EVALUATION OF THE SHELF-STABILITY OF A HIGH-CAROTENOID BREAKFAST FOOD AND ITS IMPACT ON SKIN CAROTENOID LEVELS

by

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ABSTRACT

Evaluation of the Shelf-Stability of a High-Carotenoid Breakfast Food and its Impact on Skin Carotenoid Levels

by

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Utah State University, 2016

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It has been well documented that fruit and vegetable (F/V) intake is linked to lower risk of mortality and chronic disease. Raman resonance spectroscopy is a valid indicator of F/V intake and quantifies that intake by measuring skin carotenoid levels. In this study, 46 children, participated in a 6-week feeding study wherein they were randomly assigned to one of two groups: 1) consuming a high-carotenoid (HC) breakfast/snack food, or 2) consuming a placebo bar, every day. The HC food contained 4.3mg carotenoids per serving and the placebo contained none. Skin carotenoids were measured every two weeks using a BioPhotonic scanner. The treatment group had a mean increase in scanner score of 5,802 Raman intensity units which was significantly higher than the mean increase of the placebo group, 1,771. In this study we found that consumption of 120 gm of a high-carotenoid food significantly increased skin carotenoid levels in children ages 5-18 over a 6 week period.

Packaging type as well as storage conditions play a role in preserving carotenoids which are sensitive to light, temperature, and oxygen. Care must be taken when choosing packaging and storage conditions for foods containing carotenoids. Two shelf-life studies, one at room-temperature and another in frozen storage, were conducted on the HC breakfast/snack food to determine the best method of packaging to maintain quality and preserve carotenoids. The food was randomly packed into one of three packaging types for both studies. The packages used in the frozen study were then randomly assigned to one of three freezer storage methods. Room-temperature samples were analyzed on days 3, 7, 10, and 14 and samples in frozen storage were pulled every month for 5 months. Measures of water activity, moisture content, color values ($L^*a^*b^*$, chroma, and hue), and carotenoid content were analyzed in each sample. At the conclusion of each study, L^*, b^*, chroma, and hue were significantly affected by packaging type. No significant associations were found in any other measures. From this study we draw the conclusion that cellophane packaging or packaging with a N_2 backflush would be the best options for use with this food to best retain its quality.

(109 pages)

PUBLIC ABSTRACT

Evaluation of the Shelf-Stability of a High-Carotenoid Breakfast Food and its Impact on Skin Carotenoid Levels

Dawn L. Reed

Carotenoids are pigments found throughout nature in a large variety of plants including fruits and vegetables (F/V). Carotenoids provide much of the color we see in nature but are perhaps most noticeable in plants with yellow, orange, or red flesh. Carotenoids are antioxidants that serve as a protection against harmful ultraviolet radiation and other free radical producing processes in the body and in plant tissues. Once consumed, carotenoids are deposited in the skin, particularly in fatty tissue. Raman resonance spectroscopy (RRS) can be used to quantify skin carotenoid levels and has been proven a good indicator of F/V consumption. A feeding study was conducted on children wherein they were assigned to one of two groups. They were asked to consume either a high-carotenoid food, called Breakfast Bites, or a granola bar that contained no carotenoids, every day for 6 weeks. The purpose of this study was to see if the amount of carotenoids found in Breakfast Bites was enough to change skin carotenoid levels in participants. Children were scanned via RRS four times during this study to observe any changes in skin carotenoid levels. At the end of the study, children who had consumed Breakfast Bites had significantly higher skin carotenoid levels than those who had consumed granola bars. This showed that consuming similar amounts of carotenoids as is found in Breakfast Bites is enough to increase skin carotenoid levels in children and may provide health benefits to the consumer.

In our second study, we set out to observe the effects of packaging and storage methods on the shelf-stability and carotenoid content of Breakfast Bites. Carotenoids are sensitive to light, temperature, and oxygen because of their chemical structure. This makes them rather unstable molecules and requires they be handled carefully. Packaging type and storage conditions play a major role in the preservation of carotenoids in foods and should therefore be chosen carefully. Two shelf-life studies were conducted on Breakfast Bites. The Bites were packaged in cellophane wrappers, in vacuum sealed bags, or in modified atmosphere packaging (N_2). Some of the packaged Bites were held at room temperature for evaluation. To evaluate freezer packaging, the remaining Bites were stored in the freezer (-17.8º C) on trays, in clear vacuum bags, or in foil vacuum bags. The Bites were analyzed at regular intervals for various shelf-life measures. At the end of each study the only significant result was the relationship between packaging type and color measures. This showed that Breakfast Bites could be packaged and stored in either cellophane or N_2 backflush packaging to best retain their overall quality.

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Dawn L. Reed

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LIST OF ABBREVIATIONS

24HDR	24-hour dietary recall
a^*	Hunter a^* value (red/green)
ANOVA	Analysis of variance
A_w	Water activity
b^*	Hunter b^* value (yellow/blue)
BSQ	Beverage and Snack Questionnaire
DRI	Dietary reference intake
F/V	Fruits and vegetables
FFQ	Food frequency questionnaire
HC	High carotenoid
HCV	High carotenoid vegetables
HDL	High-density lipoprotein
HPLC	High performance liquid chromatography
ICCs	Intraclass correlation coefficients
L^*	Hunter L^* value (lightness)
LDL	Low-density lipoprotein
MAP	Modified atmosphere packaging
MeOH	Methanol
RRS	Resonance Raman spectroscopy
UV	Ultraviolet
VLDL	Very low-density lipoprotein

CHAPTER 1

INTRODUCTION

INTRODUCTION

Carotenoids are lipid-soluble pigments that are found in a large variety of plants including fruits and vegetables (F/V) and are perhaps most noticeable in plants with yellow, orange, or red flesh.[1-4] As carotenoids are not produced within the human body or those of animals, carotenoids must be introduced to the body through consumption of F/V containing carotenoids.[5,6] Though there are more than 600 carotenoids found in nature,[7,8] the human diet only contains approximately 60 different carotenoids. Of those 60, only 20 are found in human plasma and tissues.[8] Carotenoids play an important role in the human body as antioxidants[4] protecting our cells from destruction by free radicals.[6] Carotenoids also serve as sources of pro-vitamin A[9] which is important to protect the body from infection and is essential for vision, growth, and normal development.[10]

It has long been said that breakfast is the most important meal of the day. According to Deshmukh-Taskar, et al., breakfast consumption among Americans is declining.[11] Over the past few decades, breakfast consumption among children has decreased as well. A trend exists suggesting that as children get older, regular breakfast consumption decreases.[12] When looking at the research related to children and regular breakfast consumption, there are many suggested benefits. For example: children who eat breakfast are more likely to have a better type two diabetes risk profile than those who do not;[13] they are more likely to maintain a healthy body weight long term;[14] and evidence suggests increased cognitive function and academic performance as a result of eating

breakfast daily, as well as an increased intake of fiber, calcium, and a decreased intake of saturated fat.[14]

Despite the supposed benefits of breakfast consumption, it has recently been observed that there is little causal evidence to support the hypothesis that breakfast consumption assists in weight loss and the maintenance of a healthy body weight.[15] Rather, it has been observed that the lifestyle of regular breakfast consumers tend to be healthier and therefore, breakfast consumption may simply be part of a healthy lifestyle that contributes to a healthy body weight.[15,16] Regular breakfast consumers tend to be non-smokers, have a higher fiber and micronutrient intake, and consume less alcohol and dietary fat. They also tend to be more physically active, particularly in the morning hours.[16] Thus, it can still be recommended that people eat breakfast as part of a healthy lifestyle, however, it no longer holds the weight it used to as part of a weight loss regimen.

Snacking frequency has increased in children and adults over the past few decades in the United States.[17,18] For children, the major source of snacking comes from desserts and sugary beverages but consumption of salty snacks and candy are on the rise.[18] One study found that the most common snacks among adults included desserts and sweets, crackers, chips, popcorn, and ready-to-eat cereals.[17] Snacking habits have previously been linked to weight gain and increased caloric intake. However, there is no conclusive evidence that snacking prevents or promotes weight gain.[17,19] Rather, it would seem that the nutritional quality of snacks and an individual's overall dietary habits have more of an effect on weight than calories consumed from snacks and the frequency of snacking.[17,19] Four snacking categories or patterns were identified as being associated with higher diet

quality in a recent study. These four categories were: vegetables/legumes, crackers/salty snacks, other grains, and whole fruit.[20] When consuming healthy snacks, it may benefit overall diet quality and may have a positive influence on weight and nutritional status.[17]

Due to the fast-paced nature of our current society, people are often snacking on-the-go or grabbing a quick breakfast to take along with them. Based on the research that has been conducted on snacking and the benefits of breakfast in children, it would be beneficial to have a healthy, wholesome, on-the-go breakfast or snack food for children and adults. In 2014, a group of Utah State University dietetics students tested a product developed by the Center for Human Nutrition Studies called Breakfast Bites. Breakfast Bites are a nutrient dense, on-the-go breakfast replacement and snack item. Due to the many health benefits supplied through Breakfast Bites, the dietetic students undertook a study to determine if promoting the health benefits increased the overall acceptability of the Bites.

The Bites promotion included the following: good source of protein and fiber according to Food and Drug Administration standards (good source indicates a food contains 10-19% of the recommended daily amount), provided vitamin A and folate, contained omega-3s for heart health, naturally sweetened without refined sugar, and made with all-natural, local ingredients. The health promotion of the Bites was presented to just over half of the 289 Utah State University students, faculty, and staff that participated in the study. Each participant was given two Bites, one containing chocolate and one without. They were then asked to complete a survey that rated the appearance, aroma, texture, and overall acceptability of the Bites. Samples containing chocolate had a significantly higher acceptability ($P = 0.016$) than those without and the overall

acceptability of the Bites was significantly higher (P=0.015) when the samples were prefaced with the promotion. Findings also included that with increased hunger, overall likeability and rating of the aroma of chocolate samples increased ($P < 0.05$). Increased hunger levels also significantly increased the likeability of the appearance of both samples ($P < 0.05$). This indicated to the dietetics students that Breakfast Bites were an acceptable item and that the promotion of health benefits may increase overall likeability and sales of a product.

By finding out that Breakfast Bites were an acceptable product, there was interest in selling and distributing them throughout cafés on the Utah State University campus. This in turn created the need for a shelf-life study to determine the best packaging and atmosphere type for use with Breakfast Bites. There was also a particular interest in the preservation of carotenoids found in the Bites in both room-temperature and frozen settings as the carotenoids provide the main health benefit of Bites. Because Breakfast Bites contain a high amount of carotenoids, there was also interest in finding out if consuming the Bites could raise skin carotenoid levels over time in children. If so, Breakfast Bites could be considered a great on-the-go food item for children, giving them the fuel they require to live healthy lifestyles.

HYPOTHESIS

A high carotenoid shelf-stable breakfast food will increase the skin carotenoid content in school age children.

OBJECTIVES

1. Evaluate effectiveness of a high carotenoid breakfast product in increasing skin carotenoid levels in kids, 5-18 years.
    a. By comparing levels in control group to intervention group in a six week feeding study
2. Evaluate shelf-stability of a high carotenoid breakfast product.
    a. By evaluating the effect of different packaging materials and atmospheres
    b. By monitoring water activity, moisture content, and carotenoid content

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CHAPTER 2

LITERATURE REVIEW

CAROTENOIDS

Carotenoids are lipid-soluble pigments that are found in a large variety of plants including fruits and vegetables (F/V), flowers, roots, as well as various bacteria, algae, butterflies, and crayfish.[1-4] Carotenoids provide much of the color we see in nature but are perhaps most noticeable in plants with yellow, orange, or red flesh.[1,2,4,5] Carotenoids are also found abundantly in green plant tissues, however, the presence of chlorophyll masks their vibrant colors.[4] Carotenoids serve various purposes within plants that include: light harvesting, photoprotection in photosynthesis, and production of scents and flavors to assist in pollination and seed dispersal.[1,6]

As carotenoids are not produced within the human body or those of animals, carotenoids must be introduced to the body through consumption of plants, namely F/V, containing carotenoids.[7,8] Though there are more than 600 carotenoids found in nature,[3,9] the human diet contains approximately 60 different carotenoids. Of those 60, 20 are found in human plasma and tissues.[9] Carotenoids most commonly found in human tissues include beta-carotene, alpha-carotene, lycopene, beta-cryptoxanthin, lutein, zeaxanthin, phytofluene, and phytoene.[3,4,9-12] Close to 90% of all carotenoids in the body are represented by beta- and alpha-carotene, lycopene, lutein, and beta-cryptoxanthin.[13] The main food source of beta-carotene is carrots (Table 2.1).[5,9] One study[14] found that purple carrots contain higher amounts (approximately 2.3 times more) of alpha-and beta-carotene than orange carrots. The main food sources for beta-cryptoxanthin are orange

Table 2.1. Beta-carotene contents of foods[a]

Food	Content (mg/100 g wet wt)[b]
Carrots, raw	18.3
Mangos, canned	13.1
Sweet potato, cooked	9.5
Carrots, cooked	8.0
Pumpkin, canned	6.9
Kale, cooked	6.2
Spinach, raw	5.6
Spinach, cooked	5.2
Winter butternut squash	4.6
Swiss chard, raw	3.9
Apricots, raw	2.6
Pepper, red, raw	2.4
Pepper, red, cooked	2.2
Cantaloupe, raw	1.6
Lettuce, romaine, raw	1.3
Tomato paste	1.2

[a] Based on reference 5

[b] Edible portion

fruits such as oranges, papaya, and sweet red peppers.[9,13] More than 85% of lycopene consumed in the United States comes from tomato products, however, tomatoes are not the only source of lycopene in F/V (Table 2.2).[5] There are a few animal based foods that contain small amounts of carotenoids.[15] For example, lutein and zeaxanthin are found in decent amounts in egg yolks and some carotenoids are found in certain fish and crustaceans and dairy products (Table 2.3).[5,15]

Carotenoids play an important role in the human body as antioxidants[4] as some carotenoids are sources of pro-vitamin A.[13] Free radicals that cause damage to our cells are always being produced within the body as a result of cellular metabolism, mostly from the mitochondria and phagocytic cells.[4,8] Free radicals can also be a result of damage to the skin from ultraviolet (UV) light and the result of direct contact with

Table 2.2. Lycopene contents of foods[a]

Food	Content (mg/100 g wet wt)[b]
Tomato paste	29.3
Catsup	17.0
Tomato puree	16.7
Pasta sauce	16.0
Tomato sauce	15.9
Tomato soup	10.9
Tomato, canned, whole	9.7
Tomato juice	9.3
Watermelon, raw	4.9
Tomato, cooked	4.4
Tomato, raw	3.0

[a] Based on reference 5
[b] Edible portion

Table 2.3. Lutein and zeaxanthin contents of foods[a]

Food	Content (mg/100 g wet wt)[b]
Kale, cooked	15.8
Spinach, raw	11.9
Spinach, cooked	7.0
Lettuce, romaine, raw	2.6
Broccoli, raw	2.4
Broccoli, cooked	2.2
Summer squash, zucchini, raw	2.1
Corn, sweet, cooked	1.8
Peas, green, canned	1.4
Brussels sprouts, cooked	1.3
Corn, sweet, canned	0.9
Beans, green, cooked	0.7
Beans, green, canned	0.7
Beans, green, raw	0.6
Okra, cooked	0.4
Cabbage, white, raw	0.3
Egg yolk	0.3
Celery, raw	0.2
Orange, raw	0.2
Tomato paste	0.2

[a] Based on reference 5
[b] Edible portion

hazardous environmental compounds.[4,8,16] If not taken care of, free radicals will increase oxidative stress in the body which can result in the destruction of cells and cell compartments.[8] Oxidative stress of the skin due to sun exposure can result in skin irritation, premature skin aging, and ultimately diseases such as skin cancer.[4,16] Certain lifestyle factors such as smoking, inactivity, and a poor diet have also been shown to contribute to oxidative stress.[13,17]

The body has developed a protection against the damage resulting from free radicals by creating an antioxidative network. This network includes vitamins (A, C, D, and E), carotenoids, and several enzymes.[8,16] Because antioxidants form a protective chain, they stand as a marker of the antioxidative potential of the skin.[18] Antioxidants neutralize free radicals before they are able to damage the epidermis through free radical scavenging.[4,16] It is known that carotenoids lose their color once exposed to free radical oxidizing species.[5] The interaction of antioxidants and free radicals eventually leads to the destruction of antioxidants. Some are, however, able to neutralize several free radicals before being destroyed.[8] Antioxidants can act synergistically, indicating that a combination of carotenoids with any of the aforementioned compounds may be more potent than any single carotenoid by itself.[4,19] However, according to Clevidence et al.,[19] not only can carotenoids act synergistically but they may also compete for absorption and can influence the rate of metabolism of other carotenoids. Of the carotenoids, lycopene is the most efficient antioxidant, closely followed by beta-carotene.[4] Lutein is the least efficient.[4]

Recent studies have looked at the possibility of pro-oxidant effects from high doses of beta-carotene due to supplementation. One study observed increases in lung

cancer in smokers taking 20 mg beta-carotene supplements daily.[20] It has been suggested that the pro-oxidant effect could be a result of the degradation of beta-carotene to radicals, however, more research is needed in this area.[6,17] It has also been suggested that the presence of vitamin C may help reduce the risk of beta-carotene acting as a pro-oxidant.[6]

Carotenoids are the main source of vitamin A for most people around the world, especially those living in third-world countries.[4] Vitamin A protects the body from infection and is essential for vision, growth, and normal development.[21] Though 50 different carotenoids can be metabolized into vitamin A, beta-carotene is the most potent precursor for vitamin A.[5] The bioconversion of beta-carotene to vitamin A is variable between food sources and among individuals. A beta-carotene conversion to retinal of 21µg carotenoid:1 unit retinal has been seen in spinach and a 15:1 conversion has been seen in carrots.[5] Other main pre-vitamin A carotenoids include alpha-carotene, and beta-cryptoxanthin.[21,22] Due to the fact that supplementing with vitamin A has the potential for toxicity at high doses (25,000 international units/kg), carotenoids are a good alternative if supplementation of vitamin A is needed.[9] Despite the benefits from consuming carotenoids, they are not considered essential nutrients and therefore have no dietary reference intake value assigned to them.[13]

It has been well established that consuming higher amounts of F/V reduces the risk of mortality and chronic disease.[10,23] The World Health Organization has suggested that increasing individual F/V consumption to more than 600 grams per day has the potential to reduce worldwide disease burden (measured as number of cases) by 1.8%.[24] Numerous studies have shown that F/V consumption reduces the risk of cancer

(specifically skin, lung, prostate, and colorectum), cataracts, age-related macular degeneration, and chronic diseases or conditions such as diabetes, cardiovascular disease, low immune function, obesity, and other degenerative diseases.[4,9,22,25-29] Consumption of beta-carotene and lycopene have been related to reduced risk of cardiovascular disease and certain cancers, whereas, lutein and zeaxanthin have been related to prevention of eye disorders.[13,30]

CAROTENOID METABOLISM

In order for carotenoids to have any effect on the body, they must be absorbed, transported, and deposited in specific tissues.[4] Absorption and accumulation of carotenoids are known to differ greatly between individuals.[3] The reason for these differences is not well understood but it has been hypothesized that differences in ability to cleave beta-carotene to vitamin A or differences in metabolism may be involved.[10] It is assumed that carotenoids are absorbed similarly to major lipids such as triglycerides, cholesterol, and phospholipids[4,21] and that their distribution in the body is linked to one's lipid profile.[7]

The first step in carotenoid metabolism is the release of carotenoids from the food matrix.[4,30] Extraction from the food matrix begins in the stomach where carotenoids are transferred to the lipid fraction. The food is mashed via gastric muscle contractions and stomach acid and pepsin help release carotenoids from their matrix. Characteristics of the matrix and lipids as well as the lipophilic properties of the carotenoids effect the efficiency of the transfer.[4]

Carotenoids are then incorporated into critical micelles which consist of monoglycerides, phospholipids, free fatty acids, and bile acids.[4,30] The amount of carotenoids incorporated into micelles can vary depending on micellar fatty acid composition and saturation and the polarity of the carotenoid.[30] Xanthophylls such as lutein and zeaxanthin are solubilized more readily than carotenes such as lycopene and beta-carotene.[3,4] Next, carotenoids are absorbed in the duodenum via passive diffusion.[30] They are then secreted into chylomicrons and released in the lymphatic system for transport to the liver.[5,13,30] Chylomicrons containing carotenoids are delivered to the liver where they are exported in very low-density lipoproteins (VLDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL).[13,21,30] Carotenoids are then released into the blood stream and delivered to various tissues throughout the body, primarily adipose tissue.[13]

As some tissues in the body have larger lipoprotein receptors than others, carotenoid concentrations throughout the body vary from place to place. However, there is much evidence to suggest that there are many additional factors that contribute to the uptake and accumulation of carotenoids throughout the body.[21,30]

The absorption of carotenoids can vary depending on several different factors. For one, not all carotenoids are transported and absorbed the same. Half-lives of carotenoids in plasma varies. For example, beta-carotene, alpha-carotene, and beta-cryptoxanthin have half-lives of 7-14 days, lycopene 12-33 days, and lutein 33-91 days.[30] Carotenoids may also inhibit or enhance the absorption of other carotenoids as they can interact with each other at any point of metabolism.[30] Consuming carotenoids with dietary fat may help to optimize carotenoid absorption and dietary fiber may decrease absorption.[19,30]

Carotenoids may be absorbed better from cooked foods than from uncooked foods, as the cooking process somewhat liberates them from the food matrix prior to consumption.[4,19,21] One study[21] indicated that beta-carotene is efficiently absorbed at a rate of greater than 50% after being cooked as compared to absorption of less than 3% in uncooked F/V.[21] Other reasons that absorption can vary include the composition of the meal, the activity of digestive enzymes, and the nutrition status as well as genetic factors of the individual.[21] Carotenoids are more bioavailable when not bound to anything and may therefore, be absorbed more efficiently from supplements or pure carotenoids, such as carotenoid oil, than from food.[21,30] However, absorption of carotenoids levels off at doses of 20-30 mg as absorption becomes restricted due to limited micellar incorporation.[21] The quantity required to consume 20-30 mg of carotenoids in foods is widely variable. For example, 0.25-0.3 pounds of carrots, approximately ½ cup pasta sauce, 0.6-0.9 pounds fresh spinach, or 1.8-2.75 pounds of fresh red peppers would each contain approximately 20-30 mg carotenoids.[5] However, due to differences in bioavailability, one would not necessarily absorb 20-30 mg by consuming these foods.

SKIN CAROTENOIDS AS BIOMARKERS OF F/V INTAKE

Due to the fact that carotenoids are found in a wide variety of F/V but are not found in high amounts in other foods, carotenoids are currently considered the best biomarker to indicate F/V intake.[29] Since many self-reporting food records can be biased, there needed to be a clinical method that was indicative of F/V consumption.[31] In 2000, the National Academy of Sciences stated, in terms of measuring carotenoids, that plasma carotenoid levels are the best biomarkers for consumption of F/V.[23,31] Much research has

been done to observe the relationship between plasma carotenoid levels and skin carotenoid levels, skin carotenoid levels and skin resonance Raman spectroscopy (RRS) scan scores (used to measure skin carotenoid levels), and the correlation between carotenoid levels and F/V intake.[8,10,23,29-32] Plasma and skin carotenoids have been analyzed using high performance liquid chromatography (HPLC) which has been considered the gold standard for assessing carotenoid status in humans.[11] These plasma levels have been found to be positively correlated with carotenoid levels in skin, both plasma and skin biopsies being analyzed using HPLC.[11,23,32]

It has also been shown that plasma and skin carotenoid levels are highly correlated with RRS carotenoid scan scores.[10,11,23,29,31-33] Finally, serum carotenoid levels have been shown to be strongly correlated with F/V intake, therefore, skin carotenoid levels and RRS scans are also highly correlated with F/V intake.[10,11,29,31,34]

Because RRS has been validated as a reliable measure of carotenoid status in both adults and children,[10,11,23,29] it has become the preferred method for gathering data concerning carotenoid levels. Though HPLC may be considered the gold standard, several disadvantages have been linked with this method. Collecting serum and skin samples for analysis using HPLC is invasive due to the nature of venipuncture and skin biopsy, is expensive due to cost of phlebotomy, sample processing, and sample storage and analysis, requires trained personnel and specialized equipment, and can be time consuming.[11,31] In addition, serum carotenoid concentrations fluctuate in response to daily carotenoid intake and may therefore not give an accurate measurement of one's long-term F/V intake.[11,31] In contrast, RRS is quick, non-invasive, inexpensive, painless, does not require highly trained personnel, and can be portable, allowing it to be used

outside laboratory settings.[11,23] RRS has been shown to be consistent over time and is consistent across body sites (three were measured: palm and inner and outer arm).[11] The noninvasive nature of RRS makes it a good option for use in large populations and for use in children.[11,29]

Various factors have been observed to influence carotenoid concentrations in both plasma and skin. One study found skin carotenoid levels in children to be widely varied.[29] The Third National Health and Nutrition Examination Survey (NHANES III) observed relatively high serum carotenoid levels in childhood, however, those levels decreased through adolescence and into adulthood.[35] One study observed that children in grades 6 through 8 reported consuming significantly more high carotenoid vegetables than children in grades kindergarten through 5.[23] In adults, sun exposure, smoking, use of tanning lotions and/or tanning beds, air pollution, stress, illness, sleeplessness, alcohol consumption, and elevated body mass index (BMI) have all been found to have a correlation with decreased carotenoid concentrations.[8,18,19,25,29,31] One study observed a tendency for younger participants, more educated participants, and female participants to have higher carotenoid levels.[31] When looking at individual carotenoids, one study observed that increasing age was related to lower lycopene levels; women had higher levels of carotenoids, lycopene being an exception; and lutein, beta-cryptoxanthin, and alpha- and beta-carotene were all lower in past and present smokers as compared to those who had never smoked.[25] Another study looked at fluctuations in carotenoid levels across seasonal changes and found that during the summer and autumn months, carotenoid levels increased with little to no change in overall F/V consumption.[8] Researchers took this to mean that the freshness and ripeness of F/V are important in the accumulation of

carotenoids in the human body.[8] Overall, it has been shown that many factors can influence carotenoid levels in the body.

FOOD FREQUENCY QUESTIONNAIRES AND 24-HOUR DIETARY RECALL

Self-reported food recall methods, such as food frequency questionnaires (FFQ) and 24-hour dietary recalls (24HDR), often have substantial errors.[25,29,31] The ability to use RRS as a reliable indicator of F/V intake allows researchers to objectively assess the effects of F/V intake without having to rely on potentially biased data. RRS also displays for researchers a better picture of long term F/V intake, as FFQ are often reflective of a short time period, 1 week or 1 month for example, and 24HDR reflect intake one day at a time.[29] However, it can still be useful to look at FFQ and 24HDR to glean what participants are consuming and what F/V may have the greatest impact on RRS scan scores. Various studies have looked at carotenoid levels and their relationship with F/V intake reported in FFQ and 24HDR data and found them to be moderately to positively correlated with each other.[10,11,23,25] Al-Delaimy et al.[25] found FFQ data more strongly correlated with plasma carotenoid levels than 24HDR data. Aguilar et al.[23] found that both FFQ and 24HDR data were positively correlated with skin carotenoid levels.

There are several challenges that accompany the use of FFQ and 24HDR. Use of these methods in children is difficult because of their immature cognitive ability and reporting skills.[23] 24HDR may be a more precise measure of daily F/V intake than FFQ but is not valid in children 9 years and younger, as children under ten years have a poor correlation with actual intake when using FFQ.[23] Therefore, RRS becomes very useful in children.

Factors influencing the accuracy of FFQ and 24HDR in adults include: accuracy of participants' memory, variation in daily intake, inaccurately quantifying food consumption, and the desire to show greater compliance than actual to please researchers.[15,26,29,31] Other methods, such as direct observation, may be a more effective method of data gathering in relation to food consumption. However, this would be labor intensive, only being possible in small population studies, and would not likely demonstrate usual intake.[29]

RESONANCE RAMAN SPECTROSCOPY

RRS uses a light at a wavelength of 488 nm and 514.5 nm to assess skin carotenoid levels.[11,18,29,36] The 488 nm wavelength is absorbed mainly by beta-carotene and the 514.5 wavelength is absorbed mainly by lycopene.[8] Excitation by light increases the vibrational energy of the conjugated double bonds in the carotenoid backbone.[11,31] This causes a portion of the blue light to scatter inelastically, resulting in Raman scattering.[11] Raman scattering occurs when energy is exchanged between the incident light and the scattering molecules and can be influenced by total carotenoid content, laser or light penetration depth, stratum corneum thickness, and UV exposure history.[33] Carotenoids are some of the most efficient Raman scatterers, therefore, RRS is often the method of choice when working with carotenoids.[7,31] Next, a spectral fingerprint of the carotenoids is created, based on their unique molecular structure and vibrational and rotational energy levels.[11,29,31,33] The intensity of the Raman peak, or the Raman scattering, is directly proportional to the concentration of carotenoids present.[11,33] Skin

carotenoid levels are reported in Raman intensity counts with a higher count indicating higher carotenoid concentrations in the measured area.[11]

Though the palm of the hand is currently the preferred site of RRS scanning, initially scans were performed on the macula of the eye since carotenoids are highly concentrated in that area in healthy individuals.[31] Carotenoid levels in the macula are approximately two times more concentrated than in the skin. This, along with the fact that other chromophores (e.g. melanin, hemoglobin, collagen, porphyrins, etc.) are also found in the skin, were initial challenges with RRS.[31] Hata et al.[33] found that carotenoid concentration is not consistent in skin throughout different parts of the body. The forehead, dorsal head, inner and outer arm, and palm were scanned and the palm was found to have the highest mean carotenoid concentration of any of those areas.[33]

The palm is the preferred scan sight due to the relatively thick stratum corneum, or outer layer of skin.[29,31] Carotenoids tend to be stored in high lipid areas within the body due to their lipophilic nature. The palm of the hand has a high lipid to protein concentration ratio and is therefore a good storage site for carotenoids.[33] The thickness of the skin minimizes problems with other chromophores and prevents the laser from penetrating deeper soft tissues.[11,31] This helps to increase consistency and minimize additional error.[11,18] When using a laser for RRS, resulting scan scores are an average of the concentration of carotenoids in different layers of the skin (stratum corneum, epidermis, and the upper portion of the dermis).[18,33] This demonstrates that carotenoids are present not only in the uppermost layers of the skin but deeper layers as well.[33] The palm is also a good scan site due to ease of access, limited sun exposure, and because the melanin content is more constant among people of different ethnicities and races.[11,29]

CAROTENOID CHEMISTRY

Carotenoids are polyene molecules that have a common formula $C_{40}H_{56}$.[7,33] All carotenoids have a long conjugated chain of double bonds with near bilateral symmetry around the middle double bond.[7,13] Different carotenoids are the products of modifications to the basic structure. Cyclization of end groups and the introduction of oxygen gives various carotenoids their antioxidant properties and characteristic colors.[13] Two classes of carotenoids exist: carotenes, which are hydrocarbons, and xanthophylls. Beta- and alpha-carotene and lycopene are classified as carotenes and lutein, zeaxanthin, and beta-cryptoxanthin are classified as xanthophylls (Figure 2.1).[5]

Carotenoid scavenging strength is related to the number of conjugated double bonds in the molecule. Beta-carotene, beta-cryptoxanthin, and zeaxanthin are considered highly active antioxidants though lycopene is the most efficient.[17] Though lycopene is the best scavenger, it lacks a beta-ionone ring, causing it to lack pro-vitamin A activity.[17]

Due to the sensitivity of carotenoids to light and temperature, a result of the unsaturated nature of the carotenoids, exposure to light and elevated temperatures lead to a trans-cis isomerization in the polyene molecule, causing it to bend. This may also cause

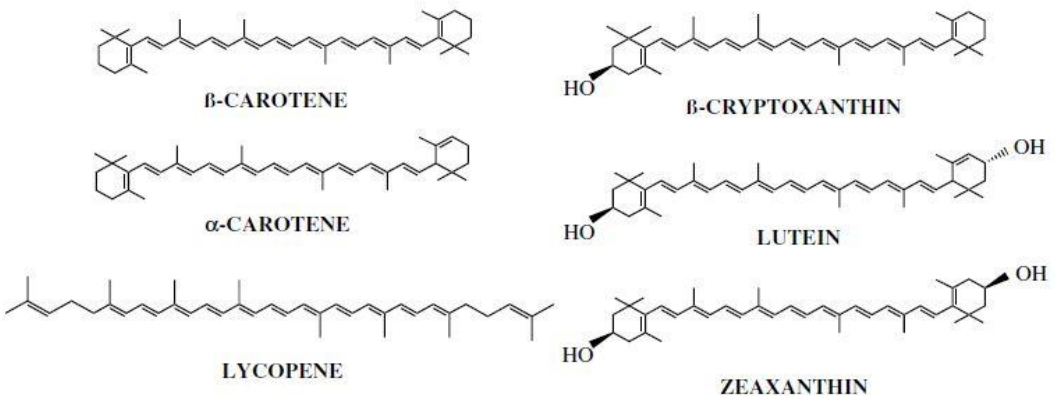

Figure 2.1. Carotenoid structures

cleavage of the molecule if oxygen is present.[7,13] As is typical of free-radical reactions, carotenoids are oxidized into secondary products that are further oxidized to other products. For example, beta-carotene degrades into epoxides, apocarotenals, and apocarotenones. These will further oxidize and become lighter, volatile carbonyl compounds such as norisoprenoids.[37] The degradation of beta-carotene into norisoprenoids contributes to the aroma compounds that can be used in the flavor industry.[37,38] These aroma compounds are found naturally in paprika, wine, black tea, and tobacco.[28,37,38]

EFFECTS OF COOKING, PACKAGING, AND STORAGE

Due to the fact that carotenoids are rather unstable molecules, there are many factors pertaining to cooking, packaging and storage that influence their stability and bioavailability in humans. In general, common household cooking methods do not greatly alter carotenoid content. For example, microwaving, boiling, and steaming do not have an extreme effect but cooking or processing methods that use extreme heat may result in the breakdown of carotenoids.[13,19] Severe heat treatments used in the processing of commercial products that contain carotenoids can alter or breakdown carotenoids through reactions such as oxidative degradation and isomerization. The amount of degradation, whether high or low, depends on the processing temperature.[22] The amount of pressure used in cooking may also influence carotenoid content. Carotenoids are relatively pressure stable at low and moderate temperatures but will degrade with higher temperatures.[26] One study found that carotenoids in nectarine puree were more influenced by pressure level than by pressure time and found that after high pressure treatment,

extraction yield of carotenoids increased.[26] Carotenoids in foods being dried or dehydrated may also be affected by drying temperature. Oven drying at 48 ºC, compared to –53 ºC freeze drying, significantly decreased the amounts of lutein and beta-carotene in daylily.[2] One study found that blanching was beneficial in preserving carotenoids found in carrot slices. This was due to the inactivation of lipoxygenase and peroxidase which catalyze the breakdown of carotenoids during storage.[39]

Many factors have an influence on the bioavailability and uptake of carotenoids from foods. The term bioavailability originally came from pharmacology and is used to describe the movement of compounds from the intestine to the vascular system.[19] Bioavailability is strongly dependent upon the dietary matrix and structure of the carotenoid in the food, what processing or cooking has been done, genetic factors and the nutritional status of the consumer, the presence of fat, and the total amount of carotenoid present.[5,15,24] Carotenoids can be found in crystalline form (alpha- and beta-carotene in carrots and lycopene in tomatoes), dissolved in oil droplets (found in orange and yellow fruits), or present in protein complexes (found in dark green vegetables such as spinach).[4] In most cases, processing or cooking increases the bioavailability of carotenoids in F/V as it likely helps to release carotenoids from these complexes.[4,19,22] For example, one study reported high levels of carotenoid bioavailability in tomato paste, approximately 80%,[40] which is reportedly 2-2.5 times higher than the bioavailability of raw tomatoes.[19] Another study fed participants a pureed vegetable soup and found that bioavailability of carotenoids from the soup was much higher than the bioavailability of raw and cooked carrots, tomatoes, and broccoli. Though there was a smaller amount of total carotenoids in the soup, when compared to other feeding studies, serum carotenoid levels were higher

when consuming soup.[24] Though cooking or processing may increase bioavailability of carotenoids, care must be taken to avoid excessive processing that could destroy the molecules altogether.[4] Differences in bioavailability also exist between F/V. 75% bioavailability has been seen for beta-carotene in carrots whereas only 17% bioavailability for beta-carotene was seen in broccoli.[40] Carotenoids have been found to be more bioavailable from supplements than from foods such as F/V.[4,19,24] For example, subjects consuming 29 mg of beta-carotene from cooked carrots had 18% of the plasma beta-carotene levels of those consuming 30 mg of beta-carotene from a supplement after 42 days of treatment.[19]

As we have seen, carotenoids are unstable molecules sensitive to light, temperature, oxygen, and acidity.[37,39] Therefore, packaging and storage are very important in preserving carotenoids as they can be exposed to light, be stored in oxygen or oxygen permeable packaging, or be part of a food that causes degradation. Proper packaging and storage are essential to retain any pro-vitamin A activity and health properties of carotenoids over time.[26,39] Different effects such as moisture, temperature, and light can have varying influences on the degradation of specific carotenoids.[22,27] Two shelf-life measures that need to be considered are water activity (A_w) and moisture content. Lower A_w has been shown to increase beta-carotene degradation.[37] One study observed greater beta-carotene loss at 0.13 A_w with carotenoid content increasing at 0.30, 0.51, and 0.76 A_w. It was also observed that temperature had a greater effect on carotenoid stability than A_w.[37] Another study observed that carotenoids are more stable within the range of 0.32-0.57 A_w. They observed maximum stability to be at approximately 0.43 A_w. Above or below these bounds, carotenoid degradation increased

slightly.[18] Sulaeman et al.[39] observed that higher moisture content during storage decreased carotenoid content in carrot chips.

The atmosphere within packaging plays a crucial role in the preservation of carotenoids in food products. The presence of oxygen increases the degradation of carotenoids.[27,39] Therefore, modified atmosphere packaging (MAP) is useful in preventing carotenoid oxidation.[27] Vacuum packing or employing a nitrogen or CO_2 backflush can help prevent ethylene production, respiration, cut-surface browning, senescence, water loss, and growth of microorganisms in fresh F/V and is useful in preventing the degradation of carotenoids.[27,37,39] It has been observed that fresh-cut carrots maintain quality and carotenoid levels with an increased presence of CO_2 and a decreased amount of oxygen in their packaging.[14,39] Overall, reduction in oxygen levels within packaging of foods containing carotenoids, helps in maintaining carotenoid levels.[14,27,39]

Packaging type, light exposure, and temperature all play a critical role in maintaining carotenoid integrity. Opaque packaging to prevent light exposure and packaging with low oxygen permeability help to protect carotenoids.[39] Storage temperatures lower than 30ºC following heat treatment and packaging in a partial vacuum along with dark storage increased the stability of carotenoids in a 180 day storage study on pumpkin purees.[22] Kale samples stored at 1ºC in the dark were more stable than those stored at 11ºC in both darkness and light.[27] Thus, we can conclude that dark storage, cooler temperatures, and MAP may be helpful in preventing the degradation of carotenoids in fresh F/V and other foods containing carotenoids.

CAROTENOID ANALYSIS

Due to the unstable nature of carotenoids, great care needs to be taken when analyzing these compounds. As carotenoids are sensitive to light, temperature, and oxygen, there are critical points throughout the analysis wherein they are at great risk of being degraded.[7] One study recommended performing analyses under a yellow fluorescent light.[14] Whether analyzing carotenoids via HPLC or spectroscopy, the carotenoids need to first be extracted from the matrix. Various studies have used methanol/chloroform mixtures, hexane/acetone/ethanol, ether/hexane, etc.[37,38,41-43] to complete this extraction. Following the completion of the extraction, the carotenoid containing solution is prepared to be separated using HPLC or is prepared to be read by a spectrophotometer.[26,27,38,39,43] Due to their intense color, carotenoids strongly absorb in the visible region, between 400 and 500 nm.[7] When carotenoids are degraded, it causes a bend in the molecule that leads to a decrease in absorption wavelengths. Carotenoids usually exhibit 3 absorption bands or two bands plus a shoulder. The longer the molecule, the stronger the light absorption and the longer the absorption wavelengths. Cooling the carotenoid solution leads to enhanced absorption.[7] Data gathered from spectroscopic or HPLC analyses can be put into formulas or compared to standards to find the amount of carotenoids present in the sample.[27,42,43]

CONCLUSIONS

In conclusion, carotenoids are found in various plants including F/V. Carotenoids cannot be synthesized in the human body and must therefore be incorporated into the body by consuming F/V. Carotenoids are beneficial as they serve a role as antioxidants in

the body, protecting cells from free radical damage. Carotenoids are absorbed in the body similar to lipids as carotenoids are lipid-soluble compounds. Carotenoids can be measured in skin by using RRS. RRS has become the preferred method of measuring carotenoid levels and has been validated as a reliable indicator of F/V intake. The use of RRS is beneficial for many reasons, one of which is that clinicians no longer have to rely solely on FFQ and 24HDR to demonstrate one's F/V intake, as these data collection methods can be biased.

Carotenoids are rather unstable compounds and can be degraded with exposure to light, oxygen, and high temperatures. This may result in the loss of their antioxidant properties. Cooking, storage, and packaging conditions can influence the bioavailability and the stability of carotenoids. Cooking or processing at high temperatures increases carotenoid degradation as does storing in oxygen permeable packaging and storing at higher temperatures. Cooking may help to increase carotenoid bioavailability in foods, however, there are other factors that contribute to carotenoid bioavailability as well.

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CHAPTER 3

6-WEEK BREAKFAST BITES FEEDING STUDY

ABSTRACT

It has been well documented that fruit and vegetable (F/V) intake is linked to lower risk of mortality and chronic disease. Raman resonance spectroscopy (RRS) has been shown to be a valid indicator of F/V intake in children and adults by quantifying skin carotenoid levels. In this study, 46 children, ages 5-18, participated in a 6 week feeding study wherein they were randomly assigned to one of two groups: 1) treatment (n=21), consuming 120 gm of a high-carotenoid breakfast/snack food, called Breakfast Bites, or 2) placebo (n=25), consuming 72 gm of a placebo bar every day. Children were otherwise asked to maintain their normal diet. Breakfast Bites contained 4.3 mg carotenoids (calculated) per 120 gm serving and the placebo contained no carotenoids. At weekly clinic visits, participants filled out surveys concerning items known to influence skin carotenoid levels and filled out 3 food frequency questionnaires (FFQ) throughout the study. Skin carotenoids were measured every two weeks using a BioPhotonic scanner. Repeated measures analysis of variance (ANOVA) was used to assess the changes in skin carotenoid levels. Weight was included as a covariate in the equation as it was a predictor of scanner score. The treatment group had a mean increase in scanner score of 5,802 Raman intensity units, which was 4,031 units higher than the mean increase of the placebo group, (P= 0.022). In this study we found that daily consumption of 120 gm of a high-carotenoid food significantly increased skin carotenoid levels in children ages 5-18 over a 6 week period.

INTRODUCTION

It has been well established that consuming higher amounts of fruits and vegetables (F/V) reduces the risk of morality and chronic disease.[1-5] Numerous studies have shown that F/V consumption reduces the risk of cancer (specifically skin, lung, prostate, and colorectum), cataracts, age-related macular degeneration, and chronic diseases or conditions such as diabetes, cardiovascular disease, low immune function, obesity, and other degenerative diseases.[3-10]

Carotenoids are lipid-soluble pigments that are found in a large variety of plants including F/V, flowers, roots, as well as various bacteria, algae, butterflies, and crayfish.[8,11-13] Carotenoids provide much of the color we see in nature but are perhaps most noticeable in plants with yellow, orange, or red flesh.[8,11,12,14] They are also present in dark, leafy green vegetables.[1] As carotenoids are not produced within the human body or those of animals, carotenoids must be introduced to the body through consumption of F/V containing carotenoids.[15-16] Carotenoids most commonly found in human tissues include beta-carotene, alpha-carotene, lycopene, beta-cryptoxanthin, lutein, zeaxanthin, phytofluene, and phytoene.[1,4,8,13,17,18]

Carotenoids play an important role in the human body as antioxidants[8] as some carotenoids are sources of pro-vitamin A.[19] Free radicals that cause damage to our cells are always being produced within the body and can be a result of damage from ultraviolet (UV) light.[8,16] If not taken care of, free radicals will increase oxidative stress in the body which can result in the destruction of cells and cell compartments.[16] Antioxidants form a protective chain and neutralize free radicals before they are able to cause any damage.[20]

Due to the fact that carotenoids are found in a wide variety of F/V but are not found in high amounts in other foods, carotenoids are currently considered the best biomarker of F/V intake.[10] Plasma carotenoid levels have been found to be positively correlated with carotenoid levels in skin in children as well as adults[2,17,23] which, in turn, has been shown to be highly correlated with resonance Raman spectroscopy (RRS) carotenoid scan scores.[1,2,10,17,21-23] Serum carotenoid levels have been shown to be strongly correlated with F/V intake, therefore, skin carotenoid levels and RRS scans are also highly correlated with F/V intake.[1,10,17,23,24]

The purpose of this study was to examine the effect of regular consumption of Breakfast Bites, a high-carotenoid food containing a known amount of carotenoids, on skin carotenoid levels in school-aged children over a 6-week period.

METHODS AND MATERIALS

Participants

A total of 48 healthy schoolchildren aged 5-18 were recruited to participate in this study. These children were from Cache County, UT, and were recruited in March 2015 through local elementary and secondary schools. Parents at a local elementary school received a recruitment letter with the study information via email. Older study participants were recruited through word of mouth or younger siblings who attended this elementary school. Grades kindergarten through 12 were represented in the study (K-5 39.1%, 6-8 30.4%, 9-12 30.4%). 47% of participants were male and the ethnicity of the study population was reflective of the ethnicity found in Cache County schools (80% Caucasian, 17% Hispanic, less than 3% Asian and Pacific Islander).

To be included in the study, parents had to complete an online survey. Upon meeting inclusion criteria, parents were invited to an initial screening. If the child's skin carotenoid levels were between 11,000 and 33,000 (see Protocol section for reasoning), they were then asked to fill out a health history questionnaire used by the Center for Human Nutrition Studies. The questionnaire included questions regarding the child's past and present medication use, supplement use, and medical history. Children were excluded from the study if any habits or medical/health history were known to influence carotenoid levels. Exclusion criteria included use of carotenoid supplements (multivitamins were acceptable), presence of chronic disease such as type 1 diabetes mellitus or asthma, more than 2 hours per day of sun exposure without using sunscreen, use of topical self-tanning lotion, and any major illness within the 2 weeks prior to the study. Qualifying participants were also asked to taste a Breakfast Bite before agreeing to participate in the study. This was to ensure the child would be willing to consume the Bites and complete the study if put in the active group. One child was excluded from participating in the study due to an unwillingness to consume Breakfast Bites.

Child assent and parent consent were obtained in person during screening. Researchers asked participants to maintain their normal lifestyle for the duration of the study including activity, exercise habits, multivitamin use, and food intake. Participants were compensated with a total of $50, receiving $10 at clinic visits 2, 4, and 6 and $20 upon completion of the study at visit 7. The research protocol was reviewed and approved by the Utah State University Institutional Review Board.

Protocol

To qualify for the study, participants needed a BioPhotonic Scanner (NuSkin, LLC, Provo, UT) score within the range of 11,000 to 32,000 Raman intensity counts. This range ensures that participants' scanner scores fall within 2 standard deviations of the average score for this age group, approximately 22,000 Raman intensity counts (information provided by NuSkin, LLC), therefore, eliminating children with unusually high or low scores.

The study consisted of 7 weekly clinic visits to the Center for Human Nutrition Studies at Utah State University. The initial, baseline visit consisted of gathering participants' weight and height, calculating BMI (kg/m^2), obtaining skin carotenoid scores, and completion of a food frequency questionnaire and clinic survey. Weight was measured by a Detecto 758C digital scale (Webb City, MO) and height was measured by a Seca 223 digital stadiometer (Hangzhou, China).

The study coordinator randomly assigned study participants to either the group consuming high-carotenoid Breakfast Bites (n=23) or the group consuming placebo breakfast bars (n=25). Children in the Bites group were instructed to consume 6 Bites (120 gm) per day and those in the placebo group were instructed to consume 3 granola bars (72 gm) per day (any combination of the following: Chewy Chocolate Chip, Chewy Peanut Butter Chocolate Chip, or Chewy Chocolate Chunk; Quaker Oats Company, Chicago, IL). Respective groups were each asked to consume their full allotment of Bites or bars anytime within the 24 hours of a day. Participants were asked to bring Bites or bars not consumed during the week to weekly clinic visits to assist in checking compliance. Bites and bars were distributed weekly during clinic visits by trained

research assistants. Breakfast Bites were made in the Utah State University Research Kitchen by trained research assistants. Bites were made in batches at baseline and at week 3 and were held frozen until distribution. The Food Processor Nutrition Analysis Software (ESHA, 2015; Salem, OR) was used to analyze the nutrition of Breakfast Bites. Neither researchers nor study participants were blinded to the group assignment.

Skin carotenoid status was measured by trained research assistants using a BioPhotonic Scanner, a portable RRS device, at each of the following clinic visits: baseline and weeks 2, 4, and 6. To calibrate each scanner, during warm-up, a black calibration cap was placed over the scanner window. Each scanner would then self-calibrate using a proprietary process and was ready for use. Participants' palms were placed against the scanner's light window and were held there for 90 seconds. Scanners emitted a light and measured skin carotenoid status in Raman intensity counts from 0 to 70,000+. Each time scan scores were collected, participants were scanned twice using the same hand and the same scanner. Were there more than 2,000 Raman intensity count difference, children were scanned a third time to minimize individual variation in scanner score. The two scores within 2,000 counts of each other were then averaged. Participants and their parents were blinded to scanner scores.

Participants completed a clinic survey each week that consisted of various questions related to lifestyle practices within the last 7 days. Children under 10 years of age required assistance from parents to complete the survey. Weekly survey questions were related to the following items: daily Bites or bar consumption, time spent outside each day, occurrence of illness and severity, new medications taken, if any, vaccinations,

if any, use of self-tanning lotion, use of a tanning bed, consistency of diet, and amount of moderate physical activity for the week.

Dietary Assessment

By using an FFQ, data was gathered regarding the frequency of consumption of foods such as fruits, vegetables, snacks, and beverages among study participants. The FFQ used in this study was a modified version of The Beverage and Snack Questionnaire (BSQ), developed and validated by Neuhouser and colleagues.[25] The BSQ was adapted from 19 questions to 28 questions to gather additional information regarding high-carotenoid vegetables as that was more relevant to this study.

Bites and Bars

Breakfast Bites were created at Utah State University through the dietetics program as a nutritious, on-the-go breakfast or snack. The carotenoids in Breakfast Bites come from the carrots, locally grown butternut squash, and dried apricots found in Bites. The remaining ingredients in Breakfast Bites are as follows: whole wheat flour, oats, cinnamon, nutmeg, powdered skim milk, honey, egg, butter, great white northern beans, raisins, dried cranberries, dark chocolate, and protein whey crisps (Grände Custom Ingredients Group, Lomira, WI; also a previous product of food development at Utah State University). Breakfast Bites contain approximately 4.3 mg of carotenoids per 120 gm serving and provide 331 calories, 9 gm protein, 6 gm fiber, 52 gm carbohydrates, and 11 gm fat (see Table A.1 for additional nutrient information). The Chewy Chocolate Chip, Chewy Peanut Butter Chocolate Chip, and Chewy Chocolate Chunk granola bars are commercially sold through Quaker Oats Company and contain no carotenoids.

Resonance Raman Spectroscopy

RRS uses a light at a wavelength of 488 nm to assess skin carotenoid levels.[10,17,20,26] Excitation by light increases the vibrational energy of the conjugated double bonds in the carotenoid backbone.[17,23] This causes a portion of the blue light to scatter inelastically, resulting in Raman scattering.[17] Raman scattering occurs when energy is exchanged between the incident light and the scattering molecules and can be influenced by total carotenoid content, laser or light penetration depth, stratum corneum thickness, and UV exposure history.[22] Next, a spectral fingerprint of the carotenoids is created, based on their unique molecular structure and vibrational and rotational energy levels.[10,17,22,23] The intensity of the Raman peak, or the Raman scattering, is directly proportional to the concentration of carotenoids present.[17,22] Skin carotenoid levels are reported in Raman intensity counts with a higher count indicating higher carotenoid concentrations in the measured area.[17]

Statistical Analysis

In order to examine differences, explain the characteristics of the study population, and compare means across subgroups of the population (age, sex, ethnicity, weight, and experiment group), study data was analyzed using analysis of variance (ANOVA) and *t*-tests, using SPSS version 25 (SPSS Inc., Chicago, IL). To account for potential confounding factors, associations between skin carotenoid status and reported illness, multivitamin use, and time spent in the sun were analyzed at each data collection point. Within independent variables, outliers were top-coded, making them equivalent to the next closest data point. This was done in order to retain outliers found in the data. To

top-code, data points were converted to Z values,[27] which needed to be within the range of 1.96, or 3 standard deviations. Any numbers above 1.96 were considered outliers and were changed to match the value next closest to 1.96. Top-coding was used for 1-3 data points in each of the following variables: total F/V from baseline FFQ, total high-carotenoid vegetable (HCV) from baseline FFQ,[11] and total HCV from week 3 FFQ.[11] To assess the correlation of scanner scores across multiple scans obtained in one visit, intraclass correlation coefficients (ICCs) were used. To analyze skin carotenoid changes between groups, repeated measures ANOVA was used. Statistics with a P value of 0.05 or less were considered statistically significant.

RESULTS

One child was excluded from the study due to illness at the time of recruitment. Ten children were excluded due to carotenoid scans outside the acceptable limit. The study began with 48 participants, however, two did not complete the feeding study (4%), a 5 year-old male and a 6-year old female. The two children who did not complete the study did not like the taste of the Bites and felt there were too many to eat each day. They were, therefore, unwilling to continue in the study. Originally 23 children were assigned to the Bites group, 21 of which completed the study. All 25 children assigned to the bars group completed the study. The mean age of study participants was 11 ± 3.5 years.

Tests of Internal Validity

Several tests were run to guarantee the internal validity of our study was sound. There were no significant differences in mean skin carotenoid status by grade/age, sex, ethnicity, weight, or group assignment at the baseline of the study (Table 3.1). The

Table 3.1. Resonance Raman spectroscopy (RRS), fruit and vegetable (F/V), and high-carotenoid vegetables (HCV) intake, by baseline characteristics of the study population (n=46)

		mean ± standard error		
	n (%)	RRS counts (Raman intensity counts)	Baseline FFQ F/V (servings)	Baseline FFQ HCV (servings)
Grade/age (y)				
Kindergarten-5th/5-10	18 (39.1)	23,310 ± 1,209	3.4 ± 0.36	1.1 ± 0.13
6th-8th/11-13	14 (30.4)	20,845 ± 4,016	3.1 ± 0.33	1.3 ± 0.14
9th-12th/14-18	14 (30.4)	22,185 ± 5,685	2.9 ± 0.43	1.2 ± 0.18
Sex				
Male	22 (47.8)	21,418 ± 983	3.0 ± 0.30	1.2 ± 0.13
Female	24 (52.2)	22,950 ± 1,086	3.3 ± 0.31	1.2 ± 0.11
Race/ethnicity				
White	37 (80.4)	22,537 ± 846[a]	3.3 ± 0.25	1.2 ± 0.10
Hispanic	6 (13.1)	20,343 ± 1,550[b]	2.3 ± 0.39	1.0 ± 0.12
Asian	3 (6.5)	22,021 ± 3,486[a]	3.6 ± 0.95	1.3 ± 0.29
Weight (kg)				
19.2-34.4	16 (34.8)	23,939 ± 1,276	3.3 ± 0.36	1.0 ± 0.10
34.5-56.8	16 (34.8)	21,068 ± 1,165	3.1 ± 0.40	1.2 ± 0.17
56.9-104.62	14 (30.4)	21,564 ± 1,353	3.0 ± 0.38	1.4 ± 0.16
Multivitamin				
Yes	14 (30.4)	24,165 ± 1,494	3.3 ± 0.40	1.1 ± 0.14
No	32 (69.6)	21,365 ± 804	3.1 ± 0.26	1.2 ± 0.10
Treatment				
Bars	25 (54.3)	21,644 ± 1046	3.4 ± 0.30	1.3 ± 0.13
Bites	21 (45.7)	22,899 ± 1035	2.9 ± 0.30	1.1 ± 0.11

Values with different letters indicate a significant difference ($P < 0.05$)
Values without letters indicate no significant differences were seen

youngest participants (5 to 10 years) tended to have the highest Raman intensity counts of any age group in the study. The youngest participants also had the highest reported consumption of F/V, however, they had the lowest reported consumption of HCV. The children who reported taking a multivitamin supplement (n=14) initially had higher scan scores (average of 2,800 Raman intensity counts) than those who did not. The initial

difference was almost significant (P=0.08), however, by the third week of the study, there was little to no difference (P=0.88). Among children who reported ever being sick during the study (50%), there were no mean differences in scan scores. An average of 5.1 children reported being sick each week with severity of sickness ranging from 1-5 (1=not very sick, 5=very sick). There was a significant correlation in baseline scan scores in relation to sun exposure. Children who reported 2 or more hours of daily sun exposure had significantly higher scores than those reporting 30-60 minutes (P=0.044) and 60-120 minutes (P=0.007) of exposure, respectively 6,245 and 7,280 Raman intensity counts higher. No children reported being exposed to tobacco smoke during the study period. Any significant differences observed at baseline were no longer significant at weeks 2, 4, or 6.

To check the effectiveness of randomization in our study, one-way ANOVAs were run and revealed no differences at baseline for age, weight, consumption of HCV, and RRS scanner scores between the two groups. Chi-square tests were run examining the distribution of gender and found no significant differences (P = 0.97). A second chi-square test showed that participants using supplements were evenly distributed across the groups (P = 0.37). Further, chi-square analyses showed no differences existed across the groups in terms of occurrence of illness during the study (P = 0.14) and daily hours of sun exposure at baseline (P = 0.27). As no significant differences were found, there were no differences between groups at baseline.

It was also important to check compliance regarding consumption of Bites and bars. Compliance data was based on participants' self-reported consumption, gathered through weekly clinic surveys. Reported consumption was then divided by total number

of assigned Bites or bars to give weekly percent compliance. Over the course of the study, participants consuming Bites had an overall compliance of 83.9% (consumed 5 Bites per day) and those consuming bars, 93.8% (consumed 2.8 bars per day). Bites consumption decreased and bar consumption increased over time but did not correlate with change in scanner scores.

Another validity check conducted examined participants' diet over the course of the study and any changes that may have taken place. Using a repeated-measures ANOVA, it was observed that F/V and HCV consumption remained consistent throughout the study (main effect P = 0.30 and P = 0.51, respectively), based on data

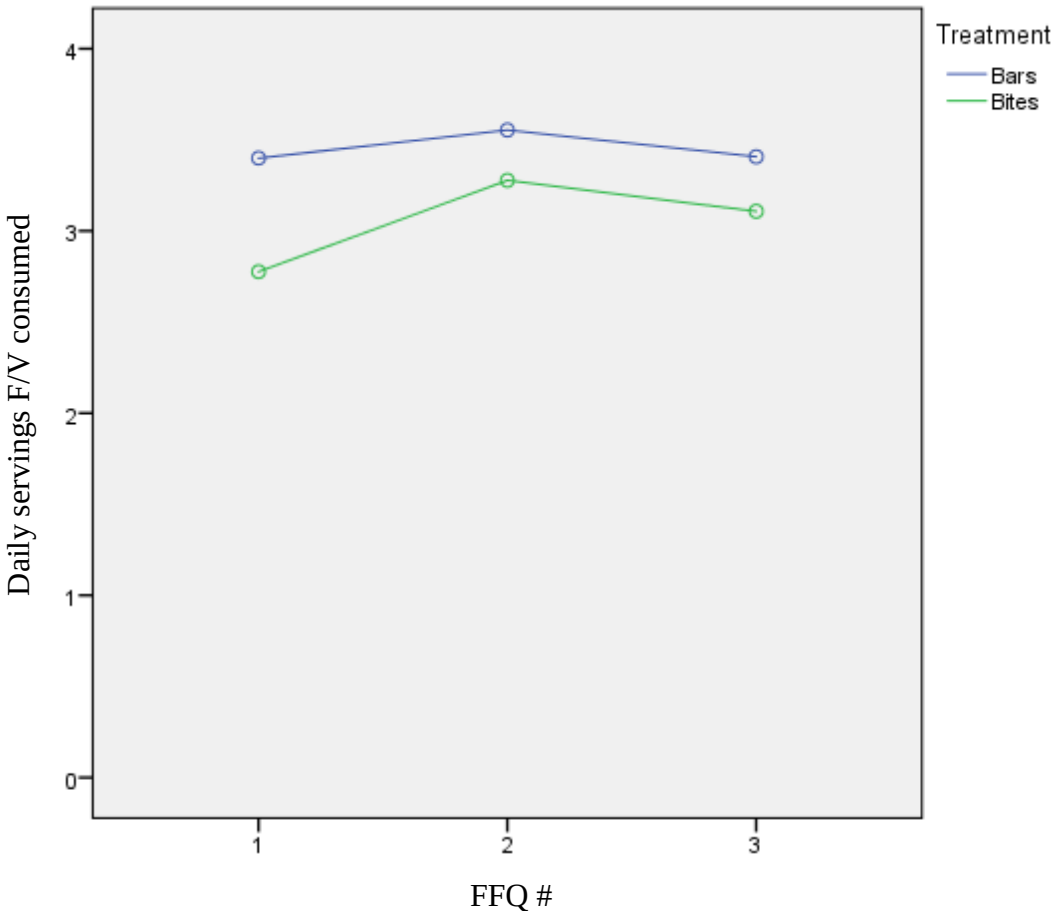

Figure 3.1. Average F/V intake at weeks 0, 3, and 6 (P > 0.05)

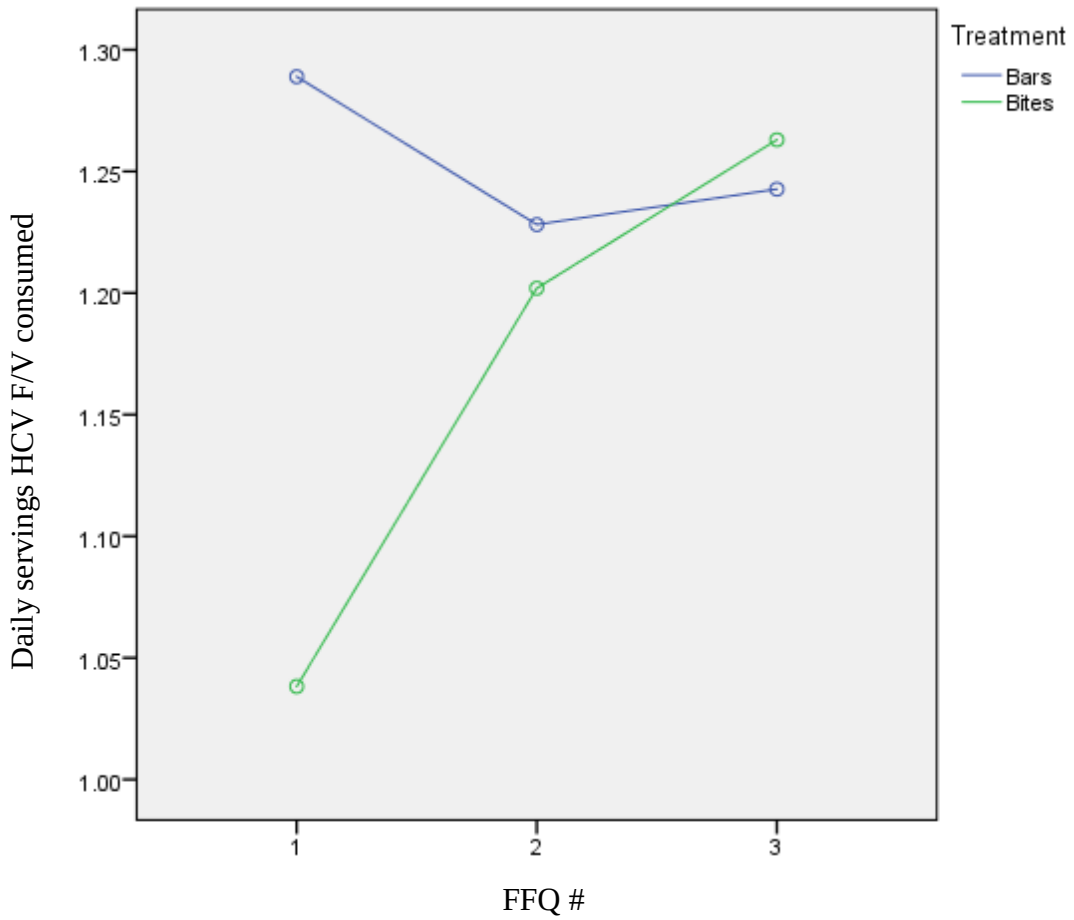

Figure 3.2. Average HCV F/V intake at weeks 0, 3, and 6 (P > 0.05)

collected through the FFQs completed by participants and their parents. There was a trend seen that children consuming bars consistently had a higher intake of F/V than those in the Bites group, though F/V consumption did increase in the Bites group over time (Figure 3.1). Neither of these trends were statistically significant. Despite having lower HCV consumption at baseline, those in the Bites group had a higher average scan score. Over the course of the study, HCV consumption tended to increase in the Bites group, though not significantly (Figure 3.2).

Finally, RRS intensity counts, taken in duplicate or triplicate if initial scores differed by >2,000 units, were highly correlated (baseline ICC=0.97, P < 0.001; week 2

ICC=0.95, P < 0.001; week 4 ICC=0.98, P < 0.001; week 6 ICC=0.94, P < 0.001). This allowed us to justify the averaging of the scores within 2,000 units of each other and suggests that the RRS scanners used in this study were reliable between clinic visits over the course of the study. In a previous study,[1] weight played a significant role in scanner score affecting how F/V intake were correlated to carotenoid levels. To avoid this, weight was considered a covariate in our study as well.

Effect of Bite Consumption

By the final week in our study, week 6, there was a significant difference in scan scores between those consuming Bites and those consuming bars (P = 0.022). After

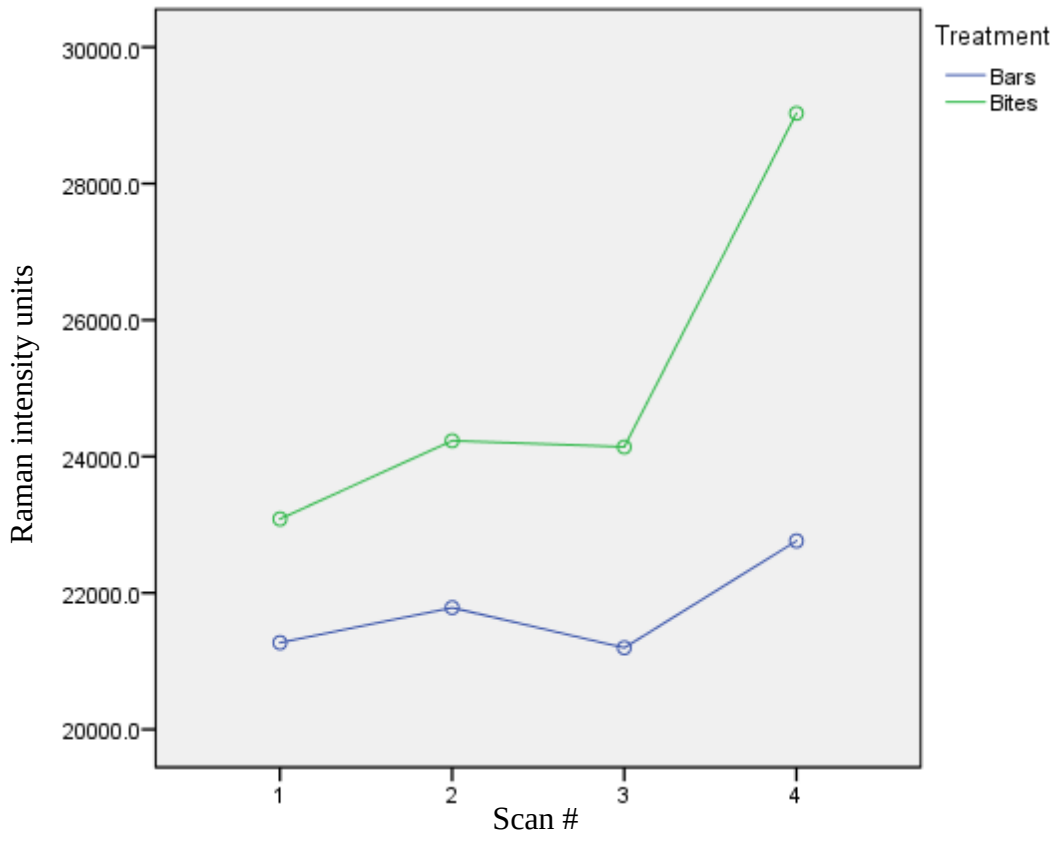

Figure 3.3. Average skin carotenoid levels at weeks 0, 2, 4 and 6 (P = 0.022)

accounting for participants' weight, the Bites and bars groups had estimated mean increases in scan scores of 5,802 and 1,771 Raman intensity units (Figure 3.3). Participants in both groups saw the largest increase in skin carotenoid status between weeks 4 and 6. We were not able to observe the effect of Bites on weight change, diet quality, or calories consumed. The only weight measurement obtained was at baseline and there were no inquiries regarding whether Bites were eaten in place of other foods or in addition to other foods normally consumed.

DISCUSSION

In this study, regular consumption of a known dose of carotenoids (4.3 mg/120 gm) from Breakfast Bites significantly increased skin carotenoid levels over a 6-week period in children aged 5-18 years. Similarly, a study of children consuming high-carotenoid juice over an 8-week period had significantly higher skin carotenoid levels.[1] Children in this study were consuming 2.75-11 mg of carotenoids per day. Those consuming 2.75-5.5 mg had a larger increase in carotenoid levels from start to finish than the participants in our study consuming 4.3 mg, respectively, 10,009 vs 5,802 Raman intensity units.[1] This is possibly due to increased bioavailability of the carotenoids as they had likely already been released from F/V through processing.[8,9,29] Similar results were seen in a study of young men consuming a F/V soup that supplied 7.9 mg of carotenoids per day.[28] Serum carotenoid levels increased significantly within three weeks with daily consumption of soup. Bioavailability was markedly higher in the soup (55% for beta-carotene and 43% for lycopene) than in raw or cooked F/V (0.1-20%). It was hypothesized that this was also due to the freeing of carotenoids through processing.[28]

The amount of carotenoids calculated to be contained in Breakfast Bites, 4.3 mg per serving, could easily be consumed by children each day. 4.3 mg could be ingested by eating approximately 50 gm, or ½ cup, of cooked carrots.[14] However, the bioavailability of carotenoids in the cooked carrots is likely different than that of Breakfast Bites and would likely not have the exact same influence on skin carotenoid levels in children.[19,29] However, there are many additional benefits from eating F/V besides consumption of carotenoids.[3,8] The World Cancer Research Fund and the American Institute for Cancer Research has recommended consuming 400-600 gm of F/V every day. This would in turn provide roughly 18 mg of carotenoids, however, the amount of bioavailable carotenoids would vary, mainly based on whether the F/V were cooked or not.[28] However, this high amount of consumption is not realistic for all individuals. This study has shown that consumption of small amounts of carotenoids can influence skin carotenoid levels and is most likely providing at least some benefit to the consumer.

The increase in carotenoid levels in the placebo group from week 4 to 6 was rather unusual. No changes in eating habits were reported, however, the school year ended in this time period and likely unconsciously influenced how the children were eating. There were also likely changes in sun exposure and physical activity which may have had an effect. One study observed that skin carotenoid concentrations increased in summer and autumn months without a significant difference in F/V intake.[16] This was thought to be a result of more local produce being consumed and therefore and increased level of carotenoids due to the freshness and ripeness of the F/V.[16] This was likely not the case in our study as June was rather early for F/V production in the Cache Valley growing season and few gardens were producing fresh produce at that time. Carotenoid

levels from week 4 to 6 seen in our study are most likely due to lifestyle changes related to the children no longer attending school. Changes in diet, physical activity, sun exposure, stress levels, and illness likely contributed to the increase we observed.

CONCLUSIONS

F/V intake is highly correlated with the prevention of various diseases and is known to increase serum and skin carotenoid levels.[1,2,23,24] RRS has been found to be a reliable method for measuring skin carotenoid concentrations and is thus a useful objective measure of F/V intake in adults and children.[1,2,10,17] RRS is sensitive to modest changes in skin carotenoid concentration.[2] In this study, children's skin carotenoid levels were monitored over a 6-week period to identify any changes related to the regular consumption of a high-carotenoid food (4.3mg/120 gm serving). Over the six weeks there was a significant difference in skin carotenoid levels between the group consuming the high-carotenoid food and the group consuming the placebo bar. This suggests that consuming 4.3 mg of carotenoids daily for 6 weeks is enough to raise skin carotenoid levels in children. 50 gm, or approximately ½ cup, of cooked carrots provides approximately 4.3 mg of carotenoids and would likely have a similar effect on skin carotenoids levels. This, however, would depend on the bioavailability of carotenoids in cooked carrots. This demonstrates that children could realistically consume similar amounts of carotenoids that were fed in this study, resulting in elevated skin carotenoid levels.

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CHAPTER 4

BREAKFAST BITES SHELF-LIFE STUDIES

ABSTRACT

Packaging type as well as storage conditions play a major role in the preservation of foods. Carotenoids are sensitive to light, temperature, and oxygen and therefore, care must be taken when choosing packaging and storage conditions for foods containing them. Two shelf-life studies, one at room-temperature and another in frozen storage, were conducted on a high-carotenoid breakfast/snack food, called Breakfast Bites, to determine the best method of packaging to maintain quality and preserve the carotenoids found in Bites. The Bites were randomly packed into one of three packaging types for both studies. The packaged Bites for the frozen storage study were then randomly assigned to one of three freezer storage methods. Room-temperature samples were analyzed on days 3, 7, 10, and 14 and samples in frozen storage were pulled at 1 month increments for 5 months. Measures of water activity (A_w), moisture content, color values (L^*,a^*,b^*), and carotenoid content were analyzed for each sample. After 2 weeks at room temperature, L^*, b^*, chroma, and hue were significantly affected by packaging type ($P < 0.0001$, $P = 0.009$, $P = 0.0159$, $P < 0.0001$, respectively). The freezer storage study saw similar results in that these measures were again significantly affected by packaging type: L^* ($P = 0.0013$), b^* ($P = 0.0197$), chroma ($P = 0.0275$), and hue ($P = 0.0007$). No significant associations were found in A_w, moisture content, or carotenoid levels in either study. From this study we draw the conclusion that Breakfast Bites could be packaged and

stored in cellophane or N_2 backflush to best retain their overall quality and could be used in combination with any freezer storage method.

INTRODUCTION

Carotenoids are lipid-soluble pigments that are found in a large variety of plants including fruits and vegetables (F/V), flowers, roots, as well as various bacteria, algae, butterflies, and crayfish.[1-4] Carotenoids provide much of the color we see in nature but are perhaps most noticeable in plants with yellow, orange, or red flesh.[1,2,4,5] Carotenoids are considered antioxidants and play an important role in the human body protecting cells from free radicals.[4,6,7]

Carotenoids are polyene molecules that have a common formula $C_{40}H_{56}$.[8,9] All carotenoids have a long conjugated chain of double bonds with near bilateral symmetry around the middle double bond.[8,10] Carotenoids are sensitive to light and temperature due to their unsaturated nature. Exposure to light and elevated temperatures leads to a trans-cis isomerization in the polyene molecule, causing it to bend. This may also cause cleavage of the molecule if oxygen is present, causing it to lose its antioxidant properties.[8,10]

In general, common household cooking methods do not greatly alter carotenoid content.[10,11] Severe heat treatments used in the processing of commercial products that contain carotenoids can alter or breakdown carotenoids through reactions such as oxidative degradation and isomerization.[12] In most cases, processing or cooking increases the bioavailability of carotenoids in F/V as it likely helps to release carotenoids from these complexes.[4,11,12] Though cooking or processing may increase bioavailability of

carotenoids, care must be taken to avoid excessive processing that could destroy the molecules altogether.[4]

Carotenoids are unstable molecules sensitive to light, temperature, oxygen, and acidity.[13,14] Therefore, packaging and storage are very important in preserving carotenoids as they can prevent exposure to light and heat and will determine how much oxygen is available to interact with the carotenoids through modified atmosphere packaging (MAP) and impermeable packaging. The atmosphere within packaging plays a crucial role in the preservation of carotenoids in food products. The presence of oxygen increases the degradation of carotenoids.[14,15] Therefore, MAP is useful in preventing carotenoid oxidation.[15] Opaque packaging to prevent light exposure and packaging with low oxygen permeability help to protect carotenoids.[14] Proper packaging and storage are also essential to retain any pro-vitamin A activity and health properties of carotenoids over time.[14,16]

Due to the unstable nature of carotenoids, great care needs to be taken when analyzing these compounds. As carotenoids are sensitive to light, temperature, and oxygen, there are critical points throughout the analysis wherein they are at great risk of being degraded.[8] Whether analyzing carotenoids via high performance liquid chromatography (HPLC) or spectroscopy, the carotenoids need to first be extracted from the matrix. Following the completion of the extraction, the carotenoid containing solution is prepared to be separated using HPLC or is prepared to be read by a spectrophotometer.[14-18] Due to their intense color, carotenoids strongly absorb in the visible region, between 400 and 500 nm.[8]

METHODS AND MATERIALS

Sample Preparation

Breakfast Bites were made in the Utah State University Research Kitchen by trained research assistants. Ingredients for the Bites were obtained through a local farmer, a local supermarket, and through US Foods. All ingredients were carefully weighed using calibrated balances (Mettler Toledo, Columbus, OH) to ensure consistency between batches. Breakfast Bites contain the following ingredients: whole wheat flour, oats, cinnamon, nutmeg, powdered skim milk, honey, egg, butter, raisins, dried cranberries, dried apricots, pureed carrots, butternut squash, and great northern white beans, dark chocolate, and protein whey crisps (Grände Custom Ingredients Group, Lomira, WI). Ingredients were combined using an electric mixer (Bosch, Stuttgard, Germany) and were then measured out using a #24 portion scoop. Bites were periodically weighed throughout sample preparation (approximately 20 grams per Bite, wet weight) to ensure consistency. The Bites were then baked using the "Cookie" setting on the combi-oven (Rational, Landsberg am Lech, Germany) following which, they were cooled for 30 minutes at room temperature. Samples were then ready to be packaged.

Packaging and Storage

Breakfast bites were packaged into one of three packaging types: 1) 2.5in x 0.75in x 6.5in standard, clear cellophane food-grade pouch with unmodified atmosphere, 2) 6in x 6in standard, clear vacuum grade pouch with vacuum sealing, or 3) 6in x 6in standard clear PVC food-grade pouch with nitrogen backflush. Samples to be used in the extended freezer storage study were then assigned to one of three storage methods: 1) samples

were placed on sheet trays and frozen with no additional packaging, 2) packaged in clear, master pack bags, sealed, and frozen or 3) packaged in foil-lined (light proof) master pack bags, sealed, and frozen. Samples used for the 2-week shelf-life study remained at room temperature while all other samples were put into a freezer and held at -17.8ºC.

A baseline sample was analyzed for color, water activity (A_w), and moisture content. Samples in the 2-week shelf-life study were pulled and analyzed at days 3, 7, 10, and 14. Samples in the extended freezer storage study were pulled, thawed at room temperature for 3.5 hours, and analyzed at 1 month increments for 5 months. Each sample was analyzed for color, A_w, moisture content, and carotenoid content. Two complete replicates (Breakfast Bite production, packaging, storage, and analysis) were performed. Carotenoid content measurements were only obtained from the second replicate.

Color Measurement

L^*, a^*, and b^* values were measured by a HunterLab Miniscan portable colorimeter (Reston, VA, USA). Diameter aperture was 5 mm and the colorimeter was set to use illuminant D-65. The colorimeter was standardized using black and white standard tiles. Three measurements were taken per sample. Chroma was calculated as $\sqrt{a^{*2} + b^{*2}}$ and hue angle was calculated as $\tan^{-1}(\frac{b^*}{a^*})$.

Water Activity and Moisture Content

Samples were roughly chopped by hand then placed in a Spice and Nut Grinder (Cuisinart, Stamford, CT) to finely chop the sample. A_w of 2 gm samples was measured using an Aqua Lab 4TE water activity meter (Decagon Devices, Pullman, WA, USA).

Samples were then analyzed for moisture content using an MA150 infrared moisture analyzer with a ceramic heating coil (Sartorius Weighing Technology GmbH, Goettingen, Germany), by drying at 110°C to a constant weight.

Carotenoid Analysis

Each sample pulled for analysis was freeze-dried using a Dura-Dry freeze dryer (FTS Systems, Inc. Stone Ridge, NY, USA) and held for carotenoid analysis. Following freeze-drying, samples were sealed in vacuum pouches and were stored at -80ºC until time of analysis.

For carotenoid analysis, only one replicate was tested. A modified version of the analysis by Brown et al.[19] was used. Freeze-dried Breakfast Bites were ground into a powder, again using a Spice and Nut Grinder. 50 mg samples of powder were weighed into 5 ml centrifuge tubes whereupon 500 µl of 1 methanol (MeOH) : 1 chloroform ($CHCl_3$) was added and the mixture was vortexed for 15 minutes. 250 µl of aqueous 50 mM Tris, pH=6.5, 1 M sodium chloride (NaCl) was added and vortexed. The mixture was then centrifuged for 10 minutes at 13,000 rpm. The $CHCl_3$ layer was removed and put into an amber glass vial and 400 µl 100% $CHCl_3$ was added to the remaining aqueous fraction. This was vortexed then centrifuged for 10 minutes at 13,000 g. The $CHCl_3$ layer was added to the amber vial. A third extraction was found to be lacking in carotenoids and therefore was not necessary. The $CHCl_3$ was then evaporated using a Pierce Reacti-Therm III N_2 evaporator (Thermo Fisher Scientific, Waltham, MA), without heat. Upon complete evaporation, the carotenoids were resuspended in 600 µl of MeOH and 400 µl of 30% methanolic potassium hydroxide (KOH).[20]. Vials were then vortexed and

incubated on ice for 1 hour. Following incubation, the mixture was put into a microcuvette and scanned using a UV-Vis Spectrophotometer (Shimadzu UV-2100U, Shimadzu Corp., Tokyo, Japan). The difference in absorbance values at 450 nm and 550 nm was calculated for each sample and was put into the following equation to find total carotenoid content (µg/100g).

$$\frac{\mu g}{100g}(DWB) = \frac{A_{450}-A_{550}}{2480} \times \frac{10\mu g}{\mu l} \times \frac{1000\mu l}{\text{sample wt (g)}} \times 100$$

Statistical Analysis

Analysis of variance (ANOVA) was performed using the proc glm function in SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). For the room temperature study, package type, storage time, and their interaction were evaluated as fixed factors. For the extended freezer storage study, main package type, outer (master) package type, storage time, and their interactions were evaluated as fixed factors. A 95% statistical significance level ($\alpha = 0.05$) was used. Posthoc mean comparisons were made using the Tukey-Kramer adjustment. Due to lack of replication, ANOVA could not be performed for carotenoid content, so only summary statistics are provided.

RESULTS AND DISCUSSION

Color

2-Week Shelf-Life Study

Color was the most significantly impacted measure in the 2-week shelf-life study. L^* was significantly affected by packaging type ($P = 0.0013$) as was b^* ($P = 0.0197$) (Table 4.1, Figures 4.1 and 4.2; see Appendix B for detailed statistics). L^* values

Table 4.1. Pooled average values over 14 days for each color property of Breakfast Bites in all three treatments. Values not sharing the same letter within a column are significantly different ($P < 0.05$).

Treatment	L*	a*	b*	Chroma	Hue-angle
Cellophane	48.568^{b}	11.260	28.479^{b}	30.633^{b}	1.193^{b}
Vacuum	36.109^{a}	10.041	20.165^{a}	22.538^{a}	1.106^{a}
N2 Backflush	45.561^{b}	10.385	25.573^{ab}	27.613^{ab}	1.180^{b}
P-value	0.0013	0.3046	0.0197	0.0275	0.0007

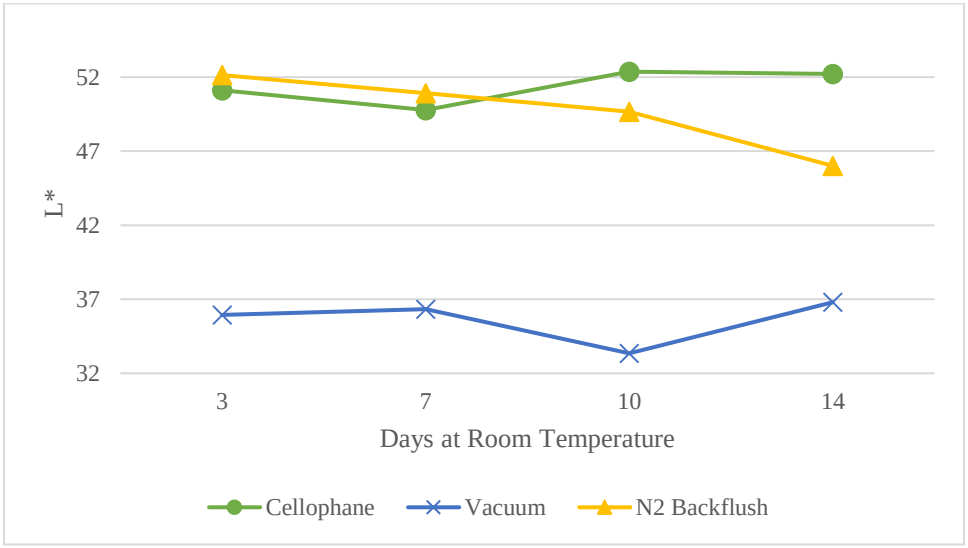

Figure 4.1. L* values of Breakfast Bites in 2-week shelf-life study

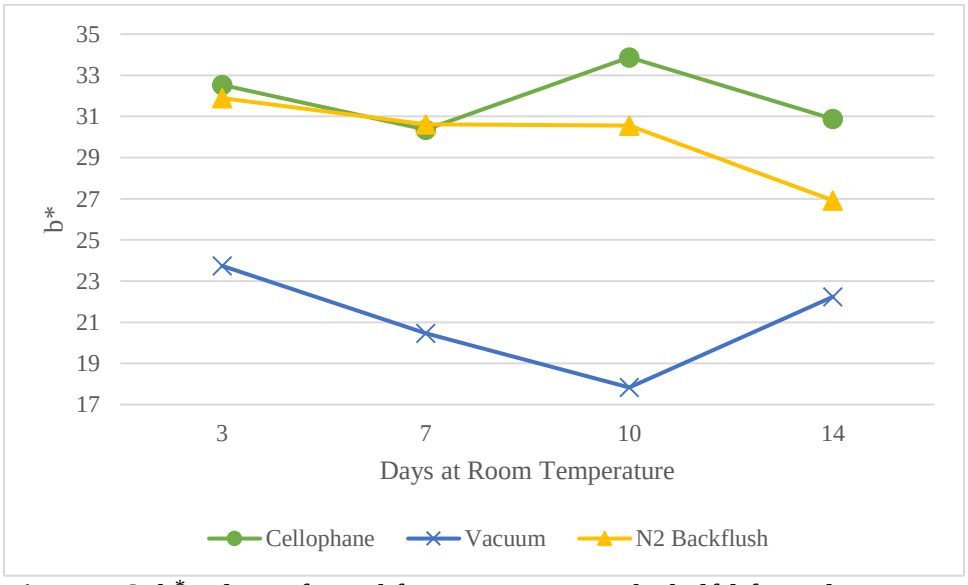

Figure 4.2. b* values of Breakfast Bites in 2-week shelf-life study

increased slightly in vacuum and N_2 packaged Bites. L^* values in vacuum packaged Bites were consistently lower than those in cellophane or N_2 packaging. This difference was visually noticeable as the vacuum packaged Bites were considerably darker. b^* values decreased overall, indicating a decrease in yellowness. By visual estimation, color appeared to be consistent through to the middle of the Bites. Sulaeman et al.[14] found that decreases in b^* values were reflective of decreasing carotenoid levels. They also observed that color degradation increased with increasing storage temperature, moisture content, and A_w. If these observations are correct, we would expect to see decreases in carotenoid values in Bites over the course of 2 weeks at room temperature. This would agree with the findings of Bechoff et al.[13] as they observed significantly higher carotenoid degradation at higher temperatures. Hue and chroma were also significantly affected by packaging type ($P = 0.0007$ and $P = 0.0275$; see Appendix B for additional figures). The vacuum packed Bites consistently had lower b^*, chroma, and hue values. Time had no significant effect on the color of the Breakfast Bites.

Extended Freezer Storage Study

Color was the most significantly impacted measure in the extended freezer storage study as well. L^* was significantly affected by packaging type ($P < 0.0001$) as was b^* ($P = 0.009$) (Table 4.2, Figures 4.3 and 4.4). L^* values had a slight overall increasing trend in all packaging types and was consistently lower in vacuum packaged Bites. Again, this was apparent visually as the vacuum packaged Bites were much darker. Based on the study by Sulaeman et al.,[14] we could hypothesize that carotenoid values increased over the course of this study. However, no significant trends were seen in

Table 4.2. Pooled average values over 5 months for each color property of Breakfast Bites in all three treatments. Values not sharing the same letter within a column are significantly different (P < 0.05).

Treatment	L*	a*	b*	Chroma	Hue-angle
Cellophane	47.307[b]	10.916[b]	27.758[b]	29.837[b]	1.193[b]
Vacuum	35.419[a]	9.488[a]	19.872[a]	22.033[a]	1.121[a]
N2 Backflush	46.374[b]	10.614[ab]	26.669[b]	28.714[b]	1.188[b]
P-value	<0.0001	0.0290	<0.0001	0.0002	<0.0001

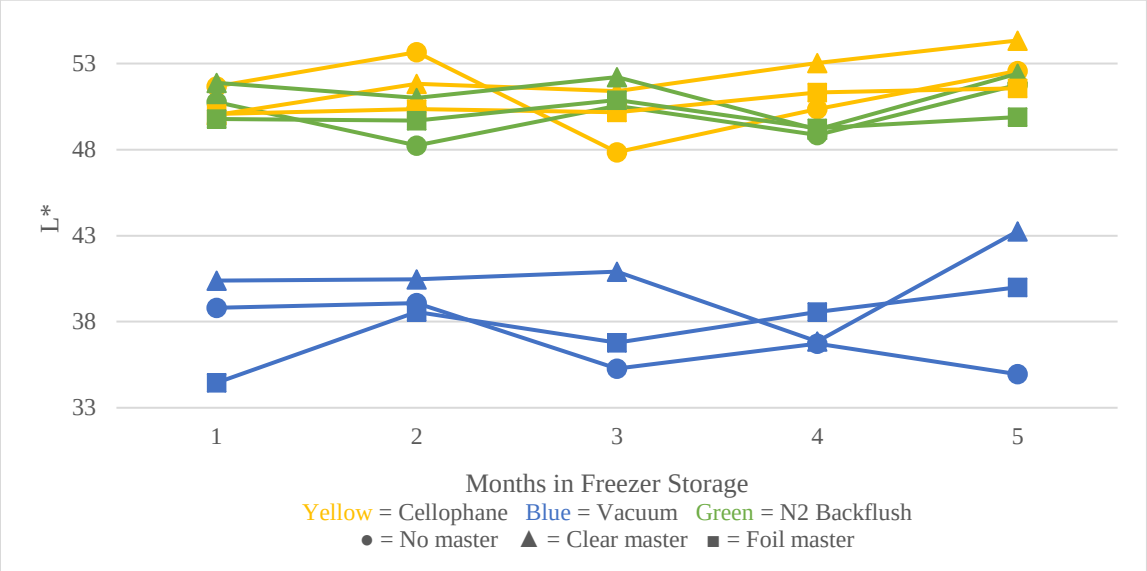

Figure 4.3. L* values of Breakfast Bites in extended freezer storage study

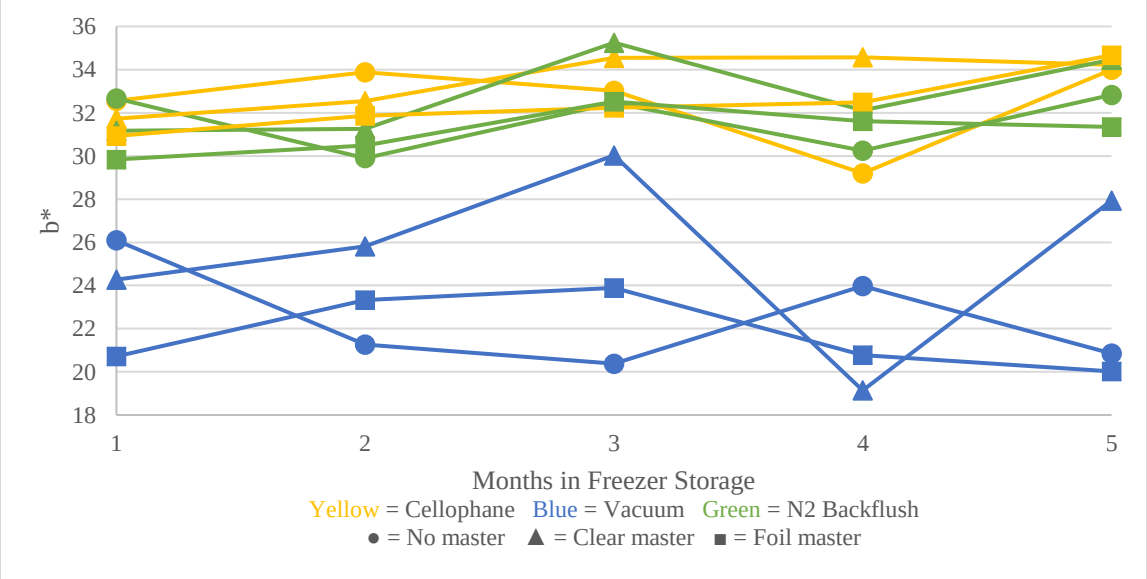

Figure 4.4. b* values of Breakfast Bites in extended freezer storage study

our carotenoid analysis. Hue and chroma were also significantly affected by packaging type ($P < 0.0001$ and $P = 0.0159$, respectively). The vacuum packed Bites consistently had lower b^*, chroma, and hue values (Figure 4.4, Appendix B). Time had no significant effect on the color of the Breakfast Bites.

Water Activity and Moisture Content

2-Week Shelf-Life Study

Neither A_w nor moisture content were significantly associated with packaging type or time. There was a general downward trend in the interior A_w and a general upward trend in external moisture content. Sulaeman et al.[14] found that increased moisture content during storage decreased carotenoid content but saw no significant changes in moisture content and A_w in deep-fried carrot chips when packaged in similar situations. There was no moisture migration seen within the Bites, indicating no staling

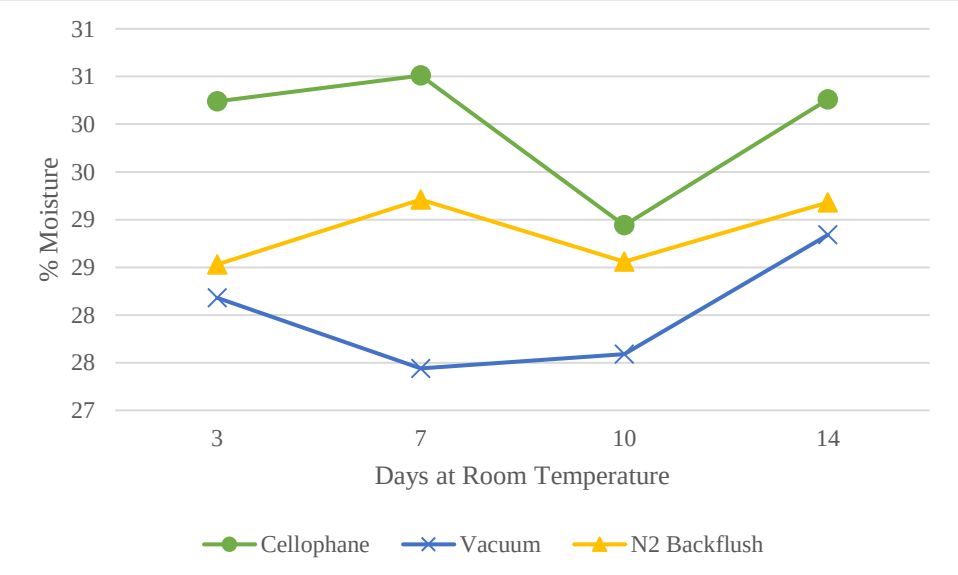

Figure 4.5. Interior moisture content (%) of Breakfast Bites in 2-week shelf-life study

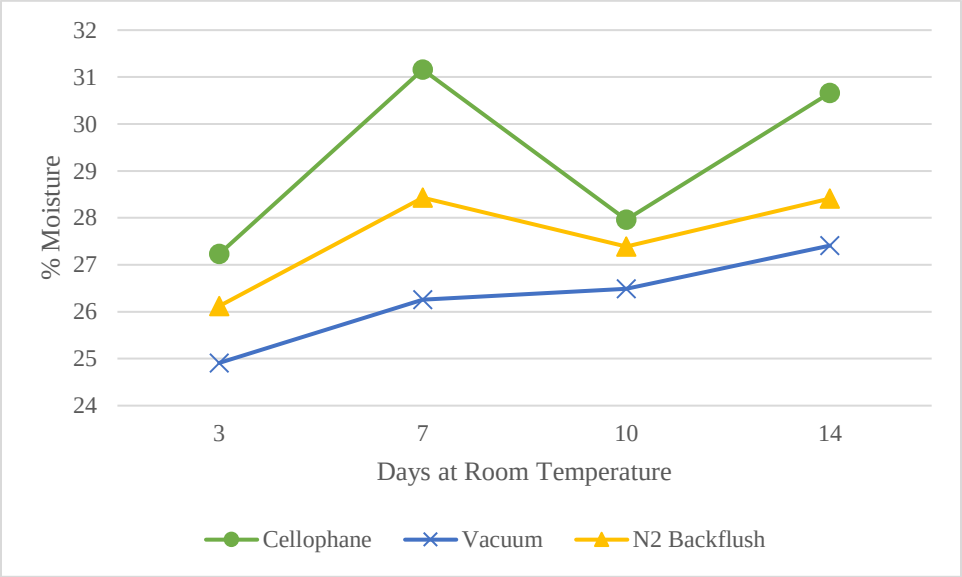

Figure 4.6. Exterior moisture content (%) of Breakfast Bites in 2-week shelf-life study

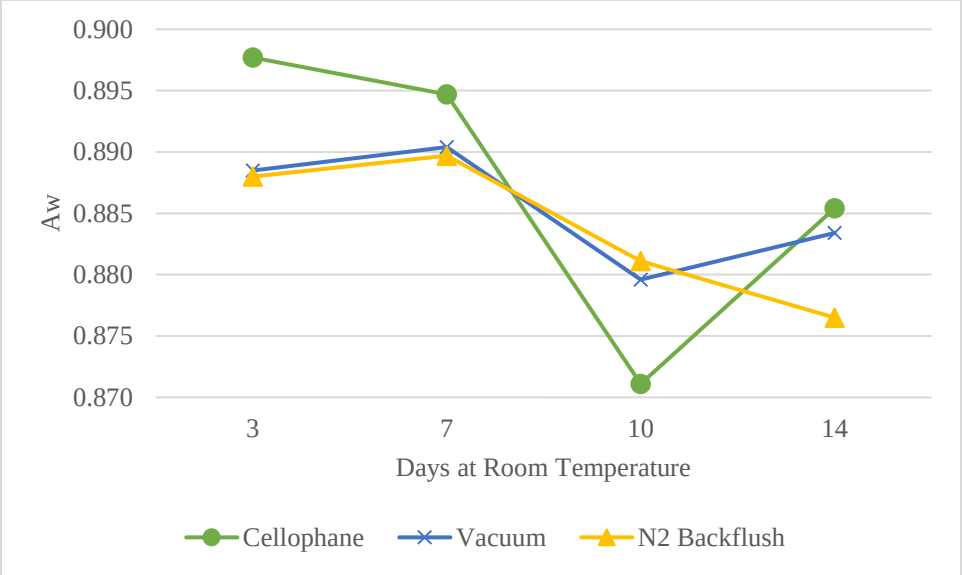

Figure 4.7. Interior A_w of Breakfast Bites in 2-week shelf-life study

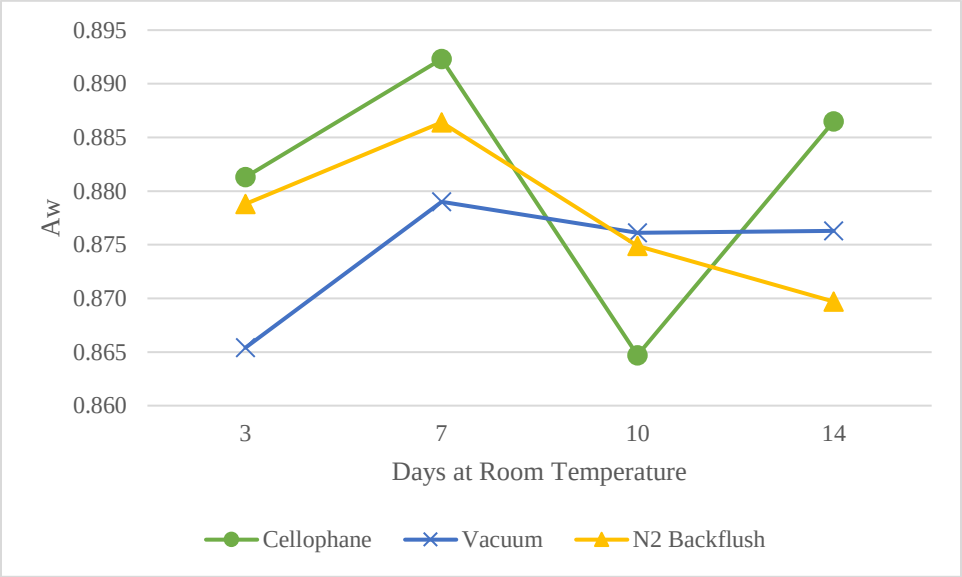

Figure 4.8. Exterior A_w of Breakfast Bites in 2-week shelf-life study

occurred.[21] The interior A_w of the Bites averaged 0.895 and the exterior A_w averaged 0.881 (see Figures 4.5-4.8 for changes in moisture content and A_w). Microorganisms are able to grow at such high A_w and can result in food spoilage and food borne illnesses.[22] The majority of bacteria, with the exception of *Staphylococcus aureus*, will not grow below A_w 0.9. Molds, however, will grow as low as A_w 0.7 and some down to A_w 0.6.[22] Mold growth was seen on day 14 in the cellophane packaging in the first replicate. This suggests that it may be beneficial to perform a study to observe microbial growth on Bites in various packages at room temperature.

Extended Freezer Storage Study

Neither A_w nor moisture content were significantly associated with packaging type or time. There was a slight downward trend in the interior A_w. Again, there was no moisture migration in the Bites. Interior A_w of the Bites averaged 0.903 and exterior A_w averaged 0.879 (see Figures 4.9-4.12 for changes in A_w moisture content). A_w was high

enough to allow for mold growth but none was seen due to storage at freezing temperatures.

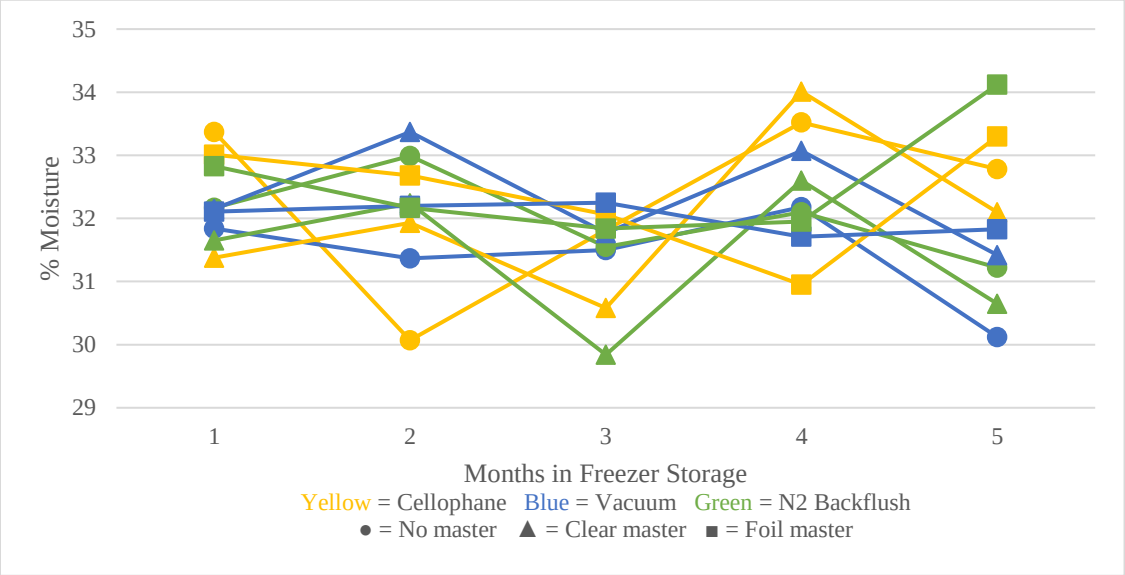

Figure 4.9. Interior moisture content (%) of Breakfast Bites in extended freezer storage study

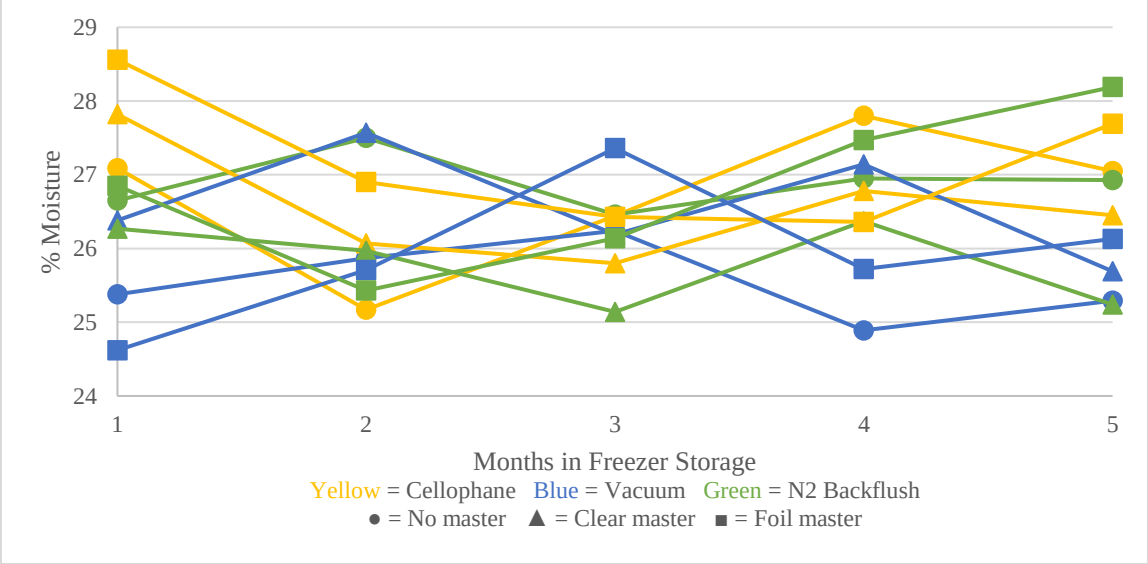

Figure 4.10. Exterior moisture content (%) of Breakfast Bites in extended freezer storage study

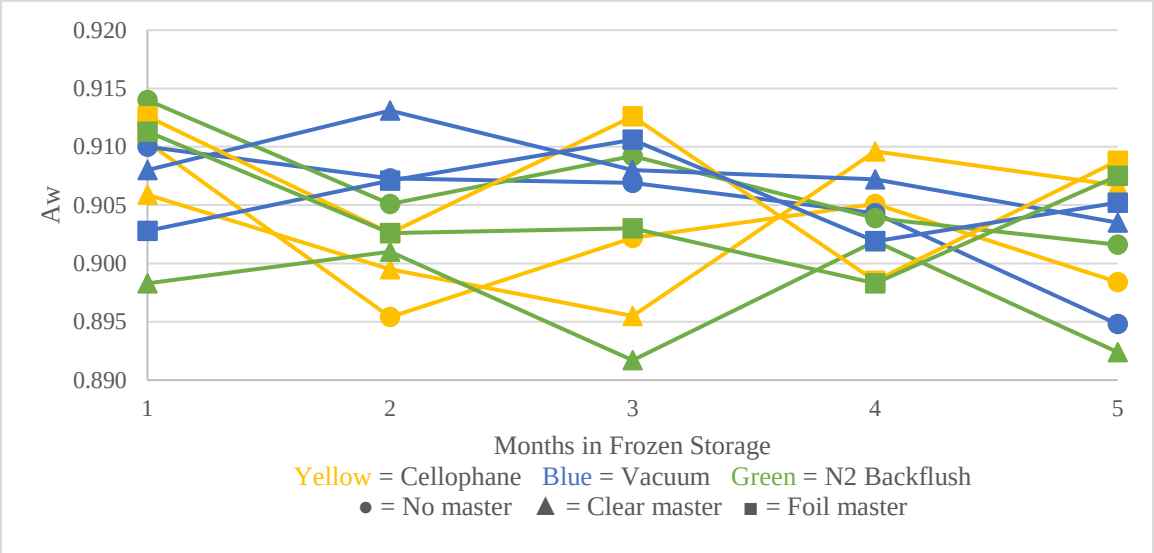

Figure 4.11. Exterior A_w of Breakfast Bites in extended freezer storage study

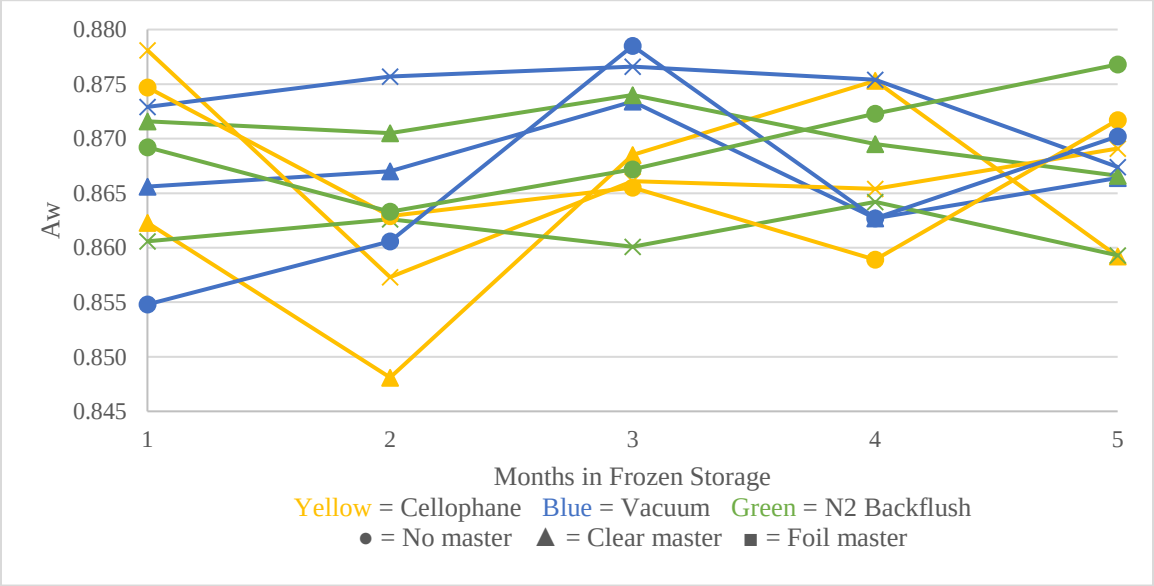

Figure 4.12. Interior A_w of Breakfast Bites in extended freezer storage study

Carotenoid Content

In both studies, little to no trend was seen in carotenoid levels. Tables 4.3 and 4.4 show carotenoid content throughout the study. Also see Figures 4.13 and 4.14 for visual representation of carotenoid content. The variability in carotenoid levels may have been

Table 4.3. Carotenoid content of Breakfast Bites in 2-week shelf-life study (mg/120gm)

Treatment	Day 3	Day 7	Day 10	Day 14
Cellophane	3.18	2.55	2.39	2.46
Vacuum	2.68	1.92	1.85	3.55
N2 Backflush	0.59	2.11	2.94	3.33

Table 4.4. Carotenoid content of Breakfast Bites in extended freezer storage study (mg/120gm)

Treatment	1 Month	2 Month	3 Month	4 Month	5 Month
Cellophane	3.74	2.21	2.32	2.28	2.88
Vacuum	1.78	2.49	2.64	2.51	1.14
N2 Backflush	0.82	2.85	0.70	2.00	1.56
Cellophane Clear	2.35	2.03	0.14	2.70	3.67
Vacuum Clear	3.06	1.88	2.25	2.48	3.36
N2 Clear	2.37	1.66	0.89	2.05	2.53
Cellophane Foil	2.04	2.82	2.63	2.45	2.70
Vacuum Foil	0.53	3.52	1.45	3.35	3.79
N2 Foil	2.40	1.85	2.36	1.53	0.64

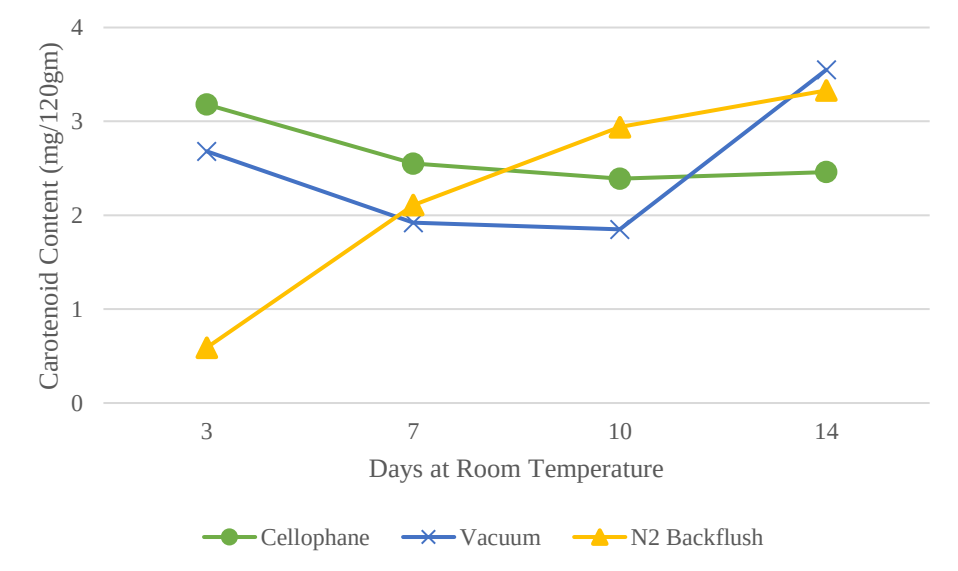

Figure 4.13. Carotenoid content of Breakfast Bites in 2-week shelf-life Study (mg/120gm)

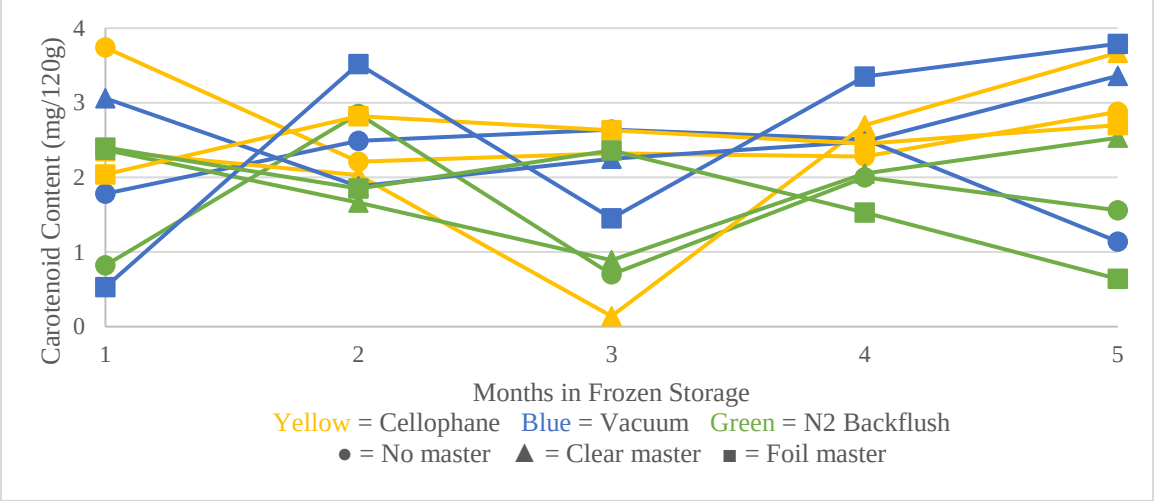

Figure 4.14. Carotenoid content of Breakfast Bites in extended freezer storage study (mg/120gm)

due to the instability of carotenoids during analysis. Variation may also be due to carotenoid variability within samples. Though samples were homogenized as well as possible, not every weighed sample would have had a truly representative amount of carotenoids. Using The Food Processor Nutrition Analysis Software (ESHA, 2015; Salem, OR), it is estimated that Breakfast Bites contain 4.3 mg carotenoids per serving (120 gm). The highest carotenoid content observed in any Breakfast Bites sample was 3.79 mg per serving, indicating that our analyses were consistent with the ESHA's estimation. The lowest carotenoid content observed was 0.14 mg per serving in the cellophane packaging stored in a clear master bag at month 3. However, a relatively high value of 3.67 mg per serving was observed in that packaging at month 5. This reaffirms that carotenoids can be degraded due to instability or can be non-homogenous throughout samples when analyzing carotenoids.

The calculated 4.3 mg carotenoids in Bites could similarly be consumed by eating approximately 50 gm, or ½ cup, cooked carrots.[5] This demonstrates that this amount of

carotenoid could easily be consumed by both adults and children each day. However, bioavailability may differ between these two food items and does not necessarily mean that 4.3 mg will be absorbed. In another portion of our study, consumption of Breakfast Bites significantly raised skin carotenoid levels in children over a 6-week period (Chapter 3). Skin carotenoid levels are indicators of F/V intake with higher levels indicating higher F/V intake.[23] It has been well established that consuming higher amounts of F/V reduces the risk of morality and chronic disease.[24,25] Therefore, we can assume the carotenoid content in Breakfast Bites is beneficial for consumption and could potentially offer health benefits to consumers.

CONCLUSIONS

Based on color and appearance, we can conclude that cellophane and N_2 backflush packaging appear to be the best option. Our data suggests that both of these packaging types could be used to store Bites at room temperature for 2 weeks or in combination with any freezer storage method at -17.8ºC for 5 months.

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CHAPTER 5

CONCLUSIONS

A feeding study was conducted in children, ages 5-18, to observe the change in skin carotenoid levels while regularly consuming Breakfast Bites or placebo bars over a 6 week time period (Chapter 3). Children were asked to consume 6 Bites or 3 bars per day. Children presented to the clinic for weekly visits where they received their ration of Bites or bars and filled out weekly questionnaires for the duration of the study. Food frequency questionnaires were filled out by participants or their parents three times at baseline, week 3, and week 6. Skin carotenoid levels were taken at baseline and at weeks 2, 4, and 6 by scanning the participants' palms using a BioPhotonic scanner. After 6 weeks of consuming Bites or bars scan scores in the Bites group were significantly higher ($P = 0.022$) than the scan scores of those in the placebo group. The Bites group had a mean increase in scan scores of 5,802 Raman intensity units. This suggests that regular consumption of a high-carotenoid food ($\geq$ 4.3 mg/120 gm) could result in increased carotenoid levels in the skin, in turn increasing antioxidant levels throughout the body. Follow up considerations for this study might include conducting another feeding study to observe how many Breakfast Bites children would consume when given the opportunity to choose. This would assist in determining what age groups or audiences would be with most prone to consuming Breakfast Bites. It could also be beneficial to conduct a feeding study involving additional flavors of Breakfast Bites to see if incorporating additional ingredients affects the absorption and deposition of carotenoids into the skin.

Two shelf-life studies were conducted on Breakfast Bites, one at room temperature and one in long-term freezer storage, to observe changes in various shelf-stability measures in different packaging types (Chapter 4). Bites were packaged in one of three packaging types: 1) standard clear polyvinylchloride (PVC) food-grade pouch, unmodified atmosphere; 2) standard clear PVC food-grade pouch with nitrogen backflush; and 3) standard clear vacuum-grade pouch with vacuum sealing. Packaged Bites were then randomized to one of three storage methods: 1) freezing with no additional packaging; 2) packaging in a clear master pack bag, sealed, and frozen; and 3) packaging in a foil-lined master pack bag, sealed, and frozen. Samples were then pulled at regular intervals and analyzed for internal and external water activity (A_w) and moisture content, L^*,a^*,b^* color changes, and carotenoid levels. Over the 2 week shelf-life study, it was found that L^* was significantly associated with packaging type ($P = 0.0013$) as was b^* ($P = 0.0197$), hue ($P = 0.0007$), and chroma ($P = 0.0275$). No significant changes were found in the other measures. The freezer storage study had similar findings with color related values being significantly affected by packaging type with no other significant findings. Carotenoid values showed no trend, likely due to their unstable nature during analysis and the inconsistent carotenoid content between samples. Thus, we can draw the conclusion that based on color and appearance, cellophane and packaging with N_2 backflush would be the best options to use when storing Breakfast Bites at room temperature for up to 2 weeks. These two packaging methods could be used with any freezer storage method for long-term storage.

Future research related to Breakfast Bites should include a sensory panel to see if Bites in different packaging types are preferred more than others by consumers. It would

also be advisable to perform tests relating to food safety and microbial growth in Breakfast Bites stored at room temperature. Both of these studies would be beneficial in getting Breakfast Bites on café shelves across the Utah State University campus by finalizing a product that consumers can safely enjoy.

APPENDICES

APPENDIX A

STATISTICS AND FIGURES FOR CHAPTER 3

Table A.1. Breakfast Bites nutritional facts (per serving)

Basic Components	
Gram Weight (g)	120
Calories (kcal)	331
Calories from Fat (kcal)	98
Calories from SatFat (kcal)	26
Protein (g)	9
Carbohydrates (g)	52
Dietary Fiber (g)	6
Fat (g)	11
Saturated Fat (g)	3
Mono Fat (g)	2
Poly Fat (g)	4
Trans Fatty Acid (g)	0
Cholesterol (mg)	41
Starch (g)	0
Soluble Fiber (g)	1
Insoluble Fiber (g)	3
Total Sugars (g)	25
Monosaccharides (g)	12
Galactose (g)	0
Glucose (g)	6
Fructose (g)	6
Disaccharides (g)	5
Lactose (g)	3
Sucrose (g)	1
Maltose (g)	0.2
Vitamins	
Vitamin A - RAE (RAE)	234.4
Vitamin B1 (mg)	0.2
Vitamin B2 (mg)	0.2
Vitamin B3 (mg)	1.5
Vitamin B6 (mg)	0.2
Vitamin B12 (mcg)	0.3

Table A.1. continued

Biotin (mcg)	2.7
Vitamin C (mg)	4.9
Folate, DFE (mcg)	27.4
Pantothenic Acid (mg)	0.5
Vitamin D - IU (IU)	2.4
Vitamin E - mg (mg)	0.9
Minerals	
Calcium (mg)	144.8
Iron (mg)	2.3
Potassium (mg)	428.6
Sodium (mg)	94.0
Zinc (mg)	1.0
Chloride (mg)	8.9
Chromium (mcg)	0.03
Poly Fats	
18:2 - Linoleic (g)	3.1
18:3 - Linolenic (g)	0.7
20:5 - EPA (g)	0
22:6 - DHA (g)	0

Table A.2. Fixed effects (ANOVA) for Treatment and Total F/V

		Num df	F Value	Pr > F
Total F/V baseline	Between groups	1	1.560	0.218
	Within groups	44		
	Total	45		
Total F/V week 3	Between groups	1	0.322	0.573
	Within groups	42		
	Total	43		
Total F/V week 6	Between groups	1	0.272	0.605
	Within groups	43		
	Total	44		

Table A.3. Fixed effects (ANOVA) for Gender and Total F/V

		Num df	F Value	Pr > F
Total F/V baseline	Between groups	1	0.801	0.376
	Within groups	44		
	Total	45		
Total F/V week 3	Between groups	1	0.644	0.427
	Within groups	42		
	Total	43		
Total F/V week 6	Between groups	1	0.25	0.62
	Within groups	43		
	Total	44		

Table A.4. Fixed effects (ANOVA) for Ethnicity and Total F/V

		Num df	F Value	Pr > F
Total F/V baseline	Between groups	2	1.369	0.265
	Within groups	43		
	Total	45		
Total F/V week 3	Between groups	2	3.610	0.036
	Within groups	41		
	Total	43		
Total F/V week 6	Between groups	2	3.810	0.030
	Within groups	42		
	Total	44		

Table A.5. Fixed effects (ANOVA) for Treatment

		Num df	F Value	Pr > F
Weight	Between groups	1	0.714	0.403
	Within groups	44		
	Total	45		
Age	Between groups	1	0.058	0.811
	Within groups	44		
	Total	45		
HCV baseline	Between groups	1	1.331	0.255
	Within groups	44		
	Total	45		
Baseline scan	Between groups	1	0.714	0.403
	Within groups	44		
	Total	45		

Table A.6. Fixed effects (ANOVA) for Age group and Baseline scan

		Num df	F Value	Pr > F
Baseline scan	Between groups	2	0.955	0.393
	Within groups	43		
	Total	45		

Table A.7. Fixed effects (ANOVA) for Age group and Baseline F/V and HCV

		Num df	F Value	Pr > F
Total F/V baseline	Between groups	2	0.550	0.581
	Within groups	43		
	Total	45		
HCV baseline	Between groups	2	0.230	0.795
	Within groups	43		
	Total	45		

Table A.8. Fixed effects (ANOVA) for Gender and Baseline measures

		Num df	F Value	Pr > F
Total F/V baseline	Between groups	1	0.801	0.376
	Within groups	44		
	Total	45		
HCV baseline	Between groups	1	0.026	0.872
	Within groups	44		
	Total	45		
Baseline scan	Between groups	1	1.079	0.305
	Within groups	44		
	Total	45		

Table A.9. Fixed effects (ANOVA) for Ethnicity and Baseline measures

		Num df	F Value	Pr > F
Total F/V baseline	Between groups	2	1.369	0.265
	Within groups	43		
	Total	45		
HCV baseline	Between groups	2	0.512	0.603
	Within groups	43		
	Total	45		
Baseline scan	Between groups	2	0.488	0.617
	Within groups	43		
	Total	45		

Table A.10. Fixed effects (ANOVA) for Weight and Baseline measures

		Num df	F Value	Pr > F
Total F/V baseline	Between groups	2	0.221	0.803
	Within groups	43		
	Total	45		
HCV baseline	Between groups	2	1.130	0.332
	Within groups	43		
	Total	45		
Baseline scan	Between groups	2	1.525	0.229
	Within groups	43		
	Total	45		

Table A.11. Fixed effects (ANOVA) for Treatment and Baseline measures

		Num df	F Value	Pr > F
Total F/V baseline	Between groups	1	1.560	0.218
	Within groups	44		
	Total	45		
HCV baseline	Between groups	1	1.331	0.255
	Within groups	44		
	Total	45		
Baseline scan	Between groups	1	0.714	0.403
	Within groups	44		
	Total	45		

Table A.12. Fixed effects (ANOVA) for Multivitamin and Baseline scan

		Num df	F Value	Pr > F
Baseline scan	Between groups	1	3.202	0.080
	Within groups	44		
	Total	45		

Table A.13. Fixed effects (ANOVA) for Multivitamin at Scan points

		Num df	F Value	Pr > F
Baseline scan	Between groups	1	3.202	0.080
	Within groups	44		
	Total	45		
Week 2 scan	Between groups	1	0.806	0.374
	Within groups	44		
	Total	45		
Week 4 scan	Between groups	1	0.360	0.552
	Within groups	42		
	Total	13		
Week 6 scan	Between groups	1	0.024	0.877
	Within groups	44		
	Total	45		

Table A.14. Fixed effects (ANOVA) for Illness at baseline

		Num df	F Value	Pr > F
Baseline scan	Between groups	1	0.837	0.365
	Within groups	44		
	Total	45		

Table A.15. Fixed effects (ANOVA) for Illness at week 2

		Num df	F Value	Pr > F
Week 2 scan	Between groups	1	0.004	0.951
	Within groups	44		
	Total	45		

Table A.16. Fixed effects (ANOVA) for Illness at week 4

		Num df	F Value	Pr > F
Week 4 scan	Between groups	1	0.774	0.384
	Within groups	42		
	Total	43		

Table A.17. Fixed effects (ANOVA) for Illness at week 6

		Num df	F Value	Pr > F
Week 6 scan	Between groups	1	1.691	0.200
	Within groups	44		
	Total	45		

Table A.18. Fixed effects (ANOVA) for Sun exposure at baseline

		Num df	F Value	Pr > F
Baseline scan	Between groups	3	4.492	0.008
	Within groups	42		
	Total	45		

Table A.19. Fixed effects (ANOVA) for Sun exposure at week 2

		Num df	F Value	Pr > F
Week 2 scan	Between groups	3	0.231	0.874
	Within groups	42		
	Total	45		

Table A.20. Fixed effects (ANOVA) for Sun exposure at week 4

		Num df	F Value	Pr > F
Week 4 scan	Between groups	3	1.512	0.226
	Within groups	40		
	Total	43		

Table A.21. Fixed effects (ANOVA) for Sun exposure at week 6

		Num df	F Value	Pr > F
Week 6 scan	Between groups	3	0.655	0.585
	Within groups	42		
	Total	45		

APPENDIX B

STATISTICS AND FIGURES FOR CHAPTER 4

Table B.1. Type 3 tests fixed effects (ANOVA) for Inner Aw, 2-week shelf-life study

Effect	Num DF	F Value	Pr > F
time	3	2.50	0.1093
pkg	2	0.39	0.6876
time*pkg	6	0.12	0.9922

Table B.2. Type 3 tests fixed effects (ANOVA) for Outer Aw, 2-week shelf-life study

Effect	Num DF	F Value	Pr > F
time	3	0.58	0.6383
pkg	2	0.16	0.8504
time*pkg	6	0.27	0.9421

Table B.3. Type 3 tests fixed effects (ANOVA) for Inner Moisture %, 2-week shelf-life study

Effect	Num DF	F Value	Pr > F
time	3	0.18	0.9095
pkg	2	2.00	0.1780
time*pkg	6	0.53	0.7770

Table B.4. Type 3 tests fixed effects (ANOVA) for Outer Moisture %, 2-week shelf-life study

Effect	Num DF	F Value	Pr > F
time	3	0.89	0.4733
pkg	2	1.89	0.1929
time*pkg	6	0.09	0.9966

Table B.5. Type 3 tests fixed effects (ANOVA) for L*, 2-week shelf-life study

Effect	Num DF	F Value	Pr > F
time	3	0.12	0.9458
pkg	2	12.13	0.0013
time*pkg	6	0.69	0.6598

Table B.6. Type 3 tests fixed effects (ANOVA) for a*, 2-week shelf-life study

Effect	Num DF	F Value	Pr > F
time	3	0.34	0.7945
pkg	2	1.31	0.3046
time*pkg	6	0.56	0.7568

Table B.7. Type 3 tests fixed effects (ANOVA) for b*, 2-week shelf-life study

Effect	Num DF	F Value	Pr > F
time	3	0.36	0.7817
pkg	2	5.54	0.0197
time*pkg	6	0.43	0.8472

Table B.8. Type 3 tests fixed effects (ANOVA) for chroma, 2-week shelf-life study

Effect	Num DF	F Value	Pr > F
time	3	0.36	0.7810
pkg	2	4.92	0.0275
time*pkg	6	0.44	0.8386

Table B.9. Type 3 tests fixed effects (ANOVA) for hue angle, 2-week shelf-life study

Effect	Num DF	F Value	Pr > F
time	3	0.36	0.7812
pkg	2	14.04	0.0007
time*pkg	6	0.42	0.8530

Table B.10. Type 3 tests fixed effects (ANOVA) for Inner Aw, extended freezer storage study

Effect	Num DF	F Value	Pr > F
time	4	0.75	0.5633
pkg	2	0.39	0.6815
time*pkg	8	0.27	0.9717
fs	2	0.16	0.8561
time*fs	8	0.22	0.9858
pkg*fs	4	0.56	0.6911
time*pkg*fs	16	0.20	0.9994

Table B.11. Type 3 tests fixed effects (ANOVA) for Outer Aw, extended freezer storage study

Effect	Num DF	F Value	Pr > F
time	4	0.37	0.8322
pkg	2	0.07	0.9367
time*pkg	8	0.09	0.9994
fs	2	0.01	0.9942
time*fs	8	0.06	0.9998
pkg*fs	4	0.18	0.9456
time*pkg*fs	16	0.03	1.0000

Table B.12. Type 3 tests fixed effects (ANOVA) for Inner Moisture %, extended freezer storage study

Effect	Num DF	F Value	Pr > F
time	4	0.66	0.6211
pkg	2	0.34	0.7129
time*pkg	8	0.35	0.9400
fs	2	0.04	0.9649
time*fs	8	0.67	0.7142
pkg*fs	4	0.91	0.4667
time*pkg*fs	16	0.46	0.9545

Table B.13. Type 3 tests fixed effects (ANOVA) for Outer Moisture %, extended freezer storage study

Effect	Num DF	F Value	Pr > F
time	4	0.09	0.9853
pkg	2	0.28	0.7575
time*pkg	8	0.15	0.9961
fs	2	0.02	0.9800
time*fs	8	0.04	1.0000
pkg*fs	4	0.09	0.9850
time*pkg*fs	16	0.05	1.0000

Table B.14. Type 3 tests fixed effects (ANOVA) for L*, extended freezer storage study

Effect	Num DF	F Value	Pr > F
time	4	0.09	0.9848
pkg	2	42.28	<0.0001
time*pkg	8	0.04	1.0000
fs	2	0.23	0.7978
time*fs	8	0.07	0.9997
pkg*fs	4	0.15	0.9605
time*pkg*fs	16	0.13	1.0000

Table B.15. Type 3 tests fixed effects (ANOVA) for a*, extended freezer storage study

Effect	Num DF	F Value	Pr > F
time	4	0.05	0.9948
pkg	2	3.83	0.0290
time*pkg	8	0.07	0.9997
fs	2	0.26	0.7739
time*fs	8	0.07	0.9998
pkg*fs	4	0.07	0.9915
time*pkg*fs	16	0.21	0.9994

Table B.16. Type 3 tests fixed effects (ANOVA) for b*, extended freezer storage study

Effect	Num DF	F Value	Pr > F
time	4	0.07	0.9917
pkg	2	11.67	<0.0001
time*pkg	8	0.03	1.0000
fs	2	0.12	0.8889
time*fs	8	0.05	0.9999
pkg*fs	4	0.08	0.9888
time*pkg*fs	16	0.11	1.0000

Table B.17. Type 3 tests fixed effects (ANOVA) for hue angle, extended freezer storage study

Effect	Num DF	F Value	Pr > F
time	4	0.36	0.8390
pkg	2	38.29	<0.0001
time*pkg	8	0.18	0.9928
fs	2	0.26	0.7728
time*fs	8	0.28	0.9701
pkg*fs	4	0.55	0.6994
time*pkg*fs	16	0.33	0.9909

Table B.18. Type 3 tests fixed effects (ANOVA) for chroma, extended freezer storage study

Effect	Num DF	F Value	Pr > F
time	4	0.06	0.9930
pkg	2	10.58	0.0002
time*pkg	8	0.03	1.0000
fs	2	0.13	0.8805
time*fs	8	0.05	0.9999
pkg*fs	4	0.07	0.9914
time*pkg*fs	16	0.11	1.0000

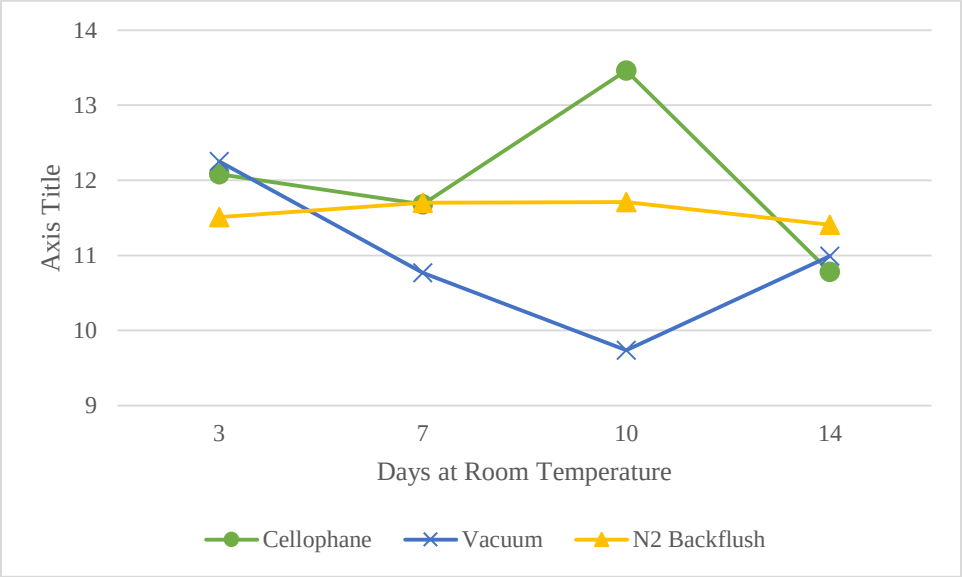

Figure B.1. a* values of Breakfast Bites in 2-week shelf-life study

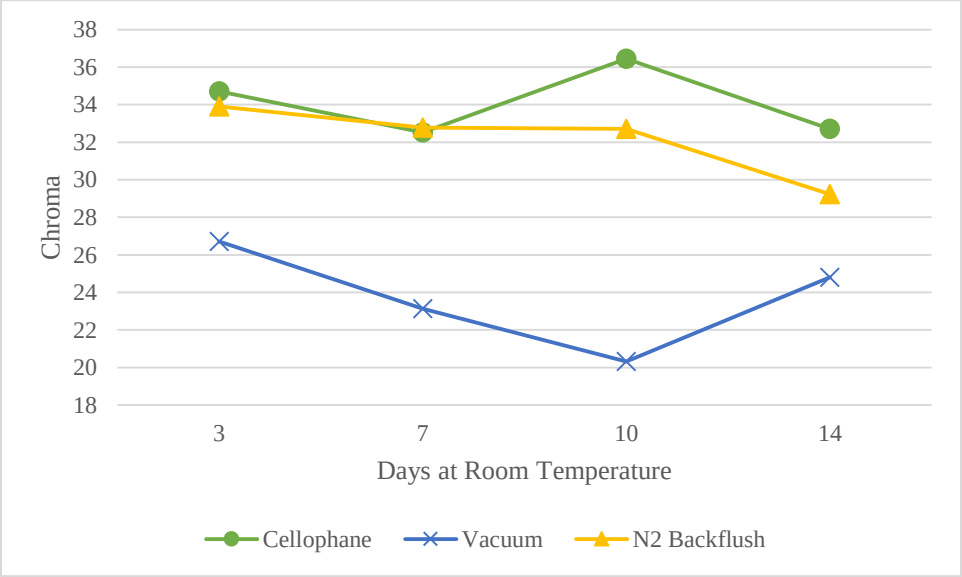

Figure B.2. Chroma of Breakfast Bites in 2-week shelf-life study

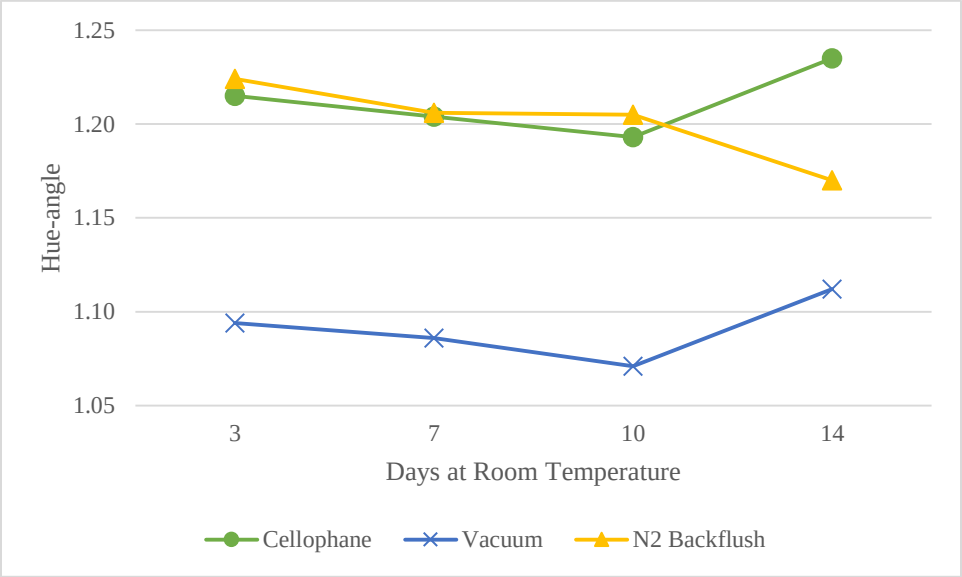

Figure B.3. Hue-angle of Breakfast Bites in 2-week shelf-life study

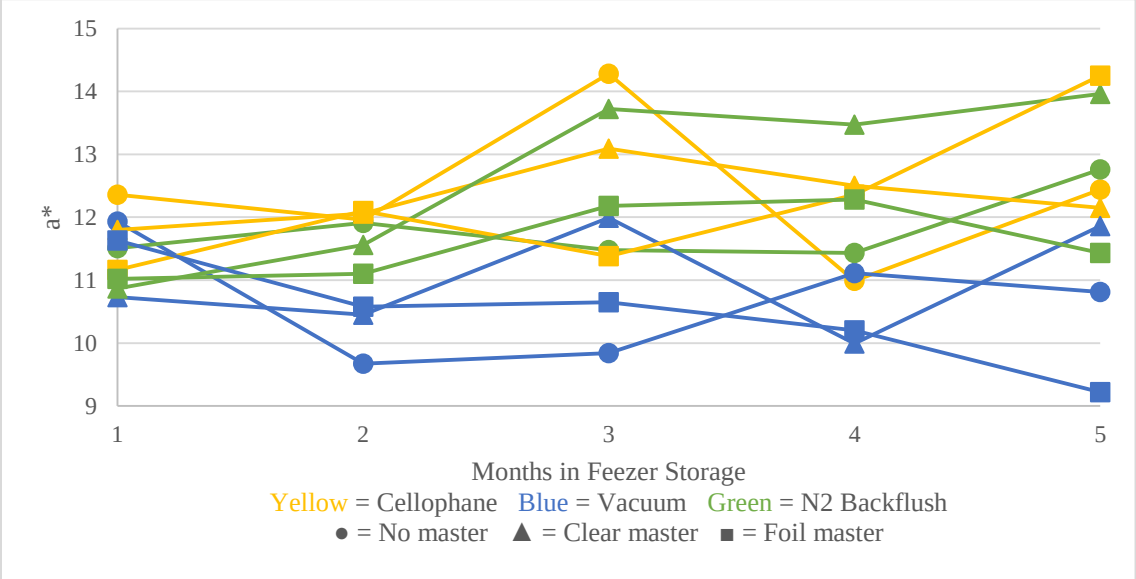

Figure B.4. a* values of Breakfast Bites in extended freezer storage study

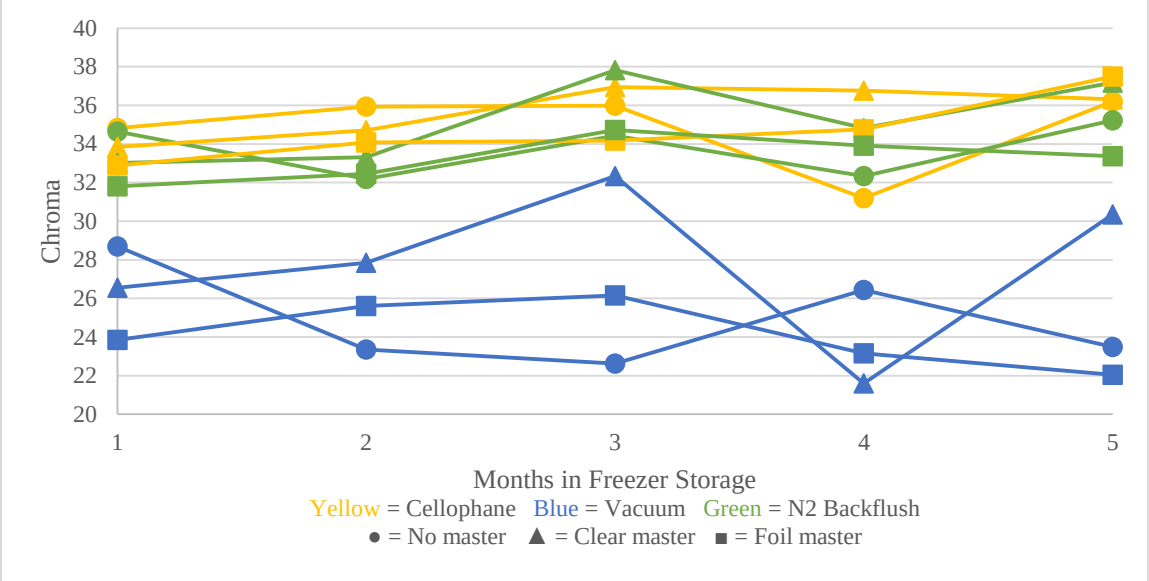

Figure B.5. Chroma of Breakfast Bites in extended freezer storage study

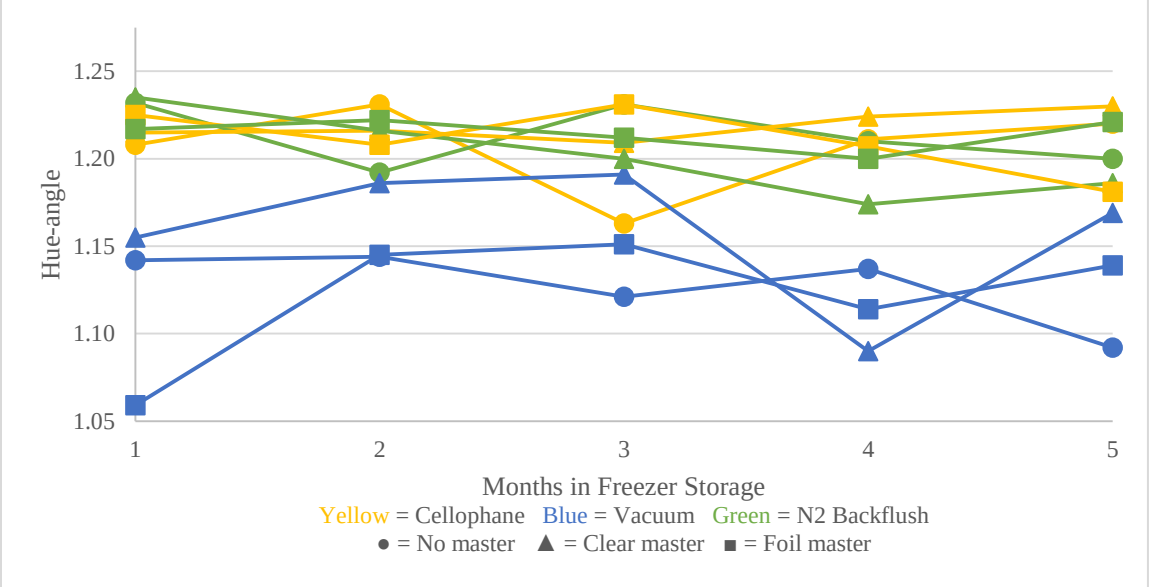

Figure B.6. Hue-angle of Breakfast Bites in extended freezer storage study