. The addition of sucrose with pure bixin did not improve the stability of bixin to the same extent as the other systems. It has a higher k-constant, implying that bixin degrades faster in this system. This has also been proven in the absorption spectra, where the degradation is much more visible compared to the other sample (Figure 3.4). This could be explained by the fact that sucrose has a low encapsulation efficiency and therefore there is a higher risk for bixin to be exposed to light. However, the presence of botanical tissue has protected the color pigment. This means that botanical tissue does contribute to the encapsulation but to a very small extent. Botanical tissue did not make a difference to the rest of the samples with MD and whey as their main constituents. That is because MD has a glassy structure which acts as a powerful oxygen barrier, preventing oxidative reactions. (Przybysz *et al.*, 2016) The high concentration of lactose (78.3%) in whey contributes to its barrier function. (Keogh and O'Kennedy, 1999)

Table 4.1: A summary of all the decay constants and quantum yields of the samples.

	Samples		K-constant/slope	Standard error	R value	Quantum Yield
Without Carbohydrates	Pure Bixin	Fe	-39.45E-07	4.0E-07	0.89	135.18E-09
	Bixin+BT	Ge	-54.28E-07	2.0E-07	0.98	90.66E-09
	Bixin+BT(enc)	Go	-97.36E-07	4.0E-07	0.98	188.25E-09
Bixin+Carbohydrates						
	MD	Ao	-3.10E-07	0.5E-07	0.81	0.16E-09
	MD+GA	Bo	-6.57E-07	0.8E-07	0.85	0.14E-09
	MD+CMC	Co	-2.22E-07	0.3E-07	0.84	0.05E-09
	MD+Pectin	Do	-6.36E-07	0.8E-07	0.91	0.17E-09
	Whey	Eo	-4.64E-07	1.0E-07	0.70	0.14E-09
	Sucrose	Fo	-12.23E-07	1.0E-07	0.93	0.31E-09
NF Bixin +Carbohydrates						
	MD	Ho	-3.46E-07	0.4E-07	0.87	0.19E-09
	MD+GA	Ae	-4.16E-07	0.4E-07	0.91	0.22E-09
	MD+CMC	Be	-4.56E-07	1.0E-07	0.73	0.17E-09
	MD+Pectin	Ce	-4.05E-07	0.6E-07	0.81	0.12E-09
	Whey	De	-1.99E-07	0.6E-07	0.55	0.07E-09
	Sucrose	Ee	-1.99E-07	0.6E-07	0.66	0.06E-09

4.3 Quantum Yield

One interesting observation is that even though the decay constants show very little variance from each other, the quantum yield values show much more difference within the samples. The main reason for this could be that the decay constants were obtained from a set of normalized data while the quantum yield was calculated for the absolute amount of bixin present in the well. Even with that, the concentration of bixin in the well was fairly similar between the encapsulation systems except for the reference systems, so this could not have had a major influence in the difference between the samples. Although it could account to the difference in the magnitude between the quantum yields and decay constants. While the vast difference in the quantum yield values between the reference samples and the encapsulation systems could be due to the difference in bixin concentration. The degradation constant might indicate how bixin concentration reduces over time but the quantum yield of photo-bleaching seems to be more specific towards light. It shows how the photons produced from the light are absorbed and to what extent this affects the carotenoid. In reality, the light reaches only the surface of the samples in the wells and ideally the photons absorbed will reduce layer by layer through the sample. For practical reasons, the photons are assumed to be absorbed the same throughout the sample and therefore it has been integrated over the entire thickness. There is a possibility of light passing through the sample completely to the other end, but thankfully the high reflectance of the table prevents any such loss of light. The distribution of bixin in the capsules is uneven within each sample, this was noticed in a SEM

examination. Therefore, it is important to keep in mind that the non- uniformity of the encapsulated particles mean that they absorb light to a different extent within the samples and this can also affect the yields.

In comparison to the study made by Jørgensen and Skibsted, the magnitude of the calculated quantum yields is very high. In their study, it was in the range of 10^{-5}, while in this study, the samples show much less yield. This could be explained by the fact that the other study involved the use of lutein and ß-carotene as the carotenoids and the concentration of these carotenoids used was on average around 100 times lower than the bixin concentration. Also, their samples were exposed to monochromatic light while they were present in air saturated photolysis solution adding on to their degradation. (Jørgensen and Skibsted, 1990) This is in contrast to the direct bixin exposure to a wide spectrum of light with a much higher pigment concentration and without any photolysis solution. The light source used only emits light in the visible spectra, therefore the samples had very less exposure to the UV light. This was taken into consideration during the calculation of quantum yields. Exclusion of UV light actually does have a significant improvement on the color stability of bixin. Exposure to UV light is estimated to be about 100 times more harmful in terms of photo-oxidation compared to visible light. Even though visible light has a lower impact on the photo-degradation, a prolonged exposure (30 days in this case) does lead to photo-oxidation and bleaching in turn (Jørgensen and Skibsted, 1990), this is clear from the results.

It is obvious from the results that the reference samples have the highest quantum yields, this means they have the least protection against light. The reference sample Ge has a relatively lower yield compared to the other reference samples. Although, it makes sense that bixin is protected to a greater extent when surrounded by the botanical tissue, the other reference sample encapsulated with botanical tissue has a higher yield. One explanation for could be the change in the structure of the bixin crystals during encapsulation. The natural non-filtered bixin has botanical tissue covering the entire bixin as a thin layer in a complex way. During encapsulation, the bixin crystals which are thin and long get difficult to contain within the capsules due to the structural modification that occurs while spray drying.

Apart from this, Co, De and Ee have the lowest yields among all the samples. This displays their efficiency at stabilizing bixin with respect to light. The mix of MD and CMC in Co exhibits better stability without botanical tissue. Perhaps the complex formed from the said mixture captures the color pigment in a way that it is well protected from light, and this gets disturbed in the presence of botanical tissue. While in De and Ee, botanical tissue adds on to the efficacy of whey and sucrose respectively in protecting the carotenoid from light. As it was already discussed, whey acts as a good barrier for bixin and sucrose forms poor encapsulations. Botanical tissue seems to have enhanced the barrier function of whey and improved the encapsulation efficiency of sucrose. The rest of the samples have relative quantum yields of photo-bleaching. MD does not seem to be much higher in any aspect but it does display a consistent efficiency in stabilizing bixin despite mixing with other additives.

5. Conclusion

In conclusion, the use of carbohydrates to encapsulate bixin showed significant difference in the photo-degradation compared to those without carbohydrates. Botanical tissue seems to have a slight impact on the stability of the samples. Depending on the carbohydrates used, it interacts differently to give different results. The natural non-filtered bixin is better protected with botanical tissue than pure bixin or encapsulated non-filtered bixin. The use of botanical tissue along with bixin has given it an attractive color, which was maintained till the end of 30 days even though bixin concentration had decreased to a great extent. This could be beneficial and can be used in a wide range of industrial applications. Overall, maltodextrin and whey have shown better protection against light than sucrose. The photo-degradation of bixin follows a first order decay as confirmed by statistic tests. The barrier property of whey and sucrose were enhanced against light with botanical tissue while that of MD and CMC was higher without botanical tissue. The quantum yield of photo-bleaching describes the number of photons absorbed by the color pigments over time. It gives a hint at the mechanism of degradation of carotenoids that occurs in the presence of light.

6. Future studies

The botanical tissue can be characterized in a proper way and looked into for the countless benefits it could offer. As the botanical tissue maintains an appealing color, it would be interesting to investigate the cause for this. The encapsulated systems with carbohydrates can be examined for stability towards other environmental factors apart from light.

7. References

Al-Asheh, S, Jumah, R, Banat, F, & Hammad, S 2003, 'The Use of Experimental Factorial Design for Analysing the Effect of Spray Dryer Operating Variables on the Production of Tomato Powder', *Food and Bioproducts Processing*, 81, pp. 81-88.

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

Boon, C. S. *et al.* (2010) 'Factors Influencing the Chemical Stability of Carotenoids in Foods', *Critical Reviews in Food Science and Nutrition*, 50(6), pp. 515–532.

CEN (2011) 'EN 12464-1 - Light and lighting - Lighting of work places - Part 1: Indoor work places'.

En.wikipedia.org. (2017). *Bixin*. [online]

Available at: https://en.wikipedia.org/wiki/Bixin#/media/File:Bixin.png [Accessed 17 Aug. 2017].

Florentine, F. A. *et al.* (1997) 'Lighting High Bay Areas with Electrodeless Lamps', *Journal of the Illuminating Engineering Society*. Taylor & Francis, 26(1), pp. 27–34.

Gharsallaoui, A. *et al.* (2007) 'Applications of spray-drying in microencapsulation of food ingredients: An overview', *Food Research International*, 40(9), pp. 1107–1121.

International Organization for Standardization (2007) *ISO 11664-2:2007, CIE Colorimetry - Part 2: Standard Illuminants for Colorimetry*.

Jørgensen, K. and Skibsted, L. (1990) 'Light sensitivity of carotenoids used as food colours', *Zeitschrift für Lebensmittel-Untersuchung und Forschung*, 190, pp. 306–313.

Keogh, M. K. and O'Kennedy, B. T. (1999) 'Milk fat microencapsulation using whey proteins', *International Dairy Journal*, 9(9), pp. 657–663.

Krishnaiah, D., Nithyanandam, R. and Sarbatly, R. (2014) 'A Critical Review on the Spray Drying of Fruit Extract: Effect of Additives on Physicochemical Properties', *Critical Reviews in Food Science and Nutrition*, 54(4), pp. 449–473.

Przybysz, M. A., Onacik-Gür, S., Majtczak K. and Dłużewka E. (2016), 'The stability of spray-dried microencapsulated ß-carotene in the mixture of gum arabic, OSA-type modified starch and maltodextrin', *Italian Journal of Food Science*, 28(4), pp. 716-732.

Ranveer, R. C. *et al.* (2015) 'Microencapsulation and storage stability of lycopene extracted from tomato processing waste', *Brazilian Archives of Biology and Technology*, 58(6), pp. 953–960.

Roustapour, O., Hosseinalipour, M., Ghobadian, B., Mohaghegh, F. and Azad, N. (2009). 'A proposed numerical–experimental method for drying kinetics in a spray dryer', *Journal of Food Engineering*, 90(1), pp.20-26.

Shabde, V. and Hoo, K. (2008). 'Optimum controller design for a spray drying process', *Control Engineering Practice*, 16(5), pp.541-552.

Shahid-ul-Islam, Rather, L. J. and Mohammad, F. (2016) 'Phytochemistry, biological activities and potential of annatto in natural colorant production for industrial applications - A review', *Journal of Advanced Research*. Cairo University, 7(3), pp. 499–514.

Shu, B. *et al.* (2006) 'Study on microencapsulation of lycopene by spray-drying', *Journal of Food Engineering*, 76(4), pp. 664–669.

Turner, B. P. *et al.* (1997) 'Sulfur Lamps—Progress in Their Development', *Journal of the Illuminating Engineering Society*. Taylor & Francis, 26(1), pp. 10–16.

Wang, Y. *et al.* (2012) 'Study on the spray-drying encapsulation of lutein in the porous starch and gelatin mixture', *European Food Research and Technology*, 234(1), pp. 157–163.

Zhang, Y. and Zhong, Q. (2013) 'Encapsulation of bixin in sodium caseinate to deliver the colorant in transparent dispersions', *Food Hydrocolloids*. Elsevier Ltd, 33(1), pp. 1–9.

Appendix I

	Samples		Composition
Bixin+Carbs	MD	Ao	87.75% MD + 12.25% Bixin
	MD+GA	Bo	74.93% MD + 14.99% GA + 10.08% Bixin
	MD+CMC	Co	91.403% MD + 1.027% CMC + 7.57% Bixin
	MD+Pectin	Do	85.81% MD + 5.05% Pectin + 9.14% Bixin
	Whey	Eo	91.36% Whey + 8.64% Bixin
	Sucrose	Fo	92.62% Sucrose + 7.38% Bixin
Bixin+Botanical Tissue+Carbs	MD	Ho	75% MD + 15.42% BT + 9.58% Bixin
	MD+GA	Ae	60% MD + 15% GA + 15.2% BT + 9.8% Bixin
	MD+CMC	Be	74% MD + 1% CMC + 17.73% BT + 7.27% Bixin
	MD+Pectin	Ce	70% MD + 5% Pectin + 17.17% BT + 7.83% Bixin
	Whey	De	75% Whey + 18.49% BT + 6.51% Bixin
	Sucrose	Ee	75% Sucrose + 16.55% BT + 8.45% Bixin
Without Carbohydrates	Bixin+BT	Ge	61.68% BT + 38.32% Bixin
	Bixin+BT(enc)	Go	35.31% BT + 64.69% Bixin

Appendix II

$A_{(\lambda t)}$ is the Absorbance of the actual powder in the well and it can be calculated by:

$$A_{(\lambda t)} = \epsilon \cdot C_w \cdot \delta$$

Where:

ϵ is the absorptivity value of Bixin in the sample derived from cuvette measurements (l/mol.com)

δ is the thickness of the powder in the well (cm)

m_c is the mass of the carbohydrates in the samples (g)

ρ_c is the density of the carbohydrates in the samples (g/cm^3)

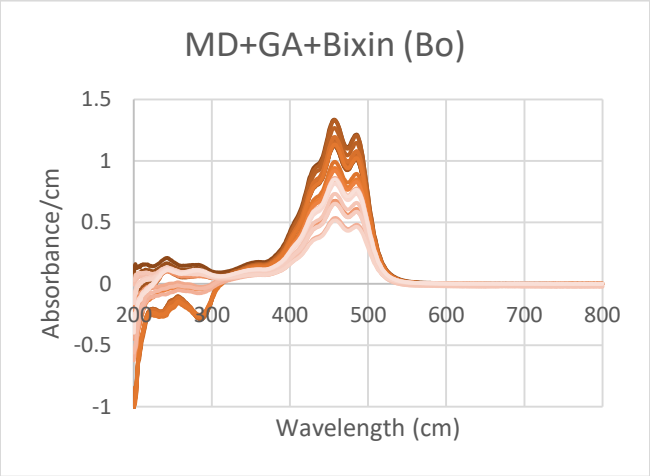
$$\epsilon = \frac{A_{cuv}}{C_{cuv}}$$

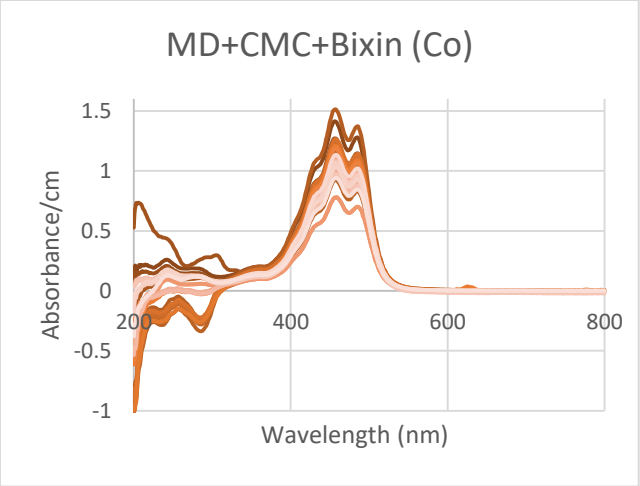
$$\delta = \frac{m_c}{a_w \cdot \rho_c \cdot 10000}$$

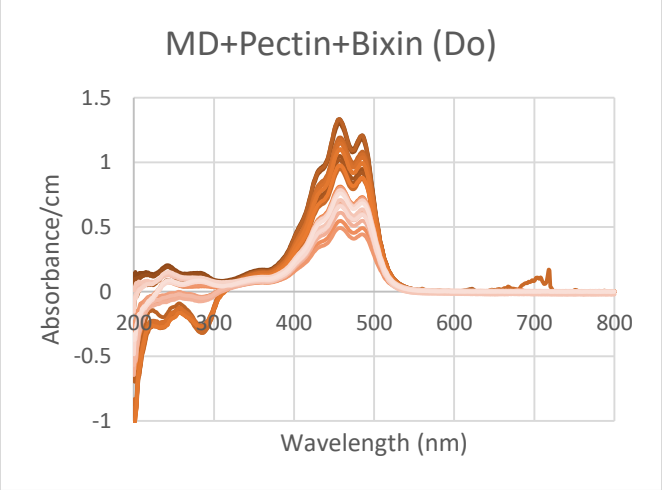
Appendix III

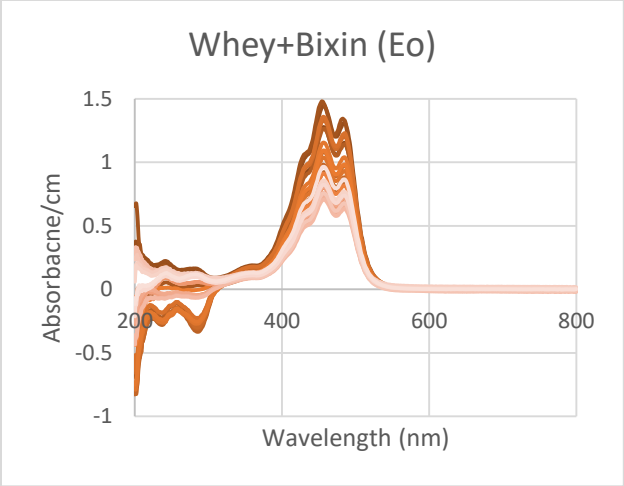
	Normality test	ANOVA			Students t test		
	Shapiro Wilk	Degrees of Freedom	Statistic	Probability	Degrees of Freedom	Statistic	Probability
Sample	P	df	F	P	df	t	P
Ao	0.963	1,24	46.52	0.0001	25	80.15	0.0001
Bo	0.938	1,24	61.529	0.0001	25	42.859	0.0001
Co	0.956	1,20	46.513	0.0001	21	104.496	0.0001
Do	0.924	1,14	67.325	0.0001	15	47.866	0.0001
Eo	0.956	1,24	23.458	0.0001	25	38.133	0.0001
Fo	0.95	1,12	81.712	0.0001	13	24.9	0.0001
Go	0.959	1,32	652.092	0.0001	33	58.491	0.0001
Ho	0.979	1,24	75.377	0.0001	25	84.63	0.0001
Ae	0.942	1,23	110.428	0.0001	24	89.306	0.0001
Be	0.956	1,20	22.613	0.0001	21	40.758	0.0001
Ce	0.962	1,24	45.253	0.0001	25	60.29	0.0001
De	0.939	1,24	10.551	0.003	25	58.42	0.0001
Ee	0.9	1,14	10.582	0.006	15	65.439	0.0001
Fe	0.972	1,32	126.821	0.0001	33	69.587	0.0001
Ge	0.963	1,30	861.047	0.0001	31	127.91	0.0001

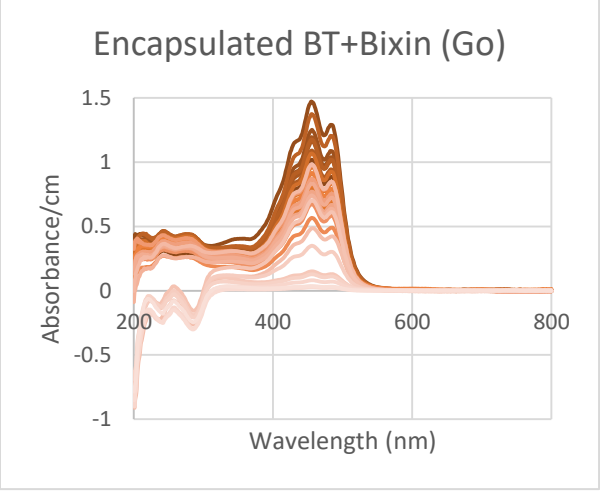
Appendix IV

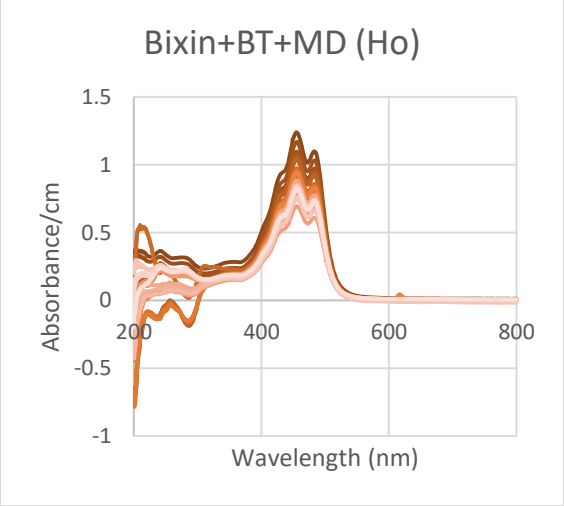

MD+GA+Bixin (Bo)
Absorbance/cm
1.5
1
0.5
0
-0.5
-1
200
300
400
500
600
700
800
Wavelength (cm)

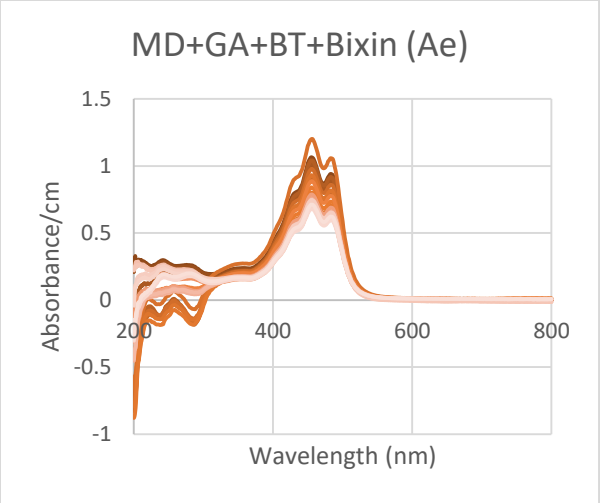

MD+CMC+Bixin (Co)
Absorbance/cm
1.5
1
0.5
0
-0.5
-1
200
400
600
800
Wavelength (nm)

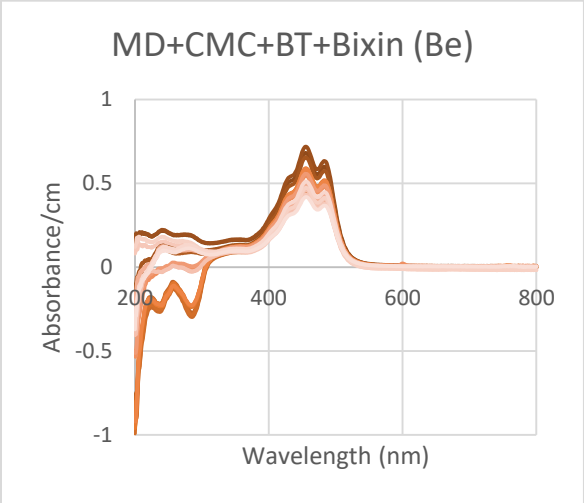

MD+Pectin+Bixin (Do)
Absorbance/cm
1.5
1
0.5
0
-0.5
-1
200
300
400
500
600
700
800
Wavelength (nm)

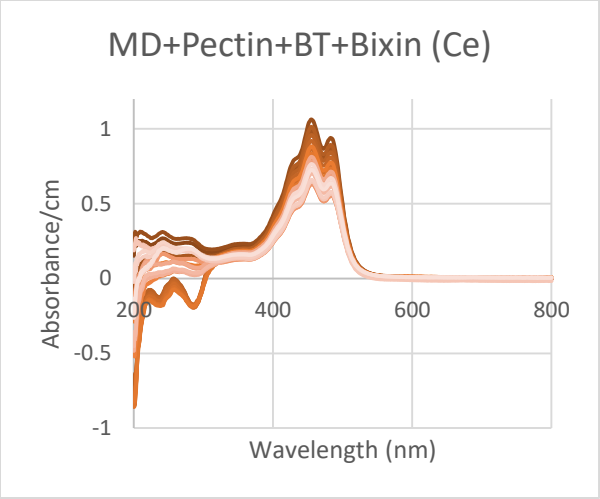

Whey+Bixin (Eo)
Absorbacne/cm
1.5
1
0.5
0
-0.5
-1
200
400
600
800
Wavelength (nm)

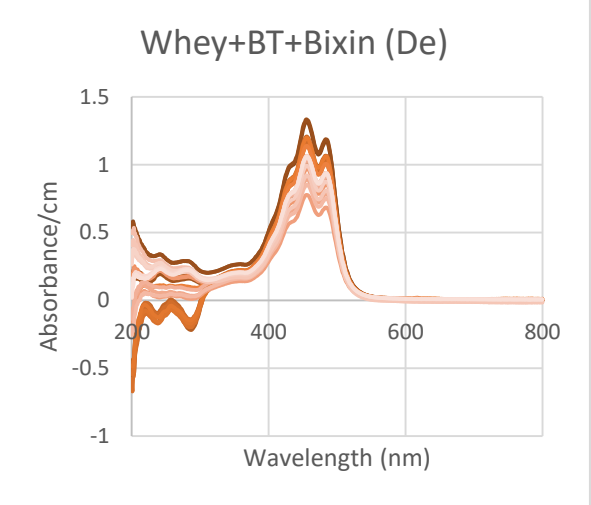

Encapsulated BT+Bixin (Go)
Absorbance/cm
1.5
1
0.5
0
-0.5
-1
200
400
600
800
Wavelength (nm)

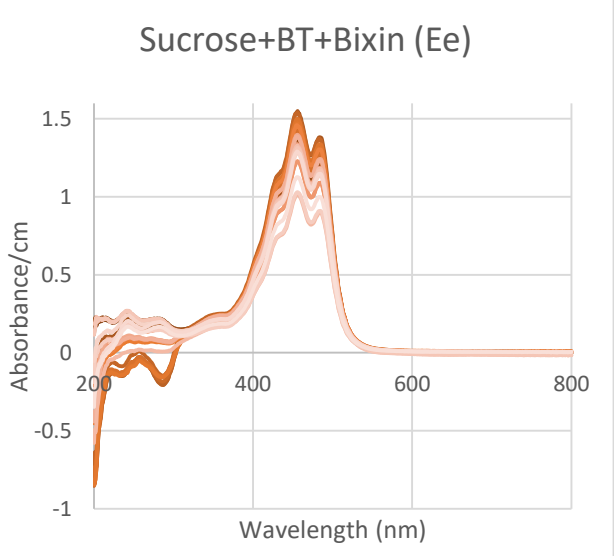

Bixin+BT+MD (Ho)
Absorbance/cm
1.5
1
0.5
0
-0.5
-1
200
400
600
800
Wavelength (nm)


MD+GA+BT+Bixin (Ae)
Absorbance/cm
Wavelength (nm)


MD+CMC+BT+Bixin (Be)
Absorbance/cm
Wavelength (nm)


MD+Pectin+BT+Bixin (Ce)
Absorbance/cm
Wavelength (nm)


Whey+BT+Bixin (De)
Absorbance/cm
Wavelength (nm)


Sucrose+BT+Bixin (Ee)
Absorbance/cm
Wavelength (nm)