Effects of resistant starch and soluble fiber on the bioaccessibility of dietary carotenoids from spinach and carrot using simulated in vitro digestion

THESIS

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Abstract

We evaluated the effects of soluble fiber, including oligosaccharides, and resistant starch on the micellarization of lutein, α-carotene, and β-carotene from a raw spinach and carrot “salad” using a simulated in vitro digestion. Fiber variables were either combined with raw salad alone or with raw salad and nonfat yogurt to simulate a more complex meal. Raw and ripe bananas were used to examine the potential impact of the natural, inherent resistant starch fiber in the banana on the micellarization of lutein, α-carotene (AC), and β-carotene (βC). It was expected that the soluble fibers, fructooligosaccharide (FOS), galactooligosaccharide (GOS), and pectin, as well as fibers displaying characteristics of soluble fiber, such as resistant starch (RS) would decrease xanthophyll and carotene micellarization as previous soluble fiber studies had shown in vitro and in vivo. The efficiency with which lutein, 13-cis-β-carotene, α-carotene, β-carotene, and 9-cis-β-carotene from the raw salad partitioned in the aqueous fraction were 54.1 ± 1.3%, 18.5 ± 0.6%, 22.0 ± 1.4%, 19.6 ± 1.0%, and 52.7 ± 2.2%, respectively. High viscosity citrus pectin significantly inhibited micellarization of lutein (α< 0.0001), 13-cis-β-carotene (α< 0.0001), α-carotene (α< 0.0001), β-carotene (α< 0.0001), and 9-cis-β-carotene (α< 0.0001) relative to the fiber free control at 4% (wt: wt). Unexpectedly, oligosaccharide and resistant starch samples (FOS, GOS, RS2, and RS3) significantly increased micellarization of lutein, AC, and βC. Micellarization of lutein in the spinach and carrot salad was significantly increased by 12%, 12%, and 14% with the addition of 2% FOS.
(α= 0.0377), 4% FOS (0.0306), and 2% RS3 (α= 0.0054), respectively. Alpha- and beta-carotene showed very similar results. Micellarization of beta-carotene was significantly increased by 41%, 60% and 49% with the addition of 2% (wt: wt) FOS (α= 0.0136), RS2 (α< 0.0001), and RS3 (α= 0.0014), respectively. Micellarization of beta-carotene cis isomers was significantly increased with the addition of both 2% and 4% (wt: wt) FOS, GOS, RS2 and RS3. The combination of yogurt and salad did not significantly affect micellarization of lutein, AC or βC. There was no significant difference in extent of micellarization of carotenoids between raw and ripe banana. The results from this study indicate that the presence of 2-4% FOS, GOS, and RS ingredients will not compromise the bioaccessibility of carotenoids in a meal.
This is dedicated to my parents, the two people who have given everything possible for the continuation of my education.
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Chapter 1: Introduction

As there is increasing evidence of the health benefits of dietary carotenoids, it is important to assess their bioaccessibility/bioavailability, which is influenced not only by the intrinsic characteristics of each carotenoid, but also by the food matrix and other dietary components. According to epidemiologic studies, a diet rich in carotenoid-containing foods is associated with a number of health benefits, such as reduced risk of cancer, cardiovascular disease, macular degeneration and cataract formation (Rock 1997; Paiva and Russell 1999; Riedl and others 1999). Dietary fiber is considered to play an important role in human diet and health (Palafox-Carlos and others 2011); however, there is evidence indicating that these complex carbohydrates interact and affect the bioaccessibility and bioavailability of dietary carotenoids (Rock and Swendseid 1992; Riedl and others 1999; Deming and others 2000; Yonekura and Nagao 2009). Intake of soluble fibers, specifically pectin and gums, have been shown to lower carotenoid absorption in humans and animals (Rock and Swendseid 1992; Riedl and others 1999; Deming and others 2000), but it remains unclear how the interactions between fibers and carotenoids lead to the inhibition of carotenoid absorption. The present study aimed to determine the effect of soluble fibers and resistant starch on dietary carotenoid bioaccessibility from raw, homogenized carrot and spinach. Research concerning the bioaccessibility of carotenoids from solid matrices is important, since only the
compounds released from the food matrix and absorbed in the small intestine are potentially bioavailable.

1.2 Statement of Problem

The consumption of fiber and carotenoids occur on a daily basis, sometimes simultaneously in a meal. Therefore, examining the effects of fiber on the bioaccessibility of carotenoids is pertinent. I hypothesized that soluble fibers such as, pectin, FOS, and GOS, as well as fibers with characteristics of soluble fiber such as resistant starch would decrease micellarization carotenoids. To test this hypothesis, the following aims were addressed.

Aim 1: To determine if micellarization of lutein, βC, and αC from spinach and carrot during simulated digestion was decreased when citrus pectin, FOS, GOS, RS2 or RS3 were added to the salad. Fiber content was tested at concentrations of 2% and 4% (wt: wt).

Aim 2: To determine if micellarization of lutein, βC, and αC from the spinach and carrot salad was changed when 4% citrus pectin, 4% FOS, or 4% GOS were added to the more complex food matrix of yogurt and salad.

Aim 3: To determine if micellarization lutein, βC, and αC during simulated digestion of spinach and carrot was altered by the presence of either a high fiber raw green banana or a lower fiber, ripe yellow banana.
Chapter 2: Literature Review

2.1 Carotenoid Background

Carotenoids are lipid-soluble plant pigments found in plants, animals and human subjects consuming plant and animal products. Carotenoids are generally responsible for the yellow, orange and red colors in fruits and vegetables. Carotenoids are isoprenoid compounds with polyene chains that may contain up to 15 conjugated double bonds. They are generally classified as either the hydrocarbon (or apolar) carotenes or the oxy-(or polar) xanthophylls. The more polar carotenoids include lutein, zeaxanthin and beta-cryptoxanthin as they contain one or more oxygen-containing functional groups. Structures of some common carotenoids are shown in Figure 1. Due to the conjugated double bond structure of carotenoids, isomerization to cis-trans isomers can occur. These isomers can be converted by light, thermal energy, or chemical reactions as when the cooking of vegetables promotes isomerization of carotenoids from the trans to the cis forms (Rock 1997). Trans isomers are more common in food and are more stable but very little is known about the biological significance of carotenoid isomerization in human health (Rao and Rao 2007). Carotenoids are sensitive to oxygen, light, acid and high temperatures which can influence the color of foods as well as their nutritional value. Typical household cooking such as steaming and boiling will not greatly alter the carotenoid content of a food, in fact mild heating increases carotenoid bioavailability.
(Deming and Erdman 1999), but excessive heat can result in isomerization and oxidative destruction of carotenoids (Thane and Reddy 1997). These structural changes decreased the vitamin A value of provitamin A carotenoids and can alter the biological properties of the carotenoids (Deming and Erdman 1999).

![Diagram of some major dietary carotenoids](image)

**Figure 1. Structure of some major dietary carotenoids (Rao and Rao 2007).**

More than 700 naturally occurring carotenoids have been identified and roughly 40 carotenoids are present in plant foods consumed by humans (Britton 1995; Rao and Rao 2007). Of those 40 present in a typical human diet, only about 20 carotenoids have been identified in human blood and tissues. The most abundant carotenoids in plasma include α-carotene (αC), beta-carotene (βC), lutein, zeaxanthin and beta-cryptoxanthin, and
relatively high concentrations of lycopene are found in populations that regularly ingest tomatoes and tomato products (Failla and Chitchumroonchokchai 2005).

2.1.1 Health Benefits of Carotenoids
Carotenoids play a major role in human nutrition and health as they are thought to assist the prevention of cardiovascular disease, eye diseases, cancer and other chronic diseases as well as providing provitamin A (Paiva and Russell 1999; Borel 2011). It has been shown that a diet rich in carotenoid-containing foods is associated with a number of health benefits, i.e., reduced risk of cancer, preventing oxidative damage, cardiovascular disease, cataract formation, and serving as precursors for vitamin A (Rock 1997; Paiva and Russell 1999; Rock and others 1998; Borel 2003; Stahl and Sies 2005; Reboul and others 2006; Yonekura and Nagao 2007). Dietary guidelines recommend increased consumption of fruits and vegetables to ward off diseases such as cancer, cardiovascular disease, osteoporosis and diabetes. Carotenoids are either classified as provitamin A or nonprovitamin A compounds. Nonprovitamin A carotenoids include lutein, lycopene and zeaxanthin. Interest in these carotenoids is growing as lycopene is thought to be involved in the prevention of prostate cancer (Giovannucci 1999) and lutein and zeaxanthin are considered to play an essential role in retinal function and have been shown to be inversely related to disorders related to the eye (Bone and others 2000; Landrum and Bone 2001; Ribaya-Mercado and Blumberg 2004). Most research involving the anticancer effects of lycopene has centered around prostate cancer but more evidence suggests that lycopene may help prevent other types of cancer as well such as breast, cervical, ovarian and liver (Rao and Rao 2007).
Provitamin A compounds can be enzymatically cleaved to yield either one (beta-cryptoxanthin and alpha-carotene) or two (βC) molecules of retinal. Retinal in turn, further reacts to form retinol and retinoic acid. Beta-carotene is the most potent source of vitamin A in the diet. The Institute of Medicine has defined the retinol equivalency ratio of b-carotene from a mixed diet to be 12 mg b-carotene to 1 mg of retinol (12:1) and of other provitamin A carotenoids to be 24:1 (Arscott and others 2010). Vitamin A is essential to maintain normal bodily functions and because humans cannot synthesize vitamin A, the body is dependent on dietary intake of it. Despite the recognition of the beneficial effects carotenoids can provide for human health, they are not considered essential nutrients and do not have a dietary reference intake (DRI) value assigned to them.

Carotenoids not only contribute to dietary vitamin A, but also act as potent antioxidants in our body. Fruits and vegetables are good sources of antioxidant phytochemicals which help to decrease the negative effects of oxidative stress. Antioxidant properties of carotenoids have been associated with cellular protection (Miller and others 1996; Biesalski and others 1997; Paiva and Russell 1999; Mayne 2003). The nonprovitamin A carotenoid, lycopene, has been shown to convey anticancer properties due to its unsaturated structure which allows it to be a potent antioxidant by trapping reactive oxygen species.

2.1.2 Vitamin A Deficiency

Vitamin A deficiency can lead to a number of symptoms and even death, particularly in women and children in developing countries. Vitamin A deficiency affects more than
100 million children and as many as 7 million pregnant women residing in more than 100 countries (World Health Organization 2009). A cycle of vitamin A deficiency disorders (VADD) exists especially in developing countries where diets are based mainly on carbohydrate-rich, micronutrient-poor food sources. Adults rely on leaves and fruits as vitamin A sources and typically do not consume the recommended amount, therefore maintaining a low pre-formed vitamin A status. This, in combination with a low-fat diet and a low-intake of vitamin E, which affects vitamin A status, results in a vitamin A deficient state. Once these deficient adults become pregnant, vitamin A needs are increased, but due to local food taboos, lack of availability and so on, low vitamin A fetal stores result. Once the baby is born, increased vitamin A needs are increased as growth continues. Breast feeding is often the recommended choice over bottle formula feeding but the mothers’ concentration of vitamin A is already so low, the vitamin A concentration in breast milk remains low, providing little to no vitamin A to the baby. This cycle of vitamin A deficiency disorders can result in serious and lethal effects (Miller and others 2002). Deficiency can result in loss of vision, altered immune function, altered embryogenesis, squamous cell metaplasia, anemia and death (Wilson and others 1953; Smith and others 1998; Zile 1998; Blomhoff and Blomhoff 2006). To control vitamin A deficiency disorders measures must be taken to encourage oral supplementation and food fortification along with the diversification of foods, controlling infectious diseases, breeding plants to contain high levels of vitamin A, and ensuring high levels of vitamin A in disaster relief kits.
2.1.3 Carotenoid and Vitamin A Sources

Dietary sources of vitamin A stem from provitamin A carotenoids in plant food and preformed vitamin A (retinol) in animal products. Vitamin A is also available from vitamin A fortified foods and pharmaceutical supplements. Some common sources of vitamin A include eggs, milk, dark-green leafy vegetables, orange and yellow fruits and vegetables, such as papaya, mango, pumpkin, squash, carrot and orange sweet potatoes (Engleberger and others 2003). Although animal foods are a good source of vitamin A, many populations do not consume eggs, milk and liver for a variety of reasons including religious beliefs, cost, availability, lactose intolerance, food allergens and preference (Engleberger and others 2003).

Fruits and vegetables constitute the major sources of carotenoids in the human diet (Rao and Rao 2007). Beta-carotene, a specific provitamin A carotenoid, can be found in a variety of fruits and vegetables but some examples are carrots, apricots, collards, sweet potatoes and cantaloupe. Another provitamin A carotenoid, alpha-carotene, can be found in many foods that contain βC such as carrots, sweet potatoes, winter squash and pumpkin. The last provitamin A carotenoid, beta-cryptoxanthin, can be found in red bell peppers, tangerines, papaya and corn. The major nonprovitamin A carotenoids consist of lutein, lycopene and zeaxanthin. Lutein is commonly found in broccoli, yellow corn, squash and leafy green vegetables such as kale, spinach and collard greens. Lycopene can be found in tomatoes, pink guava, pink grapefruit and watermelon. Zeaxanthin is often found in foods high in lutein, such as collard greens, kale, spinach and turnip greens (Rao and Rao 2007).
2.1.4 Absorption of Dietary Carotenoids

Carotenoids, retinyl esters and other fat soluble compounds are all treated in the same manner during digestion. Carotenoids are released from the food matrix during digestion. Hydrochloric acid, pepsin and gastric lipase are secreted into the gastric lumen, partially releasing the carotenoids from the food matrix into emulsified lipid droplets in the chyme. The chyme enters the small intestine and is exposed to pancreatic enzymes and bile, which are essential in the micellarization of carotenoids (Harrison and Hussain 2001). These water soluble micelles made up of bile salts, monoacylglycerides, phospholipids, cholesterol and other fat soluble compounds are key to the digestion and absorption of carotenoids. Apolar, hydrocarbon carotenoids like βC reside in the core of the droplet while polar carotenoids are distributed on the surface of micelles (Borel 1996, 2003). These mixed micelles diffuse across the unstirred water layer and deliver carotenoids to the apical surface of the mucosal epithelium. Once inside the enterocyte, βC can be converted to vitamin A.

Carotenoids and newly synthesized retinyl esters are incorporated into large, water soluble lipoproteins known as chylomicra for secretion into lymph for the transfer of lipids throughout the body (Parker 1996). Chylomicra are transported from the lymph to the plasma where triglycerides undergo rapid lipolysis by lipoprotein lipase to produce chylomicron remnants. In the liver, carotenoids can be utilized, stored or re-secreted into plasma in very low density lipoproteins (VLDL) and high density lipoproteins (HDL) (Failla and Chitchumroonchokchai 2005). The quantity of ingested carotenoid absorbed is dependent on its partitioning in mixed micelles, delivery to enterocytes, and
incorporation into chylomicrons (Furr and Clark 1997; Failla and Chitchumroonchokchai 2005).

2.1.5 Carotenoid Bioavailability

The bioavailability of nutrients is less defined than the bioavailability of pharmaceuticals; however, the concept remains similar. Essentially, bioavailability is the degree to which a nutrient is absorbed after consumption and made available to its intended target tissues for normal bodily functions. The bioavailability of a food depends on numerous factors such as the food matrix, nutritional status of the host, the mechanical processing used, if any, to break the food down, gut health and the effect of enzymes within the gut, interactions with other nutrients and drugs, and inherent variation between individuals. The bioavailability of carotenoids is generally affected by the stability of the ingested compound during digestion, delivery of the compound or its active metabolite(s) to absorptive epithelial cells lining the gastrointestinal tract, uptake across the apical surface of intestinal epithelial cells, possible metabolism within epithelial cells, and transport of the ingested compound and its active metabolite(s) across the basolateral membrane for distribution to target tissues (Failla and Chitchumroonchokchai 2005).

The first factor affecting bioavailability is the amount ingested; the greater the intake of carotenoids, the higher the plasma concentration of carotenoids. The food source, matrix and processing can greatly affect the bioavailability of carotenoids. The size of the particle, whether the material is raw or processed, and if the material is incorporated into a food or a supplement can all affect the carotenoid’s outcome. Dark green leafy vegetables were previously considered a good source of vitamin A but recent research
indicates that they do not contribute to vitamin A status as much as previously thought (de Pee 1995). The provitamin A carotenoids bind to chloroplast in green vegetables, making it much more difficult for the carotenoids to become available. Cooking/processing can help break down the food matrix but cooking too long will result in degradation. It has been shown that food processing and heating can improve alpha-and beta-carotene bioavailability from a commercial carrot puree baby food than from boiled-mashed carrots (Edwards and others 2002). Spinach βC bioavailability increased when the vegetable matrix was disrupted (Van het Hof and others 1999) and was found to be more available from liquefied spinach than from whole leaves (Castenmiller and others 1999). Alpha-carotene was more available in juice than in raw or cooked vegetables (McEligot and others 1999). It was shown that lycopene was more bioavailable from tomato paste than from fresh tomatoes, as processing broke down the cell walls making the lycopene more accessible (Gartner and others 1997). Another example when food processing was beneficial to carotenoid bioavailability was when a study established that alpha- and beta-carotene’s bioaccessibility was improved when raw carrots were homogenized and further enhanced when they were cooked (Hedren and others 2002). Dietary fat is an important component in increasing the bioavailability of carotenoids by providing an area where hydrophobic compounds can gather once released from the food matrix, which then can stimulate the secretion of bile salts and pancreatic lipases required for micellarization and inducing chylomicron synthesis (Borel 2003). Fat is essential for intestinal absorption of carotenoids as shown in previous in vivo studies (Prince and others 1993; Jalal and others 1998; Roodenburg and others 2000). Approximately 5-10
grams of fat in a meal is required for efficient absorption of carotenoids (Jayarajan and others 1980) but the type of fat can also affect carotenoid absorption *in vitro* (Borel 1996).

The transfer of carotenoids to a micelle depends on carotenoid hydrophobicity, pH, bile concentration, and carotenoid interactions (Tyssandier and others 2001). The bioavailability of lutein esters is not significantly different from that of free lutein (Bowen and others 2002). There is no apparent significant difference between alpha- and beta-carotene with regard to their bioavailability (Borel 2003). Cis isomers of βC are more readily solubilized in micelles than trans isomers (Tyssandier and others 2003), but are less efficiently absorbed than trans isomers (Ben-Amotz and Levy 1996; During and others 2002). Lycopene is less efficiently transferred into micelles than βC and lutein (Tyssandier and others 2003). Interactions between carotenoids themselves may cause positive or negative effects of carotenoid bioavailability (Tyssandier and others 2001). Beta-carotene and lutein have been seen to increase or decrease each other in human subjects while βC absorption was not affected by lycopene (Kostic and others 1995; van den Berg and van Vliet 1998). These interactions may be due to their competition for incorporation into micelles, uptake from the micelle by intestinal cells, competitive binding to beta-carotene oxygenase 1 (BCO1), and/or incorporation into chylomicra (van den Berg 1999).

2.1.6 Substances that Influence Uptake of Carotenoids

There has been research showing a variety of substances that positively and negatively affect the uptake of carotenoids. Besides fat intake and efficiency of extraction from the
food matrix, the amount and type of dietary fiber in the diet seems to determine
carotenoid bioavailability; however, extensive research is lacking (Parker 1996; Rock
1997; Borel 2003). Hemicellulose, lignin, and pectin all decreased βC utilization by
chicks when 7% of their diet was a purified fiber source (Erdman and others 1986).
These investigators also found that high methoxy apple pectin, medium methoxy apple
pectin, citrus pectin, and polygalacturonic acid all decreased storage of vitamin A in the
liver of chicks when their diets contained 7% fiber (Erdman and others 1986). It also was
reported that addition of 12 g of citrus pectin to a 500 kcal meal containing 25 mg βC
significantly reduced plasma βC 30 hours after feeding the meal to human subjects (Rock
and Swendseid 1992). Similarly, the bioavailability of lutein, βC and lycopene was
decreased in blood plasma in women fed chocolate rice pudding containing either citrus
pectin, guar gum, alginate, cellulose or wheat bran (0.15 g fiber per kg body weight)
(Riedl and others 1999). Water soluble types of dietary fiber had a stronger effect on
relative bioavailability in vivo as compared to water insoluble dietary fibers; however, an
effect was seen using insoluble fibers such as cellulose and wheat bran (Riedl and others
1999). It was further investigated whether the type of soluble fiber affected the
postabsorptive conversion of βC to vitamin A in Mongolian gerbils when their diets
contained 7% silica, pectin, or oat gum (Deming and others 2000). It was shown that
citrus pectin adversely affected the conversion of βC to vitamin A, as the hepatic vitamin
A stores were decreased while the hepatic βC stores were increased. In contrast, oat gum
resulted in significantly higher hepatic vitamin A stores and lower hepatic βC stores
compared to citrus pectin-fed stores, suggesting that oat gum does not adversely affect βC
bioavailability (Deming and others 2000). The only in vitro study related to carotenoid bioavailability in combination with fiber, showed that medium- and high-viscosity alginates and citrus and apple pectins at 200 nmol/ L inhibited micellarization of purified lutein and βC solubilized in soybean oil and saline (Yonekura and Nagao 2009). These investigators also found that these alginates and pectins inhibited Caco-2 cellular uptake of lutein and βC. Finally, lutein levels in blood plasma, liver tissue and eye tissue decreased when 200 µM lutein was administered to male adult albino Wistar lutein-deficient rats in combination with 1.25% pectin in a mixed micelle solution containing phosphate buffered saline, mono-oleoyl-glycerol, oleic acid, sodium taurocholate, cholesterol, phospholipids, and soybean oil (Mamatha and Baskaran 2011).

The decrease in carotenoid bioavailability and bioaccessibility can be explained in a variety of ways. Decreased micellarization may be due to binding of bile acids and phospholipids, inhibition of lipase activity, increased viscosity and volume of luminal contents, and increased rate of transit of enterocytes along the villus, and/or entrapment of the carotenoids (Gallaher and Schneeman 1986; Riedl and others 1999; O’Connell and others 2008; Gropper and others 2009). It has been shown that guar gum, lignin and cholestyramine are strong bile acid binding agents in vivo (Gallaher and Schneeman 1986). Cholestyramine, a bile acid sequestrant, was used as a positive control and successfully bound bile acids and phospholipids, thereby decreasing lipid solubilization (Gallaher and Schneeman 1986). Additionally, fecal bile acid secretion was increased with guar gum (Vahouny and others 1980). Previous research showed that while cellulose, wheat bran and oat bran had binding activity to bile acids, the activity was
lower in comparison to soluble fibers (Story and Kritchevsky 1976; Vahouny and others 1980; Gallaher and Schneeman 1986). As the bile acids remain bound to fibers in the cecum of the large intestine, decreased cecal bile acid reabsorption occurs. With a decrease of bile acids, more cholesterol would have to be catabolized to bile acids to maintain the bile acid pool. If cholesterol synthesis was not equal to the increased rate of catabolism, then the cholesterol pool would be reduced. Consequently, the amount of lipid, including cholesterol, solubilized within the intestine could be decreased, which could lead to reduced lipid absorption (Gallaher and Schneeman 1986). In addition to binding bile acids and phospholipids (Vahouny and others 1980; Gallaher and Schneeman 1986), guar gum, and supposedly other soluble viscous fibers, reduces phospholipid intestine concentration by increasing chyme volume as the water soluble compound forms a gel, slowing the mobility of compounds in the aqueous fraction (Gallaher and Schneeman 1986). Phospholipids are an important component of mixed micelles, which in turn, are essential for the uptake of carotenoids.

Dietary fiber may affect pancreatic lipase, which is involved in micelle formation (Redard and others 1990; O’Connell and others 2008). Dietary fiber changes the morphology of the small intestine through cell renewal (Jacobs 1983; Sigleo and others 1984; Sakata 1987). Some carotenoids may be held in the enterocytes and released in response to subsequent meals (Furr and Clark 1997). If dietary fiber is changing the small intestine morphology by increasing the rate of enterocyte turnover, then the carotenoids held in the enterocytes may be flushed out of the small intestine with shedding cells.
Carotenoids and cholesterol may share the same facilitated transport route from the mixed micelles into the epithelial cells (van Bennekum and others 2005). Cholesterol could be another factor involved in the absorption of carotenoids, interacting during the cellular uptake and during micellarization (Fernandez-Garcia and others 2007). Very few studies have been published examining the increase on bioavailability or bioaccessibility of carotenoids. One previous study showed that retinyl palmitate absorption was enhanced after 4 months of consuming soluble fiber stemming from whole foods in the diet such as lentils, beans, and oat bran (Wolever and others 1997). The investigators explained the increased absorption by an increased bile acid pool and increased micellarization due to the chronic fiber-rich diet. In a recent study, it was shown that specific food acidulants (lime juice) and antioxidant spices (turmeric/onion), increased the bioaccessibility of βC (Veda and others 2008).

2.2 Carotenoid Methods of Analysis
High-performance liquid chromatography (HPLC) is used in analytical research to separate and identify specific compounds of interest (Nikolin and others 2004). Normal phase HPLC uses nonpolar organic solvents (hexane) while reverse phase HPLC uses polar organic solvents (methanol). The mobile phase solvents are forced through high-pressure pumps onto the column. The HPLC column is the stationary phase which is made up of porous silica with specific ligands than can interact with the sample being tested. Competition between the ligand on the stationary phase and the mobile phase determines the extent of column retention. In normal phase HPLC, nonpolar molecules elute faster than more polar compounds as a nonpolar organic solvent is used for the
mobile phase. In reverse phase HPLC, polar molecules elute faster than nonpolar compounds as polar organic solvents are used. This project used reverse phase HPLC to identify and quantify carotenoids. Known concentrations of pure lutein, α-carotene and β-carotene were used to develop a standard curve to quantify the unknown samples.

2.3 Overview of Human Digestive System

Humans consume foods that contain macro and micronutrients that are essential to the growth, maintenance and repair of the human body. These nutrients are present within the food’s matrix so the food must undergo digestion before the nutrients can be utilized. The human digestive system involves the gastrointestinal tract as well as certain accessory organs. The gastrointestinal tract, or gut, is divided into the upper and lower gastrointestinal tract. The upper gastrointestinal tract includes the oral cavity, pharynx, esophagus and stomach. The lower gastrointestinal tract includes the small intestine and large intestine (cecum, ascending colon, transverse colon, descending colon, sigmoid colon and rectum). The salivary glands, liver, gall bladder and the pancreas are all accessory organs to the gastrointestinal tract which are necessary in the synthesis and storage of bile as well as the secretion of digestive enzymes (Gropper and others 2009). The digestive process begins in the oral cavity, including the mouth and pharynx which provides an entryway to the digestive tract. The teeth, jaw muscles and tongue mechanically break down, moisten, and mix the food with saliva. Three pairs of salivary glands, the parotid, sublingual and submandibular, release water, electrolytes, mucus and enzymes. The main enzyme in saliva is alpha-amylase, which hydrolyzes α1-4 bonds in starch. The pharynx propels the food from the back of the oral cavity into the esophagus.
The food and saliva, termed a bolus, travels into the esophagus which transports the food from the pharynx into the stomach via peristalsis, a wavelike muscle motion that moves the bolus. The bolus enters the stomach and is mixed with gastric juices containing acid and enzymes, chemically and physically breaking the food down, forming a thick semiliquid chyme. The main constituents of gastric juice are hydrochloric acid, enzymes and mucus. The hydrochloric acid is responsible for the low pH of about 2. Hydrochloric acid converts pepsinogen to pepsin, denatures proteins, releases nutrients from matrices, and kills many bacteria ingested along with the food. Four enzymes are found in gastric juice, salivary alpha-amylase, lingual lipase, pepsin and gastric lipase. The main enzyme, pepsin, functions as the principle proteolytic enzyme in the stomach. Pepsinogen can be converted to pepsin in an acidic environment (pH less than 5) or in the presence of previously formed pepsin. Pepsin, an endopeptidase, hydrolyzes the interior peptide bonds within proteins and performs optimally at pH 3.5. Salivary alpha-amylase is only active for a short time in the stomach as it is eventually inactivated by the very acidic pH of the gastric juice. Gastric lipase hydrolyzes short- and medium-chain triacylglycerols and is thought to perform about 20% of lipid digestion in humans. Mucus is also found in the gastric juice. Mucus, composed of mucin, glycolipids, water and bicarbonate ions (HCO₃⁻), lubricates and protects the gastric mucosa from chemical and mechanical damage. Overall, very little chemical digestion of nutrients occurs in the stomach. The stomach can only absorb water, alcohol and a few fat-soluble drugs, such as aspirin, and a few minerals. When food is swallowed, the stomach relaxes then contracts to begin the gastric emptying into the small intestine which can take between 2
and 6 hours following a meal. These contractions liquefy the food into chyme, although complete liquefaction is not necessary for gastric emptying. Gastric emptying is influenced by several factors including hormones, peptides, volume of the chyme, osmolarity of the chyme, carbohydrate concentration, protein concentration, fat concentration, salt concentration, and soluble fiber. High fat foods, salt and soluble fiber all slow gastric emptying into the small intestine (Gropper and others 2009).

The small intestine, which includes the duodenum, jejunum, and ileum, is the main site for nutrient digestion and absorption. The epithelial lining, or mucosa, of the small intestine has a large surface area to maximize the absorption of nutrients. Digestion and absorption of nutrients within the small intestine are rapid; typically nutrients are absorbed within 30 minutes after chyme has reached the small intestine. On the mucosa reside finger-like projections called villi, which emerge from crypts of Lieberkuhn out into the lumen of the intestine. Each villus is made of enterocytes, also known as absorptive epithelial cells. Enterocytes are the principle cells involved in the absorption and metabolism of nutrients. Nutrients are absorbed into enterocytes by diffusion, facilitated diffusion, active transport or endocytosis. The microvilli, hair-like extensions of the plasma membrane of the enterocytes that make up the villa, possess a surface coat, the glycocalyx. The microvilli and glycocalyx make up the brush border of the enterocytes. Covering the brush border membrane is an unstirred water layer that lies between the brush border and the intestinal lumen. Digestion of nutrients is typically completed on the brush border but it may be completed within the cytoplasm of the enterocytes. Chyme is mixed with digestive juices in the small intestine through
contractions of smooth muscles. The pancreatic secretions are rich in bicarbonate, which neutralizes the acid from the stomach (Gropper and others 2009).

The small pits, termed crypts of Lieberkuhn, lie between the villi. Epithelial cells in the crypts of Lieberkuhn, intestinal stem cells, constantly undergo mitosis. The intestinal stem cells give rise to transit cells that are pushed upwards and out of the crypt towards the tips of the villi. At the tip of the villi, these former crypt cells differentiate into epithelial cells, absorptive enterocytes, goblet cells or enteroendocrine cells. These cells eventually are sloughed off into the intestinal lumen and excreted in the feces as cell turnover is fairly rapid, approximately every 3 to 5 days (Gropper and others 2009). Paneth cells are specialized immune cells located in intestinal crypts that protect the intestinal mucosa from harmful bacteria and regulate gut flora populations through secretion of antimicrobial peptides known as defensins. Paneth cells also secrete enterochromaffin mast cells with endocrine functions and goblet cells that secrete proteins with antifungal activity and mucus. Mucus adheres to the mucosa and acts as a barrier against the acidic chyme. In addition to lubricating the mucosal cell surface, mucus facilitates the movement of chyme (Gropper and others 2009).

Immune system cells and lymphoid tissue protects the digestive tract. The lymphoid tissue, found primarily in the mucosa is called mucosa-associate lymphoid tissue (MALT) or gut-associate lymphoid tissue (GALT), if found in the non-mucosal layer. Both MALT and GALT are made up of multiple cells including T- and B-lymphocytes, plasma cells, natural killer (NK) cells, and macrophages. Macrophages destroy foreign antigens and stimulate lymphocytes to destroy antigens. Leukocytes are located between
enterocytes and make up 15% of the epithelial mucosa. The majority of the body’s plasma cells are found in the lamina propria. Plasma cells produce IgA which binds antigens ingested with foods and inhibits the growth of harmful bacteria. In addition to these cells, microfold (M) cells cover Peyer patches. These Peyer patches, aggregates of lymphoid tissue, and m cells are part of the gut immune system. M cells transport foreign antigens to MALT lymphocytes which can then start an immune response (Gropper and others 2009).

The accessory organs, the pancreas, liver and gallbladder are all essential to the digestive and absorptive processes. The pancreas contains two types of active cells: ductless endocrine cells that secrete insulin and glucagon into the blood and acinar exocrine cells that produce digestive enzymes and pancreatic juice. Pancreatic juice is composed of bicarbonate, electrolytes and enzymes. Bicarbonate is important for neutralizing the acidic chyme from the stomach and for maximizing enzyme activity within the small intestine. The pancreatic juice containing digestive enzymes are related from the bile pancreatic duct which empties into the duodenum. Enterendocrine cells secrete secretin in response to the release of acid chyme into the duodenum. Secretin stimulates the pancreas to secrete water, bicarbonate and pancreatic enzymes. These enzymes digest approximately half of all ingested carbohydrates, half of all proteins and almost all ingested fat. Secreted enzymes include alpha-amylase, pancreatic lipase, colipase, trypsinogen, chymotrypsinogen, procarboxypeptidase, proelastase, and collagenase. Enteropeptidase hydrolyzes trypsinogen to trypsin which in turn converts chymotrypsinogen to chymotrypsin. Trypsin, chymotrypsin, and other peptidases convert
proteins into peptides and amino acids which are transported across the apical surface of enterocytes (Gropper and others 2009).

The liver is made up of two lobes which contain lobules which are made up of hepatocytes. Hepatocytes synthesize bile acids from cholesterol. The main bile acids are cholic acid and chenodeoxycholic acid. The portal vein in the liver takes nutrient-rich blood away from the digestive tract and pancreas to the liver. The common hepatic duct joins the cystic duct from the gallbladder to form the common hepatic bile duct (Gropper and others 2009).

The gallbladder is located on the surface of the liver and its function is to concentrate and store the bile made in the liver until needed for fat digestion in the small intestine. Bile is made up of bile acids, salts, cholesterol and phospholipids. In addition, water, electrolytes, bicarbonate and conjugated bile pigments are secreted into bile by hepatocytes. The gallbladder concentrates the bile to allow more to be stored. Bile acts as a detergent to emulsify lipids by facilitating the formation of mixed micelles. Micelles are essential for the delivery of lipids to the brush border surface of enterocytes. When exhausted of lipid, the bile secreted into the duodenum is reabsorbed in the ileum. This circulation of bile is referred to as enterohepatic recycling or circulation. Half the cholesterol contained within the bile is taken up by the jejunum and used to form chylomicrons while the other half is excreted. Chylomicrons are secreted into the lymphatic system into systemic circulation. Lipoprotein lipase releases fatty acids from chylomicrons and once exhausted of lipids, chylomicron remnants are targeted to the liver and packaged into very low-density lipoprotein (VLDL) and low-density lipoprotein
(LDL). Certain dietary fibers present in the gut may bind to bile, making it unavailable for micelle formation (Vahouny and others 1980; Gallaher and Schneeman 1986; Gropper and others 2009).

Unabsorbed intestinal contents are passed from the ileum into the cecum of the colon. The contents move from the cecum to the ascending colon, transverse colon, descending colon, sigmoid colon and finally the rectum. Goblet cells in the crypts of Lieberkuhn secrete mucus that protects colonic mucosa and acts as a lubricant for fecal matter. The colon absorbs 90% to 95% of the water and sodium entering the colon each day. This absorption and passage of material through the colon, which usually takes about 12 to 70 hours, progressively dehydrates the material. If 1 L of chyme enters the large intestine, each day that amount is reduced to less than about 200 grams of defecated material containing sloughed gastrointestinal cells, inorganic matter, water, unabsorbed nutrients and food residue, constituents of digestive juices, and bacteria. The large intestinal microflora ranges from bacteroides, lactobacilli, clostridia, bifidobacteria, methanogens, eubacteria, and streptococci. These bacterial populations use the unabsorbed carbohydrates, and to a lesser extent, amino acids and undigested protein, as substrates for their growth and maintenance. This anaerobic breakdown of carbohydrates and protein by bacteria is known as fermentation. Various short chain fatty acids, such as lactate, acetate, butyrate and propionate, are generated from the fermentation in the large intestine. Short chain fatty acids (SCFA) serve a number of purposes in the body. They are thought to stimulate gastrointestinal cell proliferation while serving as substrates for bacteria. Butyrate may regulate gene expression and cell growth. Propionate and lactate
are absorbed in the colon and taken up for use by liver cells. Acetate is absorbed and used by muscle and brain cells. In addition to SCFA, bacteria also generate methane (CH4), hydrogen (H2), hydrogen sulfide (H2S), and carbon dioxide (CO2). These gases can be used by bacteria in the colon and if they are not used, they are excreted (Gropper and others 2009).

2.4 Starch Background

Starch is the main carbohydrate in human nutrition and is a major component in plant foods. It is the most common digestible polysaccharide in plants. Starches are polysaccharides made up of monosaccharides or glucose molecules linked together by α-D-(1-4) and/or α-D-(1-6) linkages. Starch can exist in two forms, amylose and amylopectin. Amylose is a linear or slightly branched polymer in which glucose residues are attached by α-D-(1-4) glycosidic bonds. Generally, starches high in amylose are more resistant starches. Amylopectin is a highly branched molecule with α-D-(1-4) and α-D-(1-6) linkages. High amylopectin waxy starches are typically rapidly digestible starch which means they have little to no resistance. Both amylose and amylopectin can be found in grains, potatoes, legumes, and other vegetables. Amylose contributes about 15% to 20% of total starch content while amylopectin contributes 80% to 85% of total starch content (Gropper and others 2009).

Two crystalline structures of starch exist: an ‘A’ type and ‘B’ type, which contain different amounts of amylopectin. ‘A’ type starches are found in cereals, while B type starches are found in tubers and amylose-rich starches. A third type called ‘C type’ is a mixture of both A and B and is found in legumes (Nugent 2005). The nutritional quality
of starch depends on the state of the starch and its processing. The digestion of starch within the human body can range from rapid digestion to slow digestion as is the case with resistant starch. Rapidly digestible starch (RDS) is mainly made up of amorphous and dispersed starch found in high amounts in starchy foods cooked by moist heat such as bread and potatoes (Sajilata and others 2006). This starch is completely digested in the small intestine with a rapid increase in blood glucose levels (Lehmann and Robin 2007). Slowly digestible starch (SDS) consists of physically inaccessible amorphous starch and raw starch with a type A and type C crystalline structure such as cereals and type B starch (Sajilata and others 2006). This starch, like RDS, is also completely digested in the small intestine although at a slower rate. The ratio of amylose to amylopectin in a starch product will determine its digestibility and physiological response (Lehmann and Robin 2007). Digestible starches are hydrolyzed by enzymes in the small intestine to yield glucose while nondigestible starches are resistant to small intestinal enzymes and are passed into the large intestine where they are fermented by bacteria.

The digestion of starch begins in the mouth with salivary α-amylase which hydrolyzes α 1-4 linkages in amylose and amylopectin, forming dextrins, short-chain polysaccharides. The β 1-4 bonds of cellulose and lactose as well as α 1-6 bonds of amylopectin are resistant to this enzyme. Salivary amylase action continues in the stomach until gastric acid lowers the pH and inactivates the enzyme. Dextrins are further digested in the small intestine when pancreatic α-amylase is secreted. Dextrins stemming from amylose and amylopectin are broken down into maltose but the α 1-6 bonds in amylopectin still cannot be hydrolyzed so isomaltose, a disaccharide with α 1-6 bonds, is released. On the brush
border of the small intestine, maltase, a brush border enzyme, hydrolyzes the maltose from amylose and amylopectin, forming free glucose. The remaining limit dextrins with \( \alpha 1-6 \) linkages are hydrolyzed by \( \alpha \)-dextrinase, forming glucose (Gropper and others 2009).

2.5 Fiber Background and Classification

Fiber was recognized as an important food component in the mid-1970’s as research found that dietary fiber is important for gastrointestinal tract function and for preventing and managing a variety of diseases (Topping and Clifton 2001; Anderson and others 2009). Dietary fiber refers to nondigestible (by human digestive enzymes) carbohydrates and lignin that are intact and intrinsic in plants (Food and Nutrition Board 2002). Functional fiber consists of nondigestible carbohydrates that have been isolated, extracted, or manufactured and have been shown to have beneficial physiological effects in humans (Food and Nutrition Board 2002). Some examples of both dietary and functional fibers are cellulose, pectin, lignin, gums, \( \beta \)-glucans, fructans, and resistant starches. An example of a functional fiber that is not considered to be a dietary fiber is chitin, found in the exoskeletons of crustaceans. The plant species, part of the plant (leaf, root, stem), and the plant’s maturity all influence the composition of the fiber that is consumed (Gropper and others 2009). There are 3 types of fiber overall: soluble fiber, insoluble fiber and resistant starch. Fibers that dissolve in hot water are soluble, and those that do not dissolve in hot water are insoluble. Soluble fiber is viscous and is fermented by bacteria in the large intestine. Examples include pectin, fruits and gums. Insoluble fiber promotes regularity, absorbs water, and acts as a bulking agent. Some
examples include cellulose, whole grains, lignin, bran, and flax seeds. Resistant starch has characteristics of both soluble and insoluble fiber. It is starch not absorbed in the small intestine, like soluble fiber. It also acts as a bulking agent such as insoluble fiber. Resistant starch can be found in a variety of foods including legumes and cooked and cooled starchy foods (Nugent 2005; Gropper and others 2009).

The health benefits, physiological and metabolic effects of soluble fiber are vast. Soluble fiber can bind nutrients and substances such as minerals, bile acids and lipids which can result in altered mineral balance, increased fecal bile acids and decreased lipid absorption, respectively. It has been shown that soluble fermentable fibers such as FOS and GOS can increase magnesium absorption in animals and humans (Younes and others 1996; Coudray and others 2003). Soluble fiber, such as pectin, gums, and β-glucans, can affect lipid absorption by binding or interacting with fatty acids, cholesterol, and bile acids. Fatty acids and cholesterol that are complexed to fiber cannot form micelles and negatively affects the micellarization of lipid soluble components (Phillips 1986; Anderson and others 2009; Gropper and others 2009). Additionally, when fiber binds to bile acids, these bile acids cannot be reabsorbed and recirculated in the process called enterohepatic recirculation (Gropper and others 2009). Fiber bound lipids and bile acids are not absorbed in the small intestine and are passed into the large intestine where they are degraded by bacteria or excreted.

Some fibers have been shown to lower serum cholesterol. This may occur when fiber binds to bile acids and cholesterol, causing excretion of both bile acids and cholesterol in the feces to increase. When this occurs, less bile undergoes enterohepatic recirculation,
which means cholesterol must be used for synthesis of new bile acids. As cholesterol is excreted in the feces, decreased cholesterol content occurs in liver cells. This promotes removal of LDL cholesterol from the blood and overall lower serum cholesterol. Studies have shown that gums, resistant dextrins, β-glucan and oat products, and pectin can lower serum cholesterol concentrations (Aro and others 1984; Yamashita and others 1984; Anderson and others 1994; Blake and others 1997; Brown and others 1999). In addition to lowering serum cholesterol concentrations, it has been shown that guar gum, β-glucan and oat products, and inulin improve blood lipid concentrations (Landin and others 1992; Groop and others 1993; Hallfrisch and others 1995; Jackson and others 1999).

Soluble fibers also cause gel formation which can decrease nutrient absorption, increase transit time by increasing chyme volume and viscosity, and decrease gastric emptying (Phillips 1986; Anderson and others 2009). The water-holding capacity or ability to bind water, of soluble fibers is typically high. When these soluble fibers bind water and create viscous solutions within the body several activities are decreased. There is less mixing of the gastrointestinal contents with digestive enzymes, reduced enzyme function, and decreased nutrient diffusion rate. Whether fiber directly decreases the activity of these digestive enzymes or acts by reducing the rate of enzyme penetration into the food is unclear. This can lead to both positive and negative effects. On the negative side, enzyme function is reduced and availability of digestive enzymes is decreased as the viscous gels impair nutrients from interacting with digestive enzymes. In addition, a decreased nutrient diffusion rate can prevent nutrients from being absorbed at their normal site. This can occur if the nutrient becomes trapped in the gel and then is later
released at a distal site. On the positive side, a decreased nutrient diffusion rate can attenuate the blood glucose response, which can be beneficial to people with diabetes by reducing postprandial blood glucose concentrations and insulin response. A delayed gastric emptying can also create a feeling of satiety after eating which would help in the area of weight loss. This decreased diffusion rate of nutrients is thought to be caused by increasing the viscosity and thickness of the unstirred water layer. Although soluble fibers are most known for causing gel formation and therefore delaying gastric emptying and increasing transit time, some soluble fibers can bind water and actually decrease transit time increasing frequency of defecation.

Soluble fibers are fermented in the large intestine where they promote and maintain the growth of good bacteria in the colon. When degraded by bacteria in the large intestine, short chain fatty acids are generated, such as lactate, acetate, butyrate and propionate, which acidify the lumen of the colon and increase colonic sodium and water absorption. There short chain fatty acids produced provide energy for the colonic cells, assist in normal large bowel function, stimulate gastrointestinal cell proliferation, have been shown to inhibit tumor formation and stimulate mucosal cell proliferation (Topping and Clifton 2001; Gropper and others 2009). Soluble fiber has also been shown to create morphology changes in the gut (Sigleo and others 1984).

More research has been completed on the health benefits of soluble fiber but insoluble fiber does offer health benefits as well as positive physiological effects. Insoluble fiber binds water and increases water holding capacity which causes increased fecal volume and decreased transit time leading to increased frequency of defecation. Because of this,
it is said that insoluble fiber promotes regular bowel movement by serving as a bulking agent. Although decreased transit time leads to regularity, this may also result in decreased nutrient absorption. If transit time is shortened, nutrients are in contact with enterocytes for a shorter period of time than usual. Insoluble fiber, such as cellulose and hemicellulose, can be fermented in the large intestine like soluble fiber but to a lesser extent. The fermentation of cellulose and hemicellulose is much slower than soluble fibers and resistant starches. Insoluble fiber, like soluble fiber, can affect lipid absorption by binding or interacting with fatty acids, cholesterol, and bile acids. Insoluble fibers such as lignin and chitosan can portray these properties typical of soluble fibers (Gropper and others 2009).

2.5.1 Pectin Background

Pectin is both a dietary fiber and a functional fiber. Pectins represent a complex group of naturally occurring polysaccharides which vary in methyl ester content. Pectin is rich in galacturonic acid which makes up its backbone structure as an unbranched chain of α 1-4-linked D-galacturonic acid units (Figure 2). In nature, 80% of carboxyl groups of galacturonic acid are esterified with methanol. Pectins are divided into two major groups based on their degree of esterification: high methoxyl and low methoxyl. The association of pectin chains leads to the formation of the three-dimensional networks that cause gel formation. They are water soluble and gel-forming. Pectins are very effective prebiotics and are almost completely fermented by colonic bacteria. Pectin can be found in apples, strawberries and citrus fruits. Commercially, pectins are extracted from citrus peel or apple pomace under mildly acidic conditions. Pectin is being used in the food industry as
well as the pharmaceutical industry as a potential carrier for drug delivery to the gastrointestinal tract. In the food industry, pectin is well known due to its addition to jams and jellies to promote gelling (Gropper and others 2009). Pectin, when found in its natural form in fruits and vegetables, has a typical range of 0-4% fresh weight (Baker 1997; Thakur and others 1997; Sriamornsak 2003).

![Diagram of pectin molecule](image)

Figure 2. (a) A repeating segment of pectin molecule and functional groups: (b) carboxyl; (c) ester; (d) amide in pectin chain (Sriamornsak 2003).

2.5.2 Fructooligosaccharides (FOS) Background

Fructooligosaccharides (FOS) are a type of fructan, also known as polyfructose. Fructans include inulin, oligofructose and FOS. These fructans are nondigestible oligosaccharides (NDO), which are carbohydrates that escape digestion in the stomach and small intestine
and are partly fermented in the large intestine by colonic bacterial flora (Van Dokkum 1999). FOS is formed from the hydrolysis of inulin and typically contains one moiety of glucose to which 2 to 4 fructose units are bound by a β 2-1 linkage. FOS is considered to be a type of dietary fiber as human digestive enzymes cannot hydrolyze the β 2-1 linkage and FOS escapes digestion. FOS can be broken down in the large intestine by some bacteria, such as bifidobacteria, which has β–fructosidase, which can hydrolyze the β 2-1 linkage. FOS is considered to be a type of soluble and fermentable fiber, therefore a prebiotic; however, FOS is not reported as fiber on nutrition labels and food composition databases at this time. Prebiotics serve as substrates to promote the colonic growth of selected health-promoting species of bacteria. Due to the solubility of FOS, a high degree of fermentability results, more so than other dietary fibers, aiding laxation and increasing the production of short-chain fatty acids (Schneeman 1999). In addition to providing positive effects as a prebiotic, NOD’s have been shown to increase intestinal calcium and magnesium absorption (Morohashi and others 1998; Younes and others 2001; Zafar and others 2004). Fructans do not appear to significantly increase viscosity in the gut like pectin and guar gum does (Schneeman 1999). Inulin and oligofructose appear to have a minimal effect in the small intestine, with their primary effect being to increase bulk (Schneeman 1999). Inulin and FOS do not appear to bind bile acids (Schneeman 1999), which differ greatly from fibers such as guar gum, lignin, cellulose, wheat bran and oat bran (Story and Kritchevsky 1976; Vahouny and others 1980; Gallaher and Schneeman 1986).
Americans are thought to consume up to 4 grams of FOS each day (Gropper and others 2009). FOS can be naturally found in raw chicory root, Jerusalem artichokes, globe artichokes, shallots, leeks, garlic and onions. Globe artichoke hearts in liquid contain 15 g/100 g although without the liquid that number drastically decreases to 0.13- 0.24% (Campbell and others 1997; Hogarth and others 2000). Roasted chicory root, Jerusalem artichokes, dried minced onions, garlic, shallot contain 0.42%, 5.8%, 4.2-4.5%, 0.39%, 0.85% FOS, respectively (Campbell and others 1997; Hogarth and others 2000). Out of the fruits studied, ripe bananas contain the highest amount of FOS at 0.2% (Campbell and others 1997). FOS can be found several processed products, added in a food as a prebiotic. It is commonly found in infant formula in Asian countries such as Softcurd Powdered Formula from Tokyo, which contains between 1.7 and 1.8% FOS (Campbell and others 1997). FOS is added to infant formula as a prebiotic and stool softener in formula-fed infants.

2.5.3 Galactooligosaccharides (GOS) Background

Galactooligosaccharides (GOS) are a type of nondigestible oligosaccharides (NDO), which are carbohydrates that escape digestion in the stomach and small intestine and are partly fermented in the large intestine by colonic bacterial flora (Van Dokkum 1999). GOS consists of one moiety of glucose to which 2 to 10 galactosyl monomers are typically bound by a β 1-4 bond. GOS can be found in soybeans and can be synthesized from lactose. In the United States, the primary food product containing GOS is infant formula where it is added as a prebiotic, but in some Asian countries it can be found in numerous products. Like, FOS, GOS is added to infant formula as a prebiotic and stool
softener in formula-fed infants. GOS remains intact until reaching the colon due to the high amount of β linkages present which bodily enzymes cannot break down, similar to FOS.

GOS has been shown to regulate bowel function, prevent colon cancer, increase calcium mineral absorption, and decrease pathogenic bacteria in the gut, as other dietary fibers and FOS have shown. GOS has specifically been shown to prevent E. coli attachment, salmonella infection and diarrhea (Tzortzis and others 2005; Shoaf and others 2006; Searle and others 2009; Drakoularakou and others 2009). GOS, like FOS and other NDO’s, such as inulin, has been shown to increase calcium and magnesium absorption, thought to be due to the lowering of intestinal pH (Van den Heuvel and others 2000; Younes and others 2001; Zafar and others 2004). The intestinal pH is lowered when SCFA are formed from the fiber and it has been hypothesized that the unabsorbed calcium is converted to the ionic form in the lower pH environment as well as an enlargement of the intestinal surface area leading to enhanced calcium absorption (Remesy and others 1993; Younes and others 1996). The lowered colon pH, related to SCFA production, has also been shown to lower aberrant crypt foci (Verghese and others 2002). The increased fermentation products, especially butyrate, are thought to be chemopreventive in colorectal cancer (Topping and Clifton 2001; Kilner and others 2011). Directly related to GOS, two studies have shown that GOS can significantly reduce colorectal tumors and aberrant crypt foci multiplicity in rats (Wijands and others 1999; Wijands and others 2001).
2.6 Resistant Starch Background

Resistant starch has properties of both soluble and insoluble fiber in that it is fermented in the large intestine by bacteria and it serves as a bulking agent. Resistant starch is the fraction of dietary starch and starch degradation products not digested in the small intestine by healthy individuals that pass to the large intestine to undergo fermentation by gut microflora. Resistant starch is resistant to hydrolysis by α-amylase during digestion. It is found naturally in all starch-containing foods. The amount of resistant starch in the food is dependent on the initial quantity, classification of resistant starch and how the food is processed, cooked, stored and ingested. Resistant starch is measured as the difference between total starch obtained from homogenized and chemically treated sample and the sum of rapidly digestible starch and slowly digestible starch generated from non-homogenized food samples by enzyme digestion (Sajilata and others 2006).

$$\text{Resistant Starch} = \text{Total Starch} - (\text{Rapidly Digestible Starch} + \text{Slowly Digestible Starch})$$

Most Americans are consuming within 3 to 8 grams of resistant starch per day with bread, cooked cereals/pastas, and vegetables (other than legumes) contributing 21%, 19% and 19% of total resistant starch intake, respectively (Murphy and others 2008). Resistant starch actually delivers fewer calories of digestible starch and can be used to maintain a healthy weight. The energy value has been estimated to be between 2 and 3 calories (about 8 kJ/g), depending on each individual’s metabolism while completely
digestible starch, delivers 4.2 calories (about 15 kJ/g) (Nugent 2005; National Starch 2012).

The digestion of resistant starch is very similar to fiber. The majority of resistant starch passes through the small intestine into the large intestine where it is fermented into short chain fatty acids by bacteria.

2.6.1 Resistant Starch Structure, Classification and Food Sources

The resistance of starch to digestion’s enzymes is influenced by the nature of the association between starch polymers, with higher amylose levels in the starch being associated with slower digestibility rates (Nugent 2005). Resistant starch has been classified into four subgroups: RS1, RS2, RS3 and RS4. Figure 3 summarizes their description and food sources.

<table>
<thead>
<tr>
<th>Type of RS</th>
<th>Description</th>
<th>Food sources</th>
<th>Resistance minimized by</th>
</tr>
</thead>
<tbody>
<tr>
<td>RS1</td>
<td>Physically protected</td>
<td>Whole- or partly milled grains and seeds, legumes</td>
<td>Milling, chewing</td>
</tr>
<tr>
<td>RS2</td>
<td>Ungelatinized resistant granules with type B crystallinity, slowly hydrolyzed by α-amylose</td>
<td>Raw potatoes, green bananas, some legumes, high amylose corn</td>
<td>Food processing and cooking</td>
</tr>
<tr>
<td>RS3</td>
<td>Retrograded starch</td>
<td>Cooked and cooled potatoes, bread, cornflakes, food products with repeated moist heat treatment</td>
<td>Processing conditions</td>
</tr>
<tr>
<td>RS4</td>
<td>Chemically modified starches due to cross-linking with chemical reagents</td>
<td>Foods in which modified starches have been used (for example, breads, cakes)</td>
<td>Less susceptible to digestibility in vitro</td>
</tr>
</tbody>
</table>

Figure 3. Table of the classification of types of resistant starch, food sources, and factors affecting their resistant to digestion in the colon (Nugent 2005).

As summarized in Figure 3, resistance of resistant starches can be reduced by employing various methods. Resistant starch type 1 is made less resistant by milling and chewing. Food sources include whole grain brown rice, seeds, oatmeal, barley, and legumes such
as lentils and chick peas. Resistant starch type 2 is made less resistant by food processing and cooking methods. In addition to the food sources shown in Figure 3, other examples include raw green banana flour, plantains and unmodified high amylose corn starch. Resistant starch type 3 is made less resistant by food processing methods. Resistant starch type 4 is chemically modified to purposely resist hydrolysis after food processing but each type depends on the starch base, the type and level of modification (Nugent 2005). RS1 and RS2 are considered to be dietary fibers while RS3 and RS4 are considered to be functional fibers (Food and Nutrition Board 2002; Gropper and others 2009).

RS1 is a type of resistant starch that is physically protected from digestion due to its intact cell walls found in whole grains. The structure of resistant starch type 1 can be found in Figure 4. It is measured chemically as the difference between the glucose released by enzyme digestion of a homogenized food and that released from a nonhomogenized food sample (Sajilata and others 2006). It is heat stable in under most normal cooking conditions. As this type of starch is considered physically inaccessible, mechanical force such as chewing can increase the amount of starch available to the small intestine for digestion.
RS2 is a type of resistant starch with type B crystallinity where starch is naturally tightly packed in a radial pattern limiting accessibility of digestive enzymes. Type 2 is measured chemically as the difference between the glucose released by the enzyme digestion of a boiled homogenized food sample and that from an unboiled, non-homogenized food sample (Sajilata and others 2006). Figure 4 shows the structure of RS2. RS2 is resistant to α-amylase until gelatinized.
RS3 is a type of retrograded non-granular starch. During retrogradation the starch granule is completely hydrated and amylose leaches from the granules. Upon cooling, the starch reassociates into crystalline amylose as double helices stabilized by hydrogen bonds (Wu and Sarko 1978). Resistant starch type 3 is the most resistant starch fraction out of types 1, 2 and 3. This type of resistant starch is measured as the fraction that resists dispersion by boiling and enzyme digestion. This type of starch resists enzyme digestion which means it is entirely resistant to pancreatic amylases (Sajilata and others 2006). Most moist-heated foods, like pasta salad and potato salad, contain some RS3.

RS4 is a group of starches that have been chemically modified to increase their functionality. They can be esterified or cross-linked with chemicals. They are used as thickeners, stabilizers, emulsifiers and as fiber enhancers. They are chemically modified to increase stability against the heat, acid, shear and freezing that can occur during processing (Sajilata and others 2006).

2.6.2 Resistant Starch Health Benefits

Resistant starch has been linked to a number of positive health benefits that include improved glycemic response, improved colonic health, increased satiety, increased micronutrient absorption, and improved lipid profile (Jacobasch and others 1999; Topping and Clifton 2001; Nugent 2005; Sajilata and others 2006). Resistant starches are considered to be very effective prebiotics, meaning food ingredients that are not digested by human digestive enzymes and therefore act as substrate for bacteria in the colon. Like the nondigestible oligosaccharides, FOS and GOS, resistant starches are fermentable carbohydrates which produce short chain fatty acids (SCFA). Various short chain fatty
acids, such as lactate, acetate, butyrate and propionate, are generated from the fermentation in the large intestine. These short chain fatty acids serve a number of purposes in the body and are very beneficial as they assist in normal large bowel function and prevent pathology (Topping and Clifton 2001). As SCFA are produced, the intestinal pH is lowered, which can increase calcium and magnesium absorption (Younes and others 2001). As stated previously, the lowered colon pH, related to SCFA production, has been shown to lower aberrant crypt foci (Verghese and others 2002). The increased fermentation products, especially butyrate, are thought to be chemopreventive in colorectal cancer (Asp and Bjorck 1992; Topping and Clifton 2001; Kilner and others 2011). Butyrate may regulate gene expression and cell growth, maintain a normal colonocyte population, and induce apoptosis of damaged cells (Topping and Clifton 2001; Mentschel and Claus 2003). Propionate and lactate are absorbed in the colon and taken up for use by liver cells while acetate is absorbed and used by muscle and brain cells (Gropper and others 2009). Unlike other fibers, resistant starch is one of the best substrates for the production of butyrate and other SCFA with levels twice that of wheat fiber and four times that of pectin (Champ 2004). More on colonic health, dietary resistant starches may play a role in preventing mucin depletion and DNA damage brought on by a high protein diet (Toden and others 2006). Research surrounding resistant starch has shown a link between diet, colonic bacteria and the prevention of colon cancer with increased levels of resistant starch.

Resistant starch can play a role in weight management through fiber fortification, calorie reduction, satiety, and lipid oxidation. It has been shown that resistant starch type 2
increased post-prandial lipid oxidation, which could decrease fat accumulation (Higgins and others 2004; Keenan and others 2006). Replacement of 5% dietary carbohydrate with resistant starch significantly increased a prolonged, sustained, post-prandial lipid oxidation (Higgins and others 2004). Resistant starch has also been shown to stimulate GLP-1 and PYY, gut-secreted peptides that play a role in satiety as anti-diabetes and anti-obesity hormones (Keenan and others 2006; Zhou and others 2008). These two hormones are naturally secreted in response to meal ingestion but are degraded rapidly. Research is being done to conclude how to maintain substantial high plasma levels of both hormones. Resistant starch was shown to stimulate both hormones as fermentation in the gut produced SCFA that subsequently increased proglucagon, PYY gene expression, and improved the glucose tolerance of the resistant starch fed diabetic mice (Zhou and others 2008). While the mechanism of resistant starch increasing GLP-1 and PYY is unknown, it has been suggested that fermentation of resistant starch is most likely the primary mechanism, which would broaden this research to consider all fermentable fibers. The production of propionate or other short-chain fatty acids may lower serum cholesterol concentrations by inhibiting fatty acid synthesis, cholesterol synthesis or both but propionate fed to humans has had varying effects on serum cholesterol concentrations (Nishina and Freeland 1990; Todesco and others 1991; Lin and others 1995; Gropper and others 2009). A variety of factors allow resistant starch and other fibers to help aid in weight management.

Resistant starch, considered to be a dietary fiber, has a strong impact on glycemic management related to blood glucose, insulin and the potential effect of dietary fiber on
diabetes. The slow digestibility of resistant starch gives resistant starch products a low glycemic index. Additionally, incorporating resistant starch as an ingredient reduces the amount of total digestible carbohydrates available, creating fewer calories and a lowered glycemic response. Resistant starch has been shown to reduce postprandial glucose and/or insulin response (Grandfeldt and others 1995; Behall and Hallfrisch 2002; Yamada and others 2005). Previously, a human trial added maize-based resistant starch into beverages, which subsequently lowered postprandial glycemic and insulinenmic responses without any change in palatability (Kendall and others 2008). This application of resistant starch displays its versatility in dietary strategies to control diabetes and other chronic diseases.

2.6.3 Resistant Starch Application and Intake

Resistant starch can be used in a variety of applications within the food industry, especially since there are 4 types, one of which can be chemically modified in numerous ways. Resistant starch can be used as an added fiber ingredient when low bulk is desired. It can also be used in a variety of applications where low caloric value is needed for health-focused products related to weight management, diabetes and more. Certain types of resistant starch can be used to increase viscosity, form gels, bind water, and as a stabilizer under extreme processing conditions or free/thaw conditions. Resistant starches can be used as a texture modifier, crisping agent, or as an expander in extruded foods. Resistant starch is typically a neutral white color, small in size and bland in flavor. All these are desirable characteristics as to not change the appearance, texture or flavor of the end product. Additionally, it can be listed simply on nutrition labels as
cornstarch, resistant cornstarch, maltodextrin, modified food starch, dextrin, or starch (National Starch 2012). Its applications range from baked goods such as bread, cake, muffins, and brownies, to frozen foods such as waffles and French toast, cereal, extruded snacks, dried pasta, mashed potatoes, casseroles, mixed entrees, and opaque health beverages (Sajilata 2006; National Starch 2012).

The general recommendation for adequate intake (AI) of fiber in the United States is 14 grams/1000 kcal (Anderson and others 2009). This adequate intake recommendation includes resistant starch, lignin and other associated substances but public health authorities have not set recommended levels specific for resistant starch consumption. If the energy guideline was used of 2000 kcal/day for women and 2600 kcal/day for men, the recommended daily dietary fiber intake would be 28 grams/day for adult women and 36 grams/day for adult men (Anderson and others 2009). Resistant starch is a fraction of dietary fiber and most Americans aged 1 year and older were estimated to consume approximately 4.9 grams resistant starch/day (Murphy and others 2008). Most Americans are consuming within 3 to 8 grams of resistant starch per day with bread, cooked cereals/pastas, and vegetables (other than legumes) contributing 21%, 19% and 19% of total resistant starch intake, respectively (Murphy and others 2008). The Italian diet, like the American diet, includes large volumes of pastas, rice, breads, potato and legumes. Using consumption data from the 1980’s, it was determined that the average intake of resistant starch in Italy was about 8.5 grams/day. Most Italians are consuming within 7 to 9 grams of resistant starch per day with cereals (pasta, rice, bread and bread products, and pastries), potatoes, and legumes contributing as the sources of resistant
starch (Brighenti 1998). The resistant starch intake in Australia and New Zealand, based on national nutrition surveys in the mid-1980s for Australia and early 1990s for New Zealand, shows an average of 4.0 g RS/100 g starch for men, 4.7 g RS/100 g starch for women, and 4.5 g RS/100 g starch for children (National Health and Medical Research Council 2006).

2.7 Yogurt Background

Yogurt is a dairy product produced by the bacterial fermentation of milk. The yogurt cultures, such as Lactobacillus, Acidophilus, and Bifidobacteria, ferment lactose into lactic acid. Cow’s milk is typically used to make yogurt but milk from buffalo, goats, sheep and other animals can be used as well. To make yogurt, milk is heated to 165°F to kill any pathogenic bacteria and to denature the milk proteins. The milk is then cooled to 110°F ± 5°F. The bacteria culture is added and the temperature is maintained to encourage fermentation until the yogurt is firm (4-7 hours). The milk is coagulated by an increase in acidity from lactic acid produced by the bacteria. (Nummer 2002). Yogurt can contain carrying amounts of fat from 0% to 4%. Starches and gelatin can be added to yogurt to thicken it and increase the mouthfeel.
Figure 6. Nutrition facts from the nonfat plain yogurt used.

Yogurt is rich in protein, calcium and probiotics while being low in fat and calories, making it a healthy food choice (Figure 6). Probiotics are foods that contain live cultures of specific strains of bacteria. Positive health benefits can be gained if probiotic bacteria survive the passage through the upper digestive tract and then colonize themselves in the colon (Gropper and others 2009). Probiotics have been beneficial in preventing and treating diarrhea, inflammatory bowel diseases, colon cancer, infected pancreatic necrosis, and postoperative liver transplant infections (Gropper and others 2009).

2.8 Banana Background

Bananas are an important staple food for many people in the world. Bananas are loved by young children as well as adults due to their sweetness, texture, portion size, familiarity, availability, convenience, versatility and cost. There are over 30 banana
species, with around 500 varieties having edible fruits. The nutritional value of bananas depends on a variety of factors including ripeness, climatic conditions, quality of the soil, cultivar, and method of preparation (Sharrock and Lusty 2000). A cooked ripe banana is about 75% water, 20-27% carbohydrate, 0.3% fat, and 1.2% protein. Eleven vitamins have been recorded and the fruit is considered to be a good source of potassium and vitamins A, B1, B2 and C (Sharrock and Lusty 2000).

The United States bananas are not documented by cultivar name, but they are most likely Cavendish, the primary banana cultivar marketed globally (Sharrock and Frison 1999). Lutein, α-carotene, and βC can be found in bananas (Davey and others 2009; Holden and others 1999; Englberger and others 2003; Arscott and others 2010; Beck and others 2011; Hunter and others 2011). The amount of literature on banana carotenoid content is sparse and varied, probably due to the variation in the bananas’ ripeness, growing region, storage conditions and more. Overall, it should be noted that a small, yet quantifiable amount of carotenoids can originate from the banana food sample.

These bananas found in the United States contain 5 µg of α-carotene/100 g edible portion, 21 µg of βC/100 g edible portion, and 0 µg of β-cryptoxanthin, lutein, zeaxanthin, and lycopene (Holden and others 1999). Plantains are just types of bananas that are commonly starchier at ripeness; around 30% carbohydrates versus bananas’ 20% carbohydrate content. Bananas can be eaten as ripe raw fruit or cooked green or ripe (Englberger and others 2003).

Bananas, along with the majority of fruits, differ drastically when raw and when ripe. Bananas can be described using the color index number and peel color to rate the
ripeness. Figure 7 describes the stages of ripening from 1 (green) to 7 (yellow- flecked with brown).

Figure 7. The different stages of ripening using the color index number and peel color from numbers 1-7.

The major change during the ripening process is the conversion of starch to sugar. The two main components of banana starch are amylose and amylpectin, present in a 1:5 ratio which does not change during ripening (Garcia and Lajolo 1988). Raw green bananas contain 20-30% starch (Cordenunsi and Lajolo 1995; Akerberg and others 1998; Sharrock and Lusty 2000), which can decrease to 0.8% in fully ripe fruit (Cordenunsi and Lajolo 1995; Sharrock and Lusty 2000). This starch-sucrose transformation during ripening involves several enzymes and pathways. Starch hydrolysis can be accomplished by α- and β-amylases and α 1-4 and α 1-6 glucosidases (Garcia and Lajolo 1988). At the
same time, soluble sugars increase from less than 1% in raw green bananas up to 20% in ripe bananas (Kayisu and others 1981; Cordenunsi and Lajolo 1995). During ripening, the sugars are in an approximate ratio of glucose 20: fructose 15: sucrose 65. Sucrose accumulation can be due to sucrose synthase, and sucrose phosphate synthase (Cordenunsi and Lajolo 1995). The total lipid content remains unchanged during ripening with the composition of fatty acids being an exception (Sharrock and Lusty 2000).

Out of the 31.6 ± 3.2 g total potentially available starch in a raw green banana, it contains 72.1 ± 5.7 g resistant starch type 2/ 100g total starch (Akerberg and others 1998). The reported amount of resistant starch varies, most likely due to the different degrees of maturity of the bananas tested. Resistant starch content in a raw green banana is reported as 54.2 g /100 g total starch (Englyst and Cummings 1986; Faisant and others 1995) and as 47.3 g /100 g total starch (Berry 1986; Faisant and others 1995). This amount decreases to 0% when the banana is freshly cooked but increases to 8.1% when the banana is cooked and then cooled (Englyst and Cummings 1986). Ripe bananas have about 1-2% fiber with extremely low amounts of lignin and cellulose. For the most part, cellulose remains unchanged during ripening although there is a slight change from 0.38% cellulose dry weight basis in green banana pulp to 0.28% dry weight basis in ripe banana pulp (Kayisu and others 1981). Hemicellulose is found at 1.61% dry weight basis in raw green bananas which decreases to 0.96% dry weight basis in ripe bananas (Kayisu and others 1981).
Bananas have high amounts of fructooligosaccharides in comparison with other fruits (Campbell and others 1997; Hogarth and others 2000). Raw green bananas contain 3.1 mg/g FOS dry matter or 0.7 mg/g FOS as is (Campbell and others 1997). Ripe bananas contain 10.9 mg/g FOS dry matter or 2.0 mg/g FOS as is (Campbell and others 1997).

Bananas are harvested when the fruits are fully developed, at about 75% mature when the angles are becoming less prominent. Generally, this stage is reached 75 to 80 days after the opening of the first hand. Bananas are typically ripened in storage rooms with 85 to 95% relative humidity, 58° to 75°F with 2 to 3 exposures to ethylene gas or 6 hourly applications for 1 to 4 days (Figure 8). Ethylene accelerates the ripening process. Harvested bananas allowed to ripen naturally at room temperature do not become as sweet and flavorful as those ripened artificially with ethylene. After removal from storage and during delivery, the fruit must be kept cool at 56° to 60°F and 80 to 85% relative humidity to prevent rapid spoilage (Morton 1987).

Figure 8. An example of a ripening schedule used in ethylene storage rooms.
2.9 In vitro Digestion Model

The simulated in vitro digestion method was used to mimic the human digestive process. In vitro models have been developed as simple, inexpensive, non-invasive and reproducible tools to study digestive stability, micellarization, intestinal transport and metabolism, and to predict the bioavailability of different food components (Granado-Lorencio and others 2009). The simulated in vitro digestion model consists of three separate phases: the oral, gastric and small intestinal phases. The model used must be presented as “static” as simulated in vitro digestion models are not influenced by the many factors that can affect digestive and absorptive processes in vivo (Failla and Chitchumronchokchai 2005). It has been shown that the bioavailability of carotenoids assessed with the in vitro model in conjunction with Caco-2 cells is well correlated with human derived results (Reboul and others 2006). Although the bioavailability and bioaccessibility of carotenoids may be approached using in vitro methods, host-related factors and different physiologic processes, including health and nutritional status, first-pass metabolism, intracellular regulation, and homeostatic control, may limit the comparability and predictive value of in vitro models (Granado and others 2006). There is a significant positive relationship between percentage of carotenoids transferred into micelles in the in vitro model and percent of carotenoids transferred into micelles observed in vivo, suggesting that the in vitro model is suitable for predicting the bioaccessibility of carotenoids from foods (Reboul and others 2006). Although this positive correlation exists, it is important to note that bioaccessibility is not the only factor which affects carotenoid absorption (Borel 2003; Reboul and others 2006).
Simulated gastric and small intestinal digestion has been widely used to examine the digestion of starch (Englyst and others 1999), lipids (Fouad and others 1991), polyphenols (Perez-Vicente and others 2002; Veda and others 2008), transgenic plant DNA (Netherwood and others 2004), and recombinant proteins (Richards and others 2003). Simulated \textit{in vitro} digestion has also been used to investigate carotenoid stability, bioaccessibility, and portioning during the digestion of foods, meals and supplements (Garrett and others 1999a, 1999b, 2000; Ferruzzi and others 2001; Chitchumroonchokchai and others 2004; Serrano and others 2005; Granado-Lorencio and others 2009; Yonekura and Nagao 2009). In general, the carotenoids derived from the digestions are stable (Garrett and others 1999a; Granado-Lorencio and others 2007).

In the oral phase, the carotenoid food sample is homogenized with lipid, α-amylase, synthetic saliva and mucin. It is then placed in a shaking water bath at 37°C for 10 minutes. In the gastric phase, the pH is lowered to around pH 2.5 with hydrochloric acid and pepsin is added. This portion is placed into a shaking water bath at 37°C for 1 hour. After the gastric phase, the small intestinal phase begins by increasing the pH to around pH 6.5. It is in the small intestinal phase where pancreatin, a mix of pancreatic amylase, lipase, and protease, crude bile extract, and pancreatic lipase are added. The samples are placed in a shaking water bath at 37°C for 2 hours. After the completion of the three phases, the digesta is centrifuged for 45 minutes to isolate the aqueous fraction. After being centrifuged, the aqueous fraction is passed through a 0.22 micrometer filter to separate possible microcrystalline aggregates from micelles. After filtering the sample, the micellarized fraction is left. Carotenoids are extracted from the raw starting material,
digesta, and aqueous filtrate which are then analyzed by HPLC to determine digestive stability and efficiency of micellarization.
Chapter 3: Materials and Methods

3.1 Materials

Spinach (Purchased at Giant Eagle on November 11, 2011, Giant Eagle Nature’s Basket Organic Baby Spinach Organic Washed and Ready to Eat, Net Weight 16 oz., 454 g, “Best if used by” date of November 17, 2011, Product Code 3003430392, Lot Number TFRS307A17) and baby carrots (Purchased at Giant Eagle on November 11, 2011, The Farmers Market Baby-Cut Carrots, Net Weight 2 lbs., 908 g, “Best if used by” date of November 27, 2011, Product Code 3003430055, Lot Number 29RB0957BN111R8) were purchased to prepare a salad. Types and sources of fibers were as follows: citrus peel pectin (P9135, galacturonic acid more than 74% dried basis, Sigma-Aldrich, St. Louis, MO); NutraFlora® FOS P-95 (Fructooligosaccharides, P-95, more than 95% FOS Dry Basis, NuVim Inc. Paramus, NJ); GOS Purimune™ (Galactooligosaccharides, GO-P 90, 90.5% GOS Dry Basis, Product ID 113001-156, Lot 15271, Manufactured 12/16/2008 in Korea, GTC Nutrition, Golden, CO); type 2 resistant starch (Hi-maize 260, 60% total dietary fiber, National Starch, Bridgewater, NJ); and type 3 resistant starch (Novelose 330, 30% total dietary fiber, National Starch, Bridgewater, NJ).
3.2 Methods

3.2.1 Salad Preparation

All samples were processed under filtered lights to minimize photodecomposition of the carotenoids. The foods were selected to provide sources of lutein, α-carotene (AC), and βC. Whole baby spinach leaves were weighed into a beaker to 100.0 g. Whole baby carrots were chopped with a knife and weighed into a beaker to 124.0 g. One half of the spinach and carrot were added to a blender (Cuisinart, SmartPower Premier 600-Watt Blender, Model CBT-500, East Windsor, NJ) and blended on “Ice Crush, High” for 3 minutes. The other half of the spinach and carrot were added to the blender and blended on “Ice Crush, High” for 7 minutes until a smooth, homogenous, consistency was reached. It was then further homogenized (Ika T25 digital Ultra-Turrax, S25N-10G 01.594489 tip, Staufen im Breisgau, Germany) for 6 minutes to disperse the larger particles. As there was no water present in the blending of the spinach and carrot, large particles of vegetables were still present in the mixture. The mixture was homogenized after it was blended to break down the particles further and create a more homogenous mixture.

Homogenized salad was dispersed in salts solution (120 mM NaCl solution, 6 mM CaCl₂, 5 mM KCl) before transferring 1.0 g to 50 mL polypropylene screw cap tubes (CELLTREAT Scientific Products, 229421, Lot No. 101209-060). Samples were flushed with nitrogen, capped and frozen at -20°C.
3.2.2 *In Vitro* Digestion: Oral Phase

Artificial saliva, pH 6.5±0.2, was prepared according to Oomen and others 2003. Artificial saliva contains salts, α-amylase (Type VI-B: From porcine pancreas, 075K0672, 23 units/mg solid, Sigma-Aldrich, St. Louis, MO), mucin (Type I-S, bovine submaxillary glands, M3895-1G, Lot # 039K7003V, Sigma-Aldrich, St. Louis, MO), sodium urate (U2875-5G, Sigma-Aldrich, St. Louis, MO), and urea (BP169-500, Lot No. 996194, electrophoresis grade, Fisher Scientific, Hampton, NH). The detailed composition is provided in the Appendix.

Either salad alone (1.0 g), or salad (1.0 g) plus fat-free yogurt (1.0 g), or green or ripe banana (2.0 g) plus salad (1.0 g) was added to a 50 mL polypropylene tube and salts solution (120 mM NaCl solution, 6 mM CaCl$_2$, 5 mM KCl) was added (30 mL) and mixed well. Also, test fibers were weighed to provide the “meals” with either 2 or 4% fiber/1.0 g salad. Amounts of RS2 and RS3 added to salad were normalized to 100% fiber to offset the contribution of non-fiber components. Soybean oil (3%/1.0 g salad) would be typically added at this point in the experiment but for the experiments reported, it was added at the start of the gastric phase. Artificial saliva (7 mL) was pipetted into each tube containing the salad alone or with the other test components, blanketed with nitrogen, capped, and sealed with parafilm. Tubes were placed horizontally in shaking water bath (Allied Versa-Bath S Model 224, Fisher, Hampton, NH) at 37°C and shaken at 85rpm for 10 minutes.
3.2.3 In Vitro Digestion: Gastric Phase

Pepsin (80 mg/50mL final volume) (porcine gastric mucosa, 1064 units/mg protein, P7000-100G, 109K1541, Sigma-Aldrich, St. Louis, MO) was solubilized in 2 mL 100 mM HCl. For the digestion of the salad with either yogurt or banana, pepsin content was doubled to 160 mg/50 mL reaction. Soybean oil (3% oil/ 1.0 g salad) was added to each tube before the initiation of the gastric phase. pH was (Corning pH meter 445) adjusted to 2.5±0.1 with addition of 1.0 M HCl with stirring. Volume of HCl added was recorded and 2 mL pepsin solution was added to each reaction tube. Tubes were blanketed with nitrogen, capped and sealed with parafilm wax. Tubes were placed horizontally in shaking water bath at 37°C and shaken at 85rpm for 1 hour. To terminate the gastric phase reaction, tubes were placed on ice and the pH was immediately raised to 6.0±0.2 with 1 M sodium bicarbonate.

3.2.4 In Vitro Digestion: Small Intestinal Phase

Porcine pancreatin (Lot # 017K0769, P1750-100G, activity equivalent to 4 x U.S.P. specifications, Sigma-Aldrich, St. Louis, MO) and porcine pancreatic lipase (Type II, EC No 232-619-9, contains approx. 25% protein, L3126, 100g, Lot 21K1443, Sigma-Aldrich, St. Louis, MO) were solubilized in 100 mM NaHCO₃. The amounts used per standard reaction were 20 mg pancreatin/reaction and 10 mg lipase/reaction introduced in 2 mL in 100 mM NaHCO₃. Porcine bile extract (B8631, 031M0106V, Sigma-Aldrich, St. Louis, MO) was solubilized in 100 mM NaHCO₃ to provide each reaction with 120 mg bile/reaction in 3mL volume. For digestion of mixtures of salad with either yogurt or
banana, the amounts of pancreatin, lipase and bile extract were doubled to 40 mg/reaction, 20 mg/reaction, and 240 mg/reaction, respectively.

The pH was adjusted to approximately 6.5 and added volume of NaHCO₃ was recorded. Pancreatin/lipase solution (2 mL) and bile extract (3 mL) were added to each tube. pH was further adjusted to 6.9±0.1 using 1 M HCl or 1 M NaHCO₃ as needed. Salts solution was added to increase final volume to 50mL. Tubes were blanketed with nitrogen, capped and sealed with parafilm. Tubes were placed horizontally in shaking water bath at 37°C and shaken at 85rpm for 2 hours. Tubes were then placed on ice to terminate small intestinal phase of digestion.

3.2.5 Preparation of Aqueous Fraction

Tubes were mixed well and aliquots of digesta were transferred to 10 mL centrifuge tubes (Beckman Life Sciences, Brea, CA). Tubes were centrifuged (Beckman Coulter, Avanti J-E Centrifuge, JA 20.1, 115 rotor, Palo Atlo, CA) at 12,000 rpm (18,600g) for 45 minutes at 4°C. The remaining digesta was blanketet with nitrogen and stored at -20°C for analysis. Using an 18 G 1 ½ 1” needle (Becton Dickinson, Fisher Scientific, Hampton, NH), plastic 10mL syringe (Becton Dickinson, Fisher Scientific, Hampton, NH), and sterile 33 mm, 0.22 µm syringe driven filter unit (Millex-GP, Fisher Scientific, Hampton, NH), the aqueous fraction (micellar fraction) was collected from centrifuge tubes and transferred into clean 50 mL tubes. Aqueous fraction was blanketed with nitrogen, capped and tubes stored at -20°C for until analysis.
3.2.6 Extraction of Carotenoids

**Raw Salad**

Homogenized spinach and carrot raw salad was extracted for calculation of efficiency of micellarization during simulated digestion. The salad mixture was weighed (5.0 g) and 1.0 g calcium carbonate and 4.0 g celite were added to the puree as a filtering aid. Methanol (50 mL) was added to the puree and the mixture was allowed to stand for 1 minute. The mixture was homogenized for 1 minute before filtering through Watman papers #1 and #42 (#42 on bottom). Filtrate was collected and saved. Acetone: Hexane (1:1, 50mL) was added and the mixture was allowed to stand for 1 minute. The mixture was homogenized once again for 1 minute and the sample was filtered as above. Filtrate was saved and acetone: hexane extraction was repeated a total of three times. The solid filtering agents were discarded. To the combined filtrate, 30% KOH in methanol (75 mL) was added to the filtrate and stirred for 30 minutes. The layers were washed with 20 mL distilled water twice with the bottom layer being discarded. The organic top layer was passed through sodium sulfate, rinsed with hexane, and passed into a 100 mL volumetric flask with adjustment to volume using hexane. Measured volumes were transferred into vials and were dried under nitrogen to remove the solvent. Dried samples were frozen at -20°C until HPLC analysis.

**Processing of Aqueous Fraction**

Tubes containing frozen digesta samples were thawed in water and mixed well. Aliquots were added to a 50 mL tube. Equal amounts of 30% potassium hydroxide (KOH, Fisher Scientific, Hampton, NH) in methanol were added to degrade chlorophyll in each tube.
Tubes were sealed with parafilm and placed in shaking water bath at 40°C and shaken at 85rpm for 30 minutes.

Tubes were removed and placed on ice. Hexane: Acetone (1:1) was added to each tube. The ratio of added volume of the organic solvent mixture to test sample was 3:2. Tubes were vortexed (Vortex Genie 2, Cat No 12-812, Fisher, Hampton, NH) for 60 seconds. Petroleum ether (2.75 vol.) was next added to tubes along with known quantity of internal standard (70 µL β-Apo-8-carotenal of 146.375 µg/vial). Tubes were vortexed 60 seconds before centrifugation (Marathon 3200R, Fisher Scientific, Hampton, NH) at 3000 rpm (177x-g) for 10 min at 4°C. The upper organic layer was collected and transferred into glass labeled 11 mL tubes using a plastic transfer pipet (graduated 3mL, Lg bulb, Samco Scientific). Tubes were dried using a nitrogen dryer (OA-SYS Heating system, N-EVAP 111 Nitrogen Evaporator, Organomation Associates, Inc. Berlin, MA) under a stream of nitrogen and blanketed under nitrogen for freezing at -20°C until re-solubilization for injection into HPLC.

3.2.7 Carotenoid Analysis by HPLC: Quantification

Dried samples were solubilized in 500 µL 1:1 methanol: methyl tert-butyl ether (MTBE) with 250 µL methanol being added first. The sample was sonicated for 10 seconds and vortexed for 5 seconds. Sample was transferred into HPLC vials with inserts using 10 mL syringes and nylon syringe filters (0.2 um x 13 mm, Fisher Scientific, Hampton, NH). The injection volume was 20 µL.

All-trans carotenoids and their cis isomers were separated using a Waters 2695 module HPLC and identified by UV-visible absorbance spectra recorded using a Waters 2996
photodiode array detector. Samples were injected onto a Waters YMC Carotenoid S-5 C30 reversed phase column (4.6 x 150 mm; particle size 5 µm). Molecules were separated as follows. Solvent A consisted of 98% methanol and 2% ammonium acetate solution. Solvent B was 100% MTBE (methyl tert-butyl ether). The gradient started at 85% A and decreased in a linear fashion to 40% A by 20 minutes. It increased to 85% A by 25 minutes. The flow rate was 0.6mL/min and the total run time was 25 minutes.

DAD detector collected data from 200-500nm. Limitation of detection is 5 ng/mL. An internal standard was used to standardize any slight variation of sample quantity which could be due to solvent flow rate, amount injected, and sample loss during extraction and re-solubilization. The recovery of the internal standard was 96.6 ± 3.2.

Carotenoids were identified by their retention time and spectral profiles compared to published characteristics and pure standards. Quantification of carotenoids was achieved by comparison of AUC of separated carotenoids in a sample with a standard curve obtained from using either pure all-trans lutein (Wako Chemical, Neuss, Germany), βC (Sigma-Aldrich, St. Louis, MO), or AC (Wako Chemical, Neuss, Germany) standard. Cis isomers of βC were quantified by comparison of AUC in a sample with the pure βC standard curve. The following equations were used for lutein, α-carotene and βC:
y=186346x with 6 points of standard; and y=1000006x with 6 points of standard;
y=839740x with 4 points of standard, respectively. The molar absorptivity of lutein was 149 600 L mol⁻¹ cm⁻¹ in methanol and 147 300 L mol⁻¹ cm⁻¹ in MTBE. The maximum absorbance of lutein was at 444 nm (Craft 1992). The molar absorptivity of AC was 146 269 L mol⁻¹ cm⁻¹ in ethanol. The maximum absorbance of AC was at 447 nm (Craft
The molar absorptivity of \( \beta C \) was 136 400 L mol\(^{-1}\) cm\(^{-1}\) in methanol and 139 000 L mol\(^{-1}\) cm\(^{-1}\) in MTBE. The maximum absorbance of \( \beta C \) was at 450 nm (Craft 1992; Sowell and others 1994). The efficiency of micellarization of each carotenoid was calculated as follows:

\[
\% \text{ Micellarization} = \frac{(\text{Carotenoids in aqueous fraction/mL} \times 50 \text{ mL})}{\text{Quantity per g salad digested}} \times 100\%
\]

3.2.8 Statistical Analysis

Statistical analyses consisted of one- or two-factor analysis of variance (ANOVA) with Tukey’s post hoc multiple comparisons to identify specific differences in the various factor levels. All analyses were performed using SAS version 9.2 and a 5% significance level. Significance was determined by comparing p-values for each inference to 0.05. All variables were tested with at least 3 independent digestions on a day and each experiment was repeated at least once for a minimum of \( n=6 \) independent digestions and analyses. Data represent mean ± SEM.
Chapter 4: Results and Discussion

4.1 Analysis of Carotenoids and Detection with HPLC

Homogenized carrot and spinach mixture was extracted and analyzed using HPLC to identify and quantify abundant carotenoids.

![Representative chromatogram of carotenoids in spinach and carrot salad.](image)

Carotenoids detected in the salad were lutein, α-carotene, β-carotene and cis isomers of βC (Figure 9). The concentrations of lutein, α-carotene, and βC present per gram (net weight) of salad mixture were 28.5 ± 0.36, 21.9 ± 0.71, and 52.5 ± 1.31 µg/g,
respectively. The amounts of lutein, AC and \( \beta C \) are consistent with literature, although the range varies greatly, as a result of seasonal changes, location purchased, time of storage and transportation, and pre-farmed practices of the spinach and carrot (Thane and Reddy 1997; Chitchumroonchokchai and others 2004; Reboul and others 2006). The stability of the carotenoids in the salad during simulated digestion exceeded 83\%. The bioaccessibility of carotenoids can be affected by a variety of factors including the amount ingested, food source, matrix, amount and type of processing, amount and type of fat co-ingested, carotenoid interactions, and other dietary components such as fiber (Borel 1996; Gartner and others 1997; Castenmiller and others 1999; McEligot and others 1999; Van Hof and others 1999; Tyssandier and others 2001; Edwards and others 2002; Hedren and others 2002).

All identified carotenoids from the raw salad were detected in the aqueous fraction following simulated digestion. The efficiency with which lutein, 13-cis-\( \beta C \), \( \alpha \)-carotene, \( \beta C \), and 9-cis-\( \beta C \) partitioned in the aqueous fraction were 54.1 ± 1.3\%, 18.5 ± 0.6\%, 22.0 ± 1.4\%, 19.6 ± 1.0\%, and 52.7 ± 2.2\%, respectively. These data agree with previously reported results that micellarization of xanthophylls was more efficient than carotenes (Garrett and others 1999; Chitchumroonchokchai and others 2004). It was shown that the efficiency of micellarization of trans-lutein in digested, microwaved spinach was 53\% (Chitchumroonchokchai and others 2004) which is comparable to the 54\% in my results. The same investigators reported micellarization of both trans- \( \beta C \) and 9-cis- \( \beta C \) to be approximately 25\% in digested, microwaved spinach. It was also reported that micellarization of lutein and \( \beta C \) in fresh spinach puree subjected to \textit{in vitro} digestion was
28% and 30%, respectively (Ferruzzi and others 2001). Similarly, when raw, pulped, freeze-dried carrots were subjected to in vitro digestion without oil, βC from carrots was 21% accessible and increased to 28% with the addition of 20% oil per gram dry matter carrot (Hedren and others 2002). It was also shown that βC in digested carrots was 16-21% bioaccessible (Veda and others 2006). In a more complex matrix, the efficiency of micellarization of lutein, AC and βC in a creamed spinach, carrot, tomato paste, and meat baby food mixture was 26%, 17% and 17%, respectively (Garrett and others 1999). Although the baby food matrix contained more components than my spinach and carrot salad, the spinach and carrots were the primary sources of lutein, AC and βC. Micellarization of lutein, AC, and βC in homogenized, stir fried spinach, carrot, tomato paste and vegetable oil was reported to be 30%, 16%, and 16%, respectively (Garrett and others 2000).

Bile salts play an essential role in the micellarization of hydrophobic compounds such as carotenoids (Parker 1996; Garrett and others 1999). In previous work, it was shown that carotenoids were not detected in the aqueous fraction when bile extract was omitted during the small intestinal phase of digestion (Garrett and others 1999). These investigators also showed that the quantities of carotenoids in the aqueous fraction were increased as bile extract was increased in a dose dependent manner over the range of 0 to 2.4 mg bile/ mL digestate with 4 g of starting material. I did not examine the impact of deletion of bile extract during the small intestinal phase of digestion in my project.
4.2 Effect of Fibers on Bioaccessibility of Carotenoids During Digestion of Spinach and Carrot Salad

Figure 10. Efficiency of micellarization of lutein during digestion of salad containing 2% fiber (wt: wt) is increased by addition of FOS and RS3. Data are mean ± SEM for n= 3.

Micellarization of lutein during digestion of the spinach and carrot salad was 54.1 ± 1.3 (Figure 10). The addition of 2% FOS to the salad significantly (α= 0.0377) increased micellarization of lutein by 12% to 60.5 ± 1.0. Similarly, addition of 2% RS3, significantly (α= 0.0054) increased micellarization of the xanthophyll by 14% to 61.9 ± 1.8. Although addition of 2% FOS and 2% RS3 to salad increased the micellarization of lutein, the differences were not significantly different from the control.
Figure 11. Efficiency of micellarization of α-carotene during digestion of salad containing 2% fiber (wt: wt) is increased by addition of RS2 and RS3. Data are mean ± SEM for n= 3.

The efficiency of micellarization of AC during small intestinal digestion of the control salad was 22.0 ± 1.4%. Addition of high methoxyl citrus pectin (2%) to salad decreased micellarization of AC, although the decline was not significantly different from control ($\alpha= 0.8027$). There was a 56% and 48% increase in the micellarization of α-carotene during digestion of salad containing 2% RS2 ($\alpha=0.0009$) and 2% RS3 ($\alpha=0.0075$), respectively. With the addition of 2% RS2 and 2% RS3, micellarization of α-carotene increased to 34.4 ± 1.8% and 32.5 ± 1.3%, respectively. Micellarization of AC during digestion of salad containing 2% FOS increased to 30.4 ± 2.0%, although the change is not statistically significant ($\alpha= 0.0616$).
Figure 12. Efficiency of micellarization of all trans βC during digestion of salad containing 2% fiber (wt: wt) is increased by addition of FOS, RS2 and RS3. Data are mean ± SEM for n= 3.

Micellarization of βC in the control salad was 19.6 ± 1.0%. As with AC, the efficiency of micellarization of βC decreased in salad containing 2% citrus pectin, but this change was not statistically significant (α = 0.8106). The addition of 2% FOS to the salad significantly increased (α = 0.0136) the efficiency of micellarization of βC by 41% to 27.6 ± 1.4%. The addition of 2% RS2 to the salad significantly increased (α = 0.0001) the efficiency of micellarization by 60% to 31.3 ± 1.6%. The addition of 2% RS3 to the salad significantly increased (α = 0.0014) the efficiency of micellarization of βC by 49% to 29.2 ± 1.0.
Figure 13. Efficiency of micellarization of lutein during digestion of salad containing 4% fiber (wt: wt) is decreased by addition of pectin and increased by addition of FOS. Data are mean ± SEM for n=3.

The amount of fiber added to salad was increased from 2% to 4% to examine the effect of the greater amount on micellarization of the carotenoids during simulated digestion of the salad. Micellarization of lutein was 54.1 ± 1.3% in the control salad (Figure 13). The efficiency of micellarization of lutein significantly decreased (α = 0.0001) by 22% to 42.1 ± 2.1% during the digestion of salad containing 4% high methoxylated citrus pectin. This result confirms previously reports that pectin decreases the bioaccessibility of carotenoids (Erdman and others 1986; Rock and Swendseid 1992; Riedl and others 1999; Yonekura and Nagao 2009; Mamatha and Baskaran 2011). Hemicellulose, lignin, and pectin also decreased βC utilization by chicks when the diet included 7% purified fiber (Erdman and others 1986). These investigators also found that high methoxy apple pectin, medium methoxy apple pectin, citrus pectin, and polygalacturonic acid all decreased storage of
vitamin A in the liver of chicks when their diets contained 7% fiber (Erdman and others 1986). It also was reported that addition of 12 g of citrus pectin to a 500 kcal meal containing 25 mg βC significantly reduced plasma βC 30 hours after feeding the meal to human subjects (Rock and Swendseid 1992). Similarly, the bioavailability of lutein, βC and lycopene was decreased in blood plasma in women fed chocolate rice pudding containing either citrus pectin, guar gum, alginate, cellulose or wheat bran (0.15 g fiber per kg body weight) (Riedl and others 1999). The only in vitro study related to the effect of fiber on carotenoid bioavailability showed that medium- and high-viscosity alginates and citrus and apple pectins at 200 nmol/ L inhibited micellarization of purified lutein and βC solubilized in soybean oil (Yonekura and Nagao 2009). These investigators also found that these alginates and pectins inhibited uptake of lutein and βC by Caco-2 cells. Finally, lutein levels in blood plasma, liver, and eye decreased when 200 µM lutein was administered to male adult albino Wistar in combination with 1.25% pectin delivered in mixed micelles containing mono-oleoyl-glycerol, oleic acid, sodium taurocholate, cholesterol, lutein with phospholipids, fat (soybean oil, 3%), or dietary fiber (pectin, 1.25%) (Mamatha and Baskaran 2011). Results from my study are similar to those reported for the in vitro digestion study with alginate and pectin (Yonekura and Nagao 2009).

With the addition of 4% FOS to the salad, there was a 12% increase (α= 0.0306) in the efficiency of micellarization of lutein to 60.7 ± 0.3%. This shows that the presence of either 2 or 4% FOS increased micellarization of lutein. In contrast, the enhanced
efficiency of micellarization of lutein during digestion of salad with 2% RS3 was not observed for salad containing 4% RS3.

![Bar chart](image)

**Figure 14.** Efficiency of micellarization of α-carotene during digestion of salad containing 4% fiber (wt: wt) is decreased by addition of pectin. Data are mean ± SEM for n= 3.

Micellarization of AC in the control salad was 22.0 ± 1.4 (Figure 14). Addition of 4% (wt: wt) citrus peel pectin per g salad decreased (α= 0.0001) micellarization of α-carotene by 76% to 5.3 ± 0.7%. There was no significant change in micellarization of AC during digestion of salad containing 4% FOS, GOS, RS2 or RS3.
Figure 15. Efficiency of micellarization of β-carotene during digestion of salad containing 4% fiber (wt: wt) is decreased by addition of pectin. Data are mean ± SEM for n= 3.

Micellarization of βC in the control salad was 19.6 ± 1.0% (Figure 15). Addition of 4% (wt: wt) citrus peel pectin per g salad significantly decreased (α= 0.0001) micellarization of βC by 75% to 4.9 ± 0.6%. It was previously shown that 2% RS2 and 2% RS3 increased micellarization of both hydrocarbon carotenes (Figures 11 and 12), but this effect was not observed for salads containing 4% RS2 or 4% RS3.
The effect of fibers on the bioaccessibility of βC isomers is shown in Table 1.

Micellarization of 13-cis-βC in the control salad was 18.3 ± 0.6% (Table 1). Addition of 4% (wt: wt) citrus pectin per g salad significantly decreased ($\alpha = 0.0001$) micellarization of 13-cis-βC by 71%. In contrast, the micellarization of 13-cis-βC was significantly increased during digestion of salads containing either 2% FOS, 4% FOS, 2% GOS, 4% GOS, 2% RS2, 4% RS2, 2% RS3, and 4% RS3. As there were limited significant changes in micellarization of all trans βC during digestion of salads containing either FOS, GOS, RS2 or RS3, it appears that the fibers had a greater impact on the cis isomers of βC than on all trans βC.
Micellarization of 9-cis-βC in the control salad was 51.8 ± 2.1% (Table 2). Addition of 4% (wt: wt) citrus peel pectin per g salad decreased ($\alpha = 0.0001$) micellarization of 9-cis-βC by 74%. In contrast, addition of 2% FOS, 2% GOS, 4% GOS, 2% RS2, 4% RS2, 2% RS3, and 4% RS3 significantly increased the efficiency of micellarization of 9-cis-βC. Micellarization of 9-cis-βC during digestion of salad with 4% FOS was increased, although the increase was not significantly different from control ($\alpha = 0.0620$). In general, cis isomers of βC are more readily solubilized in micelles than trans isomers (Tyssandier and others 2003), but are less efficiently absorbed than trans isomers (Ben-Amotz and Levy 1996; During and others 2002).
4.3 Effect of Yogurt on Bioaccessibility of Carotenoids in Salad

Having assessed the effect of various fibers on the bioaccessibility of carotenoids with a simple salad, I next mixed salad with non-fat plain yogurt to better simulate a more complex meal. Yogurt (1 g) was added to the homogenized spinach and carrot salad (1 g) along with either 4% citrus pectin, FOS or GOS per g salad weight and 3% soybean oil. Nonfat plain yogurt did not contribute any carotenoids to the meal. Yogurt has been used in previous in vivo studies to assess βC bioavailability as it is devoid of both carotenoids and fiber (Rock and Swendseid 1992; Parker and others 1999). Similarly, it was shown that the presence of milk does not influence the bioavailability of carotenoids from fruit juices in vivo (Granado-Lorencio and others 2009).

Because the amount of starting raw material was increased (2 g), I doubled the amount of digestive enzymes and compounds and compared micellarization for the standard amounts of digestive enzymes vs. twice the standard amount of enzymes. I first examined how enzyme concentration affects carotenoid micellarization for the simple salad only (1 g). Enzyme content significantly influenced the micellarization of lutein, α-carotene and βC (Figure 16). Micellarization of lutein was 54.1 ± 1.3% and 62.4 ± 1.4%, respectively (α= 0.0018), when the amount of digestive enzymes used was 1x vs. 2x. Similarly, micellarization of AC was 22.0 ± 1.4% and 12.7 ± 2.0% (α= 0.0017) when digestions contained 1x and 2x enzymes, respectively. Similarly, micellarization of βC was 19.6 ± 1.0% and 11.0 ± 1.6% (α= 0.0002) when digestive reactions contained 1x and 2x amounts of enzymes, respectively (Figure 16).
Figure 16. Efficiency of micellarization of lutein, α-carotene, and β-carotene during digestion of control salad containing 1x enzyme or 2x enzyme. Data are mean ± SEM for n= 6.

It has been previously reported that amounts of AC and βC, but not lutein, transferred to the aqueous fraction decreased by approximately 50% when pancreatin was deleted during the small intestinal phase of digestion (Garrett and others 1999). Thus, I expected increased efficiency of micellarization of all carotenoids in reactions with the increased amount of digestive enzymes. One significant difference between the present study and that study was the food matrix. This study used a raw spinach and carrot sample whereas Garrett and others used highly processed baby foods and meat. Additionally, Garrett and others only increased or decreased one enzyme variable at a time, while I doubled all enzymes simultaneously. Perhaps the greater quantity of digestive enzymes coagulated or formed a matrix around the salad particles that reduce access of the biliary components
and pancreatic enzymes to decrease the efficiency of micellarization. All remaining data presented were generated using 2x amounts of digestive enzymes.

The combination of non-fat yogurt and salad did not significantly affect micellarization of lutein, AC or βC (Figure 17). Micellarization of lutein ($\alpha=0.8452$), AC ($\alpha=0.2981$), and βC ($\alpha=0.2844$) during the digestion of the control salad was not significantly greater than the salad and yogurt meal (Figure 17). The absence of any significant changes may be attributable to the presence of yogurt that created a more complex food matrix. The food matrix can have a significant role on the bioavailability of carotenoids. Particle size, type and extent of processing, and whether the compound of interest is endogenous to the food or incorporated into a supplement can all affect the bioaccessibility and bioavailability of the carotenoids. It has been shown that food processing and heating improved the bioavailability of AC and βC from a commercial carrot puree baby food compared to that from boiled-mashed carrots (Edwards and others 2002). The bioavailability of βC in spinach increased when the vegetable matrix was disrupted (Van het Hof and others 1999) and was found to be more available from liquefied spinach than from whole leaves (Castenmiller and others 1999). Also, AC was more available in juice than in raw or cooked vegetables (McEligot and others 1999). Moreover, bioaccessibility of AC and βC was improved when raw carrots were homogenized and further enhanced when they were cooked (Hedren and others 2002).
4.4 Effect of Raw vs. Ripe Banana on Bioaccessibility of Carotenoids in Salad

In the previous studies, I added fiber to a food matrix lacking fiber. Review of information about foods that naturally contain high amounts of resistant starch indicated that raw bananas have a relatively high content of type 2 resistant starch. The major change during the ripening process is the conversion of starch to sugar. Raw green bananas contain 20-30% starch (Cordenunsi and Lajolo 1995; Akerberg and others 1998; Sharrock and Lusty 2000) which decreases to approximately 1% in fully ripe fruit (Cordenunsi and Lajolo 1995; Sharrock and Lusty 2000). Of the approximately 30 g total starch in a raw green banana, 50-75% is resistant starch type 2 (Berry 1986; Englyst and Cummings 1986; Faisant and others 1995; Akerberg and others 1998). This amount of resistant starch can decrease to 0% when the banana is freshly cooked but increases to

Figure 17. Efficiency of micellarization of lutein, α-carotene, and β-carotene from salad alone, salad in addition to yogurt, or salad in addition to yogurt containing 4% fiber (wt: wt). Data are mean ± SEM for n=3.
8.1% when the banana is cooked and then cooled (Englyst and Cummings 1986). Besides resistant starch, bananas contain low amounts of pectin, lignin, cellulose, fructooligosaccharides, and hemicellulose (Kayisu and others 1981; Baker 1997; Campbell and others 1997; Hogarth and others 2000). By adding banana to the homogenized spinach and carrot salad, we were able to examine the effects of the potential impact of banana’s inherent natural fiber on the micellarization of dietary carotenoids.

Banana (2 g) was added to the homogenized spinach and carrot salad (1 g). Soybean oil (3% g salad weight) was the only fat added to the food matrix. Because the amount of starting raw material was increased (3 g), the amount of digestive enzymes was twice the standard amounts as for the experiment with yogurt. The concentrations of lutein, 13-cis-βC, α-carotene, βC, and 9-cis-βC present in the 1g of raw salad mixture were 28.5 ± 0.36, 2.93 ± 0.10, 21.9 ± 0.71, 52.5 ± 1.31, and 2.82 ± 0.08 μg/g, respectively. Bananas may have contributed a small, but negligible, quantity of lutein, AC and βC to the matrix (Davey and others 2009; Holden and others 1999; Englberger and others 2003; Arscott and others 2010; Beck and others 2011; Hunter and others 2011).
The efficiency of micellarization of lutein, α-carotene, and β-carotene during digestion of salad containing raw banana was 66.7 ± 1.0%, 12.0 ± 2.2%, and 11.4 ± 1.8%, respectively. Similarly, micellarization of lutein, α-carotene, and βC during digestion of salad mixed with ripe banana was 71.7 ± 2.3%, 12.6 ± 3.2%, and 11.5 ± 2.8%, respectively. There was no significant difference in extent of micellarization of carotenoids during digestion of the meals containing raw and ripe banana. Because the banana matrix is quite complex and the actual amount and type of fiber was not characterized in the bananas, no further firm conclusions can be drawn as to why there were no significant differences between the raw and ripe sample.

The decreased micellarization of carotenoids in the experiments using yogurt and banana with 2x the normal content of digestive enzymes was unexpected (Figures 17 and 18).
The decrease in micellarization of AC and βC was not due to the greater amount of starting raw material (2 g or 3 g) because the decrease of AC and βC was also seen in the control salad without yogurt or banana digested with 2x enzymes.
Chapter 6: Discussion

Here we report the effect of varying fibers and amounts on the dietary carotenoid bioaccessibility from spinach and carrot using an in vitro digestion method. It was seen in the present study that high methoxyl citrus pectin decreased micellarization of both xanthophyll and carotene carotenoids from homogenized raw spinach and carrot. It has been shown previously that soluble fiber, such as pectin, gums, and β-glucans, can affect lipid absorption by binding or interacting with fatty acids, cholesterol, and/or bile acids. Fatty acids and cholesterol that are complexed to fiber cannot form micelles and negatively affect the micellarization of lipid soluble components (Phillips 1986; Anderson and others 2009; Gropper and others 2009). Additionally, when fiber binds to bile acids, these bile acids cannot be reabsorbed and recirculated in the process called enterohepatic recirculation (Gropper and others 2009). Fiber bound lipids and bile acids are not absorbed in the small intestine and are passed into the large intestine where they are degraded by bacteria or excreted.

Additionally, soluble fibers bind water and create viscous solutions within the body, decreasing the mixing of the gastrointestinal contents with digestive enzymes, thereby reducing enzyme function, and decreasing the nutrient diffusion rate. Whether fiber directly decreases the activity of these digestive enzymes or acts by reducing the rate of enzyme penetration into the food is unclear. Availability of digestive enzymes is decreased as the viscous gel impairs nutrients from interacting with digestive enzymes.
In addition, a decreased nutrient diffusion rate can prevent nutrients from being absorbed at their normal site. This can occur if the nutrient becomes physically trapped in the gel and then is later released at a distal site (Gropper and others 2009; Palafox-Carlos and others 2011). In the present study, viscosity of samples was not measured; however, it can be assumed that the viscosity of citrus pectin played an important role in decreasing the micellarization of lutein, AC, βC, 13-cis-βC, and 9-cis-βC. A previous in vitro digestion study measured the viscosity of carotenoid test media when different types of pectins, alginates and celluloses were added (Yonekura and Nagao 2009). These investigators found that βC uptake in Caco-2 cells was strongly and inversely related to the viscosity of the test media (Yonekura and Nagao 2009). The potential increased viscosity with the addition of pectin along with the potential binding to bile acids, fatty acids and cholesterol, may explain the decreased micellarization of dietary carotenoids from the salad when soluble fiber, citrus pectin was added.

The increased micellarization of xanthophyll and carotene carotenoids with the addition of FOS, GOS, RS2 and RS3 was unexpected. It was hypothesized that because FOS and GOS are considered to be soluble fibers, and that resistant starch has characteristics of soluble fiber, that micellarization of both xanthophylls and carotenes would decrease with the addition of these fibers. Differences between xanthophylls and carotenes, amount of fiber and type of fiber, all affected the bioaccessibility of the dietary carotenoids.

It has been previously shown that fructans do not appear to significantly increase viscosity in the gut like pectin and guar gum does (Schneeman 1999). Because fructans do not increase viscosity like pectin has been shown to do, it can be assumed that FOS
and GOS may not increase viscosity either, which may explain a lack of decreased micellarization. It has also been shown that inulin and FOS do not appear to bind bile acids (Schneeman 1999), which differs greatly from fibers such as pectin, guar gum, lignin, cellulose, wheat bran and oat bran (Story and Kritchevsky 1976; Vahouny and others 1980; Gallaher and Schneeman 1986; Gropper and others 2009). FOS, GOS, RS2 and RS3 ingredients did not exhibit decreased micellarization of carotenoids. These fibers were actually found to increase micellarization of lutein, α-carotene, trans βC and cis βC isomers. The beneficial bioaccessibility enhancing effect of these fibers in combination with carotenoids from raw, homogenized spinach and carrot can be seen in the present study. The mechanism of increased carotenoid bioaccessibility from the salad with the addition of specific amounts and types of fiber remains unknown. Reports on the effect of oligosaccharides and resistant starch on the bioaccessibility of dietary carotenoids is lacking, and, to my knowledge, this is the first report of its kind. It was shown previously that βC bioaccessibility was increased by the presence of food acidulants, amchur and lime, which was attributed to the loosening of the vegetable matrix, rendering βC more bioaccessible (Veda and others 2008). Based on this study, it can be seen that carotenoid bioaccessibility can be increased in the presence of other food components. The amounts of dietary fiber and carotenoids in the present study that were observed to bring about a beneficial influence on carotenoid bioaccessibility, are those that are normally encountered in U.S. daily diets. Thus, the presence and consumption of FOS, GOS and resistant starch in combination with carotenoid-rich vegetables, may prove to be advantageous.
When increasing the complexity of the salad food matrix with yogurt or banana in addition to the increased amount of digestive enzymes, no significant differences in micellarization were seen with type and amount of fiber. The lack of significance may be due to the many complex components and interactions possible from yogurt and banana in combination with the salad. The particular brand of nonfat plain yogurt used contained Grade A cultured nonfat milk, food starch-modified, gelatin and active cultures including *Lactobacillus*, *Acidophilus*, and *Bifidobacterium*. It also contained 5 mg cholesterol, 150 mg sodium, 460 mg potassium, 18 g carbohydrate, 13 g sugar, 10 g protein, 4% vitamin C and 35% calcium. All of these different components in the yogurt greatly increased the complexity of the food matrix and therefore could have affected the digestion and micellarization of carotenoids in numerous ways.

Further research must be done to reduce inter-sample variation as well as examine possible mechanisms, besides matrix effects, responsible for a lack of significance. Interestingly, when the standard amount of digestive enzymes was doubled, the micellarization of carotene carotenoids decreased by half. It was expected that when the amount of digestive enzymes present in the system was increased, the micellarization of all carotenoids from the salad would increase due to previous *in vitro* digestion studies (Garrett and others 1999). Although these investigators showed that with increasing bile extract, the quantities of carotenoids in the aqueous fraction were increased; they did not examine the effects of increasing all digestive enzymes simultaneously as done in the present study. In the present study, only lutein significantly increased with double the amount of enzymes in comparison to the same control salad with a single, standard dose.
of digestive enzymes. The mechanism behind the decreased micellarization with increased digestive enzymes must be further researched. Additional replicates should be completed as well as examining a possible dose dependent effect.

Although the mechanism is unknown, it can be reasoned that with higher amounts of digestive enzymes and compounds, the materials may have formed a physical barrier around the carotenes. As the shape and composition of the micelles are critical for efficient uptake, it can be possible that the carotenes may have solubilized into mal-shaped micelles that were subsequently removed by filtration during preparation for HPLC. Lastly, this decreased micellarization may be due to bile acid and lipase interactions. Although it was previously seen that with increasing bile extract, increased amounts of lutein, AC and βC were seen in micelles, these investigators only saw a slight significant increase in the carotene, βC, from 0.8 mg bile extract/ mL digestate to 2.4 mg bile/ mL (Garrett and others 1999). There was a pattern of increased AC from 0.8 mg bile/ mL to 2.4 mg/ mL but the change was not significant. The standard amount of bile extract in the in vitro assay (2.4 mg/ mL) is similar to levels present in the small intestine in the fasted state, i.e., 4-6 mmol/ L (Charman and others 1997). The investigators did not see a significant difference between the standard amount used (2.4 mg bile extract/ mL digestate) and 3.6 mg bile / mL in lutein, AC, or βC. Since the elevation of the amount of bile extract to 3.6 mg/ mL did not enhance micellarization in the investigator’s model, 2.4 mg bile extract/ mL does not appear to be limiting under the defined conditions (Garrett and others 1999). In the present study, the standard amount of bile extract was used (2.4 mg/ mL) and when the starting material was increased with the
addition of yogurt or banana, the amount of bile was increased to 4.8 mg/mL, higher than the amount (3.6 mg bile/mL) previously examined in literature. When the investigators increased the amount of bile to 3.6 mg/mL from 2.4 mg/mL there was no significant increase in the xanthophyll or carotene carotenoid content, but there was no significant decrease in carotene content either, as the present study has shown. It can be hypothesized that by increasing the bile extract content to 4.8 mg/mL in the present study, pancreatic lipase may have been partially inhibited in the small intestinal phase of the in vitro digestion.

Bile salts can efficiently displace surface active materials from the oil droplet interface and help form mixed micelles. Because they are more surface active than the material they are displacing, it makes it difficult for pancreatic lipase to adsorb to the surface, inhibiting pancreatic lipase. Lipase’s cofactor, co-lipase, is able to adsorb onto a bile salt dominated interface and complex with lipase due to electrostatic interactions with bile salts. Co-lipase facilitates the adsorption of lipase onto the lipid surface in the presence of bile salts and subsequently enhances lipid hydrolysis. The action of pancreatic lipase is dependent on the competitive adsorption process between bile salts and adsorbed material, the adsorption of lipase/colipase onto bile salt rich interface, and the removal of lipolysis products from the interface by bile salts and their portioning into mixed micelles (Wilde and Chu 2011). By doubling the amount of bile salts to 4.8 mg/mL in the present study without the sufficient amount of co-lipase, it is possible that pancreatic lipase activity may have been partially inhibited, resulting in the decreased micellarization of carotenes. Further research may include a digestion with the addition of known amounts
of co-lipase or a digestion with a single, standard dose of bile extract and an increased amount of pancreatic lipase.

In vitro digestion models are considered to be appropriate analytical tools to estimate the bioaccessibility of bioactive ingredients from different food matrices. In vitro methods are rapid, simple, non-invasive and inexpensive (Granado-Lorencio and others 2009). The amount of carotenoids that are released from the food during digestion and transferred to micelles, i.e. carotenoid bioaccessibility, is a good indicator of its availability for absorption in vivo (bioavailability) (Reboul and others 2006). There is a significant positive relationship between percentage of carotenoids transferred into micelles in the in vitro model and percent of carotenoids transferred into micelles observed in vivo, suggesting that the in vitro model is suitable for predicting the bioaccessibility of carotenoids from foods (Reboul and others 2006). Although in vitro methods provide estimates of carotenoid absorbability, absolute estimates cannot be made because the method is not subjected to the physiological factors that can affect bioavailability in vivo, such as nutritional status, homeostatic control, and individual physiologic processes (Granado and others 2006; Failla and Chitchumronchokchai 2005). Additionally, due to methodological differences between different in vitro models such as content and activity of digestive enzymes and bile salts, it is difficult to make direct comparisons with previously described studies.
Chapter 5: Conclusions

My results suggest that the presence of 2-4% fructooligosaccharides, galactooligosaccharides, and resistant starch ingredients will not compromise the bioaccessibility of carotenoids in a meal. The use of 2-4% fiber in the meal is aligned with the amounts that are normally eaten in U.S. diets. Thus, the addition of these fiber ingredients to foods to increase fiber content or improve functionality is not expected to adversely affect the bioaccessibility of carotenoids and alter dietary lipophiles such as alpha-tocopherol and other hydrophobic compounds. Because varying types of dietary fibers have been shown to affect the bioaccessibility and bioavailability of carotenoids, it can be hypothesized that fibers can also affect the absorption of alpha-tocopherol and other hydrophobic compounds.

The combination of yogurt or banana with salad did not significantly affect micellarization of lutein, AC, or βC from the spinach and carrot salad. Micellarization of AC and βC decreased in the control salad with the increased amount of digestive enzymes. The effect appears to be due to the addition of twice as much digestive enzyme as for standard simulated digestion. Further research should include increasing replicates to decrease inter-sample variation and examining the interactions of digestive enzymes in vitro. Coupling a Caco-2 cell culture model or an in vivo human or animal study would be advantageous.
References


Mentschel J, Claus R. 2003. Increased butyrate formation in the pig colon by feeding raw potato starch leads to a reduction of colonocyte apoptosis and a shift to the stem cell compartment. Metabolism 52(11):1400-1405.


Weaver GA, Krause JA, Miller TL, Wolin MJ. 1992. Cornstarch fermentation by the colonic microbial community yields more butyrate than does cabbage fiber fermentation;


**Appendix: Composition of Artificial Saliva**

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Quantity/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium Chloride (KCl, Sigma-Aldrich, St. Louis, MO)</td>
<td>0.896 g/ L</td>
</tr>
<tr>
<td>Potassium Thiocyanate (KSCN Fisher, Hampton, NH)</td>
<td>0.200 g/ L</td>
</tr>
<tr>
<td>Sodium Phosphate (NaH$_2$PO$_4$ Fisher, Hampton, NH$_3$)</td>
<td>0.888 g/ L</td>
</tr>
<tr>
<td>Sodium Sulfate (Na$_2$SO$_4$, Fisher, Hampton, NH)</td>
<td>0.570 g/ L</td>
</tr>
<tr>
<td>Sodium Chloride (NaCl, Fisher, Hampton, NH)</td>
<td>0.298 g/ L</td>
</tr>
<tr>
<td>Sodium Hydroxide (NaOH, Fisher, Hampton, NH)</td>
<td>0.072 g/ L</td>
</tr>
</tbody>
</table>

*Table 3. Composition of artificial saliva (Quantities per L DI water).*