BIOAVAILABILITY, METABOLISM, AND BIOEFFICACY OF TOMATO CAROTENOIDS

DISsertation

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

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2012

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Abstract

Carotenoids are non-polar compounds which impart the yellow, orange, and red hues to a number of commonly consumed fruits and vegetables. Some carotenoids, like β-carotene, serve as an important source of provitamin A in the human diet. In contrast, consumption of the carotenoid lycopene has been associated with a reduced risk of developing cardiovascular disease and cancer. Finally, the consumption of lycopene, in addition to the carotenoids phytoene and phytofluene, has been associated with a reduced severity of erythema after ultraviolet light exposure. A better understanding of the absorption, metabolism, and bioefficacy of these compounds is important for making future nutritional recommendations to ensure adequate vitamin A status and reduce the risk of developing specific diseases. Tomatoes have the unique ability to synthesize a variety of carotenoids, and novel varieties which accumulate β-carotene, lycopene, phytoene, and phytofluene, were used in our studies.

Our first aim was to determine if the consumption of a β-carotene-rich tomato sauce or carrots (containing both β-carotene and α-carotene) with lipid would enhance the delivery of greater quantities of vitamin A as compared to consuming the same carotene rich food without lipid. This objective was accomplished by first developing an HPLC-MS/MS method to separate and identify the carotenes and fat soluble vitamins in the test meal, and then quantititating the immediate post-prandial plasma concentrations of
β-carotene and vitamin A metabolites after healthy subjects consumed a meal with or without lipid-rich avocado in combination with a serving of tomato sauce. Consumption of the sauce with lipid-rich avocado increased β-carotene levels by 2.3 fold and increased retinyl esters (i.e. vitamin A) by 4.5 fold as compared to the same sauce meal without avocado.

An important step in elucidating the potential bioactivity of lycopene is a better understanding of its metabolism in the human body. One approach in determining if and how lycopene is metabolized is to identify the oxidative or enzymatic products of lycopene. Our second aim was to determine whether oxidative products of lycopene are present in raw and processed tomatoes and other lycopene containing foods, in addition to blood plasma of humans who have consumed processed tomato juice. Numerous lycopene metabolites were identified and quantified in raw foods and food products containing lycopene. In addition, these lycopene metabolites were also identified and quantified in the blood plasma of healthy humans who had consumed a high-lycopene tomato juice for 8 weeks.

Finally, the consumption of tomato paste and tomato oleoresin containing lycopene, phytoene, and phytofluene has been associated with reduced severity of erythema after ultraviolet light exposure in humans. Our third aim was to determine if the consumption of tangerine tomatoes, containing higher levels of the ultraviolet light absorbing carotenoids phytoene and phytofluene, would: 1) result in the accumulation of lycopene, phytoene, and phytofluene in a mouse model, and 2) reduce the incidence or severity of acute ultraviolet B irradiation-induced skin damage. An immunocompetent,
SKH-I hairless mouse model was used for this study. Animals consumed a freeze-dried tangerine tomato powder diet for ten weeks, and a subset of animals was irradiated with an acute dose of UVB light. Carotenoids accumulated in the skin of the animals which had consumed the tangerine tomato diet, and lower levels of phytoene, phytofluene, and lycopene were observed in animals which had been irradiated with UVB. Female mice consuming the tangerine tomato diet had reduced levels of DNA damage as compared to female animals consuming the control diet. Male mice consuming the tangerine tomato diet had reduced levels of inflammation and DNA damage as compared to male animals consuming the control diet.

Collectively, these results add to the body of knowledge of factors affecting carotenoid absorption and metabolism, and provide important information necessary for a further understanding of potential bioefficacy of carotenoids. In addition, this work demonstrates that novel tomato varieties may serve as a vehicle for delivering higher levels of specific carotenoids to humans and animals for future studies.
Dedicated to my family
Acknowledgements

First and foremost, I want to thank my thesis advisor, Dr. Steven J. Schwartz, to whom I will be eternally grateful. His concern for the wellbeing of his students, his ability to encourage others with confidence and support, his wisdom, and the numerous opportunities he provides for scientific and professional growth have made this a very positive and rewarding experience.

I would like to collectively thank my thesis committee for the time and thoughtful discussions which have developed my skills as a scientist, and for their various contributions to both thesis and non-thesis studies in which I have been involved. In addition, I would like to thank them each for their unique contributions to my experience. Dr. Failla’s dedication to mentoring and to high quality science always inspires me to be more for myself and to do more for others. I would like to thank Dr. Harrison for regularly sharing his depth of knowledge and passion for all things vitamin A and carotenoid related. Finally, I would like to thank Dr. Clinton, whose enthusiasm and vision for the role nutrition and dietary factors can play in disease prevention continues to inspire me.

I am truly indebted to a great number of people for all of acts of kindness and support I have received throughout this process. I would like thank Dr. Tatiana Oberyszyn and her research group for welcoming me with open arms and for sharing
their research expertise. I would like to thank Dr. Yael Vodovotz and Dr. David Francis, who have been strong sources of support and have helped guide me at key points during my studies. I want to thank Dr. Ferhan Ozadali, Marjory Renita, and Casey Philbin who served as important mentors early on in the research process and were very influential in my selection of this area of study. I am especially thankful to Dr. Ken Riedl for his countless hours of training and passing of knowledge of all things mass spectrometry related. I would also like to thank all of the past and present Schwartz lab members - whose enthusiasm and friendship has made this educational experience very enjoyable, and who have always challenged me to be a better student and teacher.

Finally, I would like to thank my parents and my grandparents, who encouraged me to follow my own path, and whose continuous love and guidance has been with me the entire way.
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**Abbreviations**

AMS = accelerator mass spectrometry

APCI = atmospheric pressure chemical ionization

AUC = area under the curve

BCO1 = beta-carotene oxygenase 1

BCO2 = beta-carotene oxygenase 2

BHT = butylated hydroxytoluene

BW = body weight

CID = collisionally induced dissociation

CPD = cyclopyrimidine dimer

CCD = carotenoid cleavage dioxygenase

CVD = cardiovascular disease risk

EDTA = ethylenediaminetetraacetic acid

ESI = electrospray ionization

HPLC = high performance liquid chromatography

IS = internal standard

MPO = myeloperoxidase

MS/MS = tandem mass spectrometry

MTBE = methyl tert-butyl ether

NMR = nuclear magnetic resonance

PDA = photodiode array

RAR = retinoic acid receptor
RAE = retinol activity equivalent
RDA = recommended dietary allowance
ROS = reactive oxygen species
RR = relative risk
RXR = retinoid X receptor
SR-B1 = steroid receptor class B type 1
TRL = triglyceride rich lipoprotein
UVB = ultraviolet B light
CHAPTER 1

Literature Review

1.1 Carotenoid Chemistry

Carotenoids are synthesized by all photosynthetic organisms and are also found in some bacteria, fungi, and yeasts (Liaaen-Jensen, 2004). To date, over 700 have been identified (Britton et al., 2004). The term carotenoid refers to a 40 carbon structure composed of eight isoprenoid units. The prefix “apo” is used to identify carotenoids which have been shortened in length by one or more carbons (Britton et al., 2004). There are two primary types of carotenoids: hydrocarbon carotenoids and xanthophylls. Hydrocarbon carotenoids, such as β-carotene and lycopene, are composed entirely of hydrogen and carbon. In contrast, xanthophylls, such as lutein, contain oxygen in addition to carbon and hydrogen (Britton et al., 2004), (Figure 1.1).
Figure 1.1 Commonly Occurring Carotenoids in Foods
The abundance of carbon and hydrogen in carotenoids makes this class of compounds lipophilic in nature. Carotenoids contain multiple conjugated double bonds, and this unique structure allows many of them to absorb visible light in the violet and blue region, and reflect hues from yellow to red. In fact, the colors of many common fruits and vegetables are provided by carotenoids (yellow and red peppers, orange pumpkins, squash, and carrots, etc.). β-carotene imparts the orange color to carrots, sweet potato, and orange pumpkins, and is also found in high levels in green leafy vegetables (1). Lycopene specifically imparts the red color to red tomatoes, watermelon, pink grapefruit, and guava fruit (2).

Carotenoids are also sensitive to isomerization and degradation under acidic and basic conditions and in the presence of molecular oxygen (3). This sensitivity is primarily due to the large number of double bonds, which provide the opportunity for a large number of theoretical stereoisomer configurations, in addition to making carotenoids inherently unstable. In nature, most carotenoids, including β-carotene and lycopene, are found predominantly in the all-trans configuration (4). Once extracted from the fruit, the conversion of all-trans carotenoid to various cis-isomers or degradation products can be catalyzed under a variety of conditions. For example, a review by Zechmeister details the ability of all-trans lycopene to isomerize in solution at room temperature, under reflux, and after exposure to ultraviolet and visible light (5). In addition, the author notes that relative to other carotenoids, lycopene is, “exceptionally sensitive,” to isomer conversion under these conditions (5).
1.2 Provitamin A Carotenoids

A sub-class of carotenoids, which contain an unsubstituted β-ionone ring, can be enzymatically cleaved to produce vitamin A in mammalian systems by β-carotene oxygenase 1 (BCO1) (6). Provitamin A carotenoids include β-carotene, α-carotene, β-cryptoxanthin, α-cryptoxanthin and γ-carotene. As noted in **Figure 1.2**, 1 mole of the symmetric β-carotene molecule is cleaved at the central double bond. Work by Nagao et al. (7) in a porcine model convincingly demonstrated that this reaction is stoichiometric. Thus, one molecule of β-carotene is cleaved to produce 2 moles of retinal. In contrast, 1 mole of α-carotene (or β-cryptoxanthin or γ-carotene) produces 1 mole of retinal (vitamin A).
Figure 1.2 Provitamin A Conversion to Vitamin A
Vitamin A, in the form of retinal or retinoic acid, is necessary for multiple biological functions. Retinoic acid, a ligand for the nuclear receptor known as retinoic acid receptor (RAR), is believed to be responsible for the majority of biological effects associated with vitamin A (8). It has also been suggested that retinoic acid can bind to the retinoid X receptor (RXR), a “master regulator” of a number of biological processes, but the evidence to support this is controversial (8). The biological effects of retinoic acid include growth promotion, cellular differentiation, immune function, embryonic development, and gap junction communication (9,10). The β-carotene metabolite retinal is believed to function primarily in the eye, where 11-cis-retinal bound to the opsin protein mediates the ability of the human eye to adapt to various intensities of light for proper vision (11).

Humans must obtain vitamin A from the diet, either in the form of preformed vitamin A in animal and fish products, or in the form of provitamin A carotenoids. In the American diet, it is estimated that approximately 70-75% of vitamin A is consumed in the form of the active vitamin, while the rest is consumed as provitamin A carotenoid (1). In 2001, the U.S. Institute of Medicine defined one 1 µg of all-trans retinol, 2 µg of β-carotene in oil, 12 µg of β-carotene in food, and 24 µg of β-cryptoxanthin or α-carotene in food as having the same retinol activity equivalents (1 RAE) biologically(12). In the United States, the recommended dietary allowance (RDA) of vitamin A for adult women is 700 RAE and for adult men is 900 RAE (12).

Vitamin A deficiency impacts a large percentage of certain populations, especially in the developing world. Globally, vitamin A deficiency is estimated to affect
0.9% of preschool children and 7.8% of pregnant women (13). Vitamin A deficiency is the leading cause of blindness in children, and is also associated with increased burden of infectious disease, xerophthalmia (dry eye syndrome), night blindness, and increased risk of mortality (14).

1.2.1 Bioavailability of Provitamin A Carotenoids

Bioavailability of carotenoids is defined as the amount of these micronutrients that are absorbed by the intestinal enterocytes, transported in the bloodstream and/or deposited in target tissues.
Figure 1.3 Carotenoid Absorption and Digestion, data adapted from (1,15,16).
A schematic of carotenoid digestion and absorption is shown in Figure 1.3. Once ingested, carotenoids must first be released from the food matrix before they can be incorporated into mixed micelles. Micelles contain bile salts, cholesterol, and fatty acids from the meal, and the amphiphilic nature of the micelle structure helps to keep the lipophilic nutrients soluble in the aqueous digesta (15). The micelles approach the unstirred water layer of the apical side of the intestinal cells (enterocytes), and the carotenoids may passively diffuse across the apical membrane (15). Historically, researchers believed that passive diffusion was the only route of absorption (17). However, in recent years, investigators have discovered that the absorption of lutein, lycopene, and β-carotene can be facilitated by a cholesterol membrane transporter known as scavenger receptor class B type I (SR-BI) (16,18). Once inside the enterocyte, the provitamin A carotenoids may be cleaved, esterified, and packaged into chylomicrons. In contrast, non-provitamin A carotenoids are directly packaged with other dietary lipids into chylomicrons (15). Chylomicrons are then transported across the basolateral membrane and make their way into the lymphatic system, which eventually releases chylomicrons into the blood via the thoracic duct. Thus, bioavailability of carotenoids can be monitored by measuring levels in the chylomicron-containing fraction (also called the Triglyceride Rich Lipoprotein fraction, or TRL fraction) of plasma.

1.2.2 Food Processing Effects on Provitamin A Carotenoid Bioavailability

Bioavailability can be affected by a number of factors, including food processing. Food processing is believed to break down plant cell structures to allow the carotenoids to be better absorbed. Human studies have demonstrated that carotenoids are more
bioavailable from processed foods than their raw or less processed counterparts. In a study by Rock et al. (19), healthy women consumed raw carrots and spinach for 4 weeks, and then crossed over to the consumption of processed carrots and spinach daily for 4 weeks. Blood levels of β-carotene were monitored before and after each feeding period (19). The daily consumption of the processed vegetables led to a three-fold increase in circulating plasma levels of β-carotene as compared to the raw counterparts. Similarly, a study by Edwards et al. (20) looked at the impact of progressively stronger processing treatments on β-carotene bioavailability in healthy humans. Raw chopped carrot, boiled mashed carrot, and processed carrot puree dose matched to deliver 18.6 mg of β-carotene were fed to 6 healthy humans. The carrot puree delivered ~2 fold greater β-carotene than the boiled mashed carrot. However, the amount of β-carotene absorbed from the raw carrot was in-between the amount of β-carotene absorbed from the carrot puree and the boiled mashed carrot in four of six subjects (20). In a study by van het Hof et al. (21) whole, peeled and canned tomatoes were treated with progressively stronger levels of homogenization (none, mild, and severe). Subjects (n=11) consumed each level of homogenized tomato in a 3-way crossover. A 9.2 fold increase in post-prandial AUC levels of β-carotene was observed for the severe homogenization meal as compared to the no homogenization meal (21).

### 1.2.3 Effect of Isomerization on Bioavailability

The geometric isomerization status of the carotenoids in the diet also has an impact on carotenoid uptake. The all-trans configuration is the primary geometric isomer observed for several carotenoids in most raw fruits and vegetables. However, under
certain food processing conditions, the *trans* isomer can be converted to various *cis* forms. For example, under certain processing conditions, significant isomerization can be observed for carotenoids found in tomatoes, carrots, corn, spinach, and mangoes (22–26). The isomeric profile of carotenoids in these fruits and vegetables can have a major impact on the bioavailability of these compounds in humans.

Unlu et al., demonstrated that *cis* isomers of lycopene are in fact more bioavailable in humans. Volunteers consuming tomato sauce processed to enhance cis lycopene isomer content absorbed significantly greater lycopene as measured by TRL post-prandial response as shown in **Figure 1.4** (27).
Figure 1.4 Dose-adjusted plasma TRL concentrations of total lycopene and lycopene cis-isomers following the consumption of sauce A (rich in all-trans-lycopene) and sauce B (rich in cis-lycopene) over 9.5 h. Values are means (n=12), with standard errors represented by vertical bars. (—○—), Total lycopene in sauce A; (—♦—), total lycopene in sauce B; (—○—,dotted line), cis-lycopene in sauce A; (—♦—, dotted line), cis-lycopene in sauce B. Taken from (27).
Similar observations were noted when consuming tomato sauce prepared from a tangerine variety of *Solanum lycopersicum* L. that contained 7Z, 9Z, 7′Z, 9′Z tetra-cis lycopene (28). Thus, *cis* isomerization of lycopene enhances lycopene uptake into the bloodstream. In contrast, cellular studies have demonstrated that *cis* isomers of β-carotene are not absorbed well by the enterocyte (29). *Cis* isomers of β-carotene are also not observed at high levels in the bloodstream, suggesting preferential absorption of the all-*trans* isomer of provitamin A nutrients (30).

### 1.2.4 Effects of Dietary Lipid on Carotenoid Bioavailability

One of the first studies to demonstrate that lipid fed with a β-carotene meal dramatically enhances the blood level of β-carotene was performed in adolescent boys in Rwanda (Roels et al., 1958). Subjects were kept at a hospital during the 41 days of the study, and divided into multiple feeding groups. Subjects were divided into 5 groups and consumed carotenoid and vitamin A free meals, in addition to one of the following at two daily meals: 100 g of grated carrot (containing 9.4 mg of β-carotene), 100 g of grated carrot with 10 mL of olive oil, 14 mg of carotene in 10 mL of oil, 10 mL of oil alone, or a placebo. Boys in the 10 mL of oil alone and the placebo groups showed no increase in blood carotene or vitamin A levels. Boys who consumed 100 g of carrot had very little increase in serum carotenoid levels, while boys who consumed the carrot with oil or the carotene in oil had dramatic increases in serum carotene and vitamin A levels (Roels et al., 1958)

Since this time, numerous studies have demonstrated that the consumption of a carotenoid containing meal with lipid dramatically enhances carotenoid bioavailability as
compared to a meal with no lipid. In a study by Brown et al., (31) α-carotene, β-carotene, and lycopene were consumed with a fat-free meal, a reduced-fat meal (6 g of fat), and a full-fat meal (28g of fat). Circulating concentrations of the carotenoids remained unchanged when they were given alone, whereas a step-wise increase in the area-under-the-curve of post-prandial response was seen with the reduced-fat and full-fat meals for all three carotenoids.

The effects of fat from whole avocado, a naturally lipid rich fruit, and the equivalent amount of avocado oil, on carotenoid absorption from a mixed salad have also been investigated. In this study, a basic salad meal consisting of 40 g of lettuce, 80 g of spinach, and 100 g of grated raw carrot was fed. In addition, no avocado fruit, 75 g avocado fruit, 150 g avocado fruit, or 24 g of avocado oil (equivalent to the fat content of 150 g of avocado fruit) was used as the lipid source in the meal. Subjects were fed the meal, plasma samples were taken over 10 hours, and the triglyceride rich lipoprotein (TRL) fraction was isolated. Dramatic increases in the uptake of α-carotene, β-carotene, and lutein from the salad were observed when subjects consumed the test salad with avocado or avocado oil as compared to the salad alone (Figure 1.5), as measured by TRL area-under-the-curve (AUC) values. The two high-fat meals (150 g of avocado fruit or 24 g avocado oil) produced no significant increase in carotene or lutein uptake as compared to the 75 g avocado meal. The same observations were made when a meal containing tomato salsa (containing the carotenoids lycopene and β-carotene) with and without avocado (guacamole) was fed. As with the salad carotenoids, the addition of
avocado to the meal dramatically increased the AUC of the TRL response for lycopene in healthy subjects (32).
Figure 1.5 Baseline-corrected plasma TRL concentrations of (A) α-carotene, (B) β-carotene, and (C) lutein during the 9.5 h after consumption of salad alone (n=11), with 75
g avocado (n=8), with 150 g avocado (n=11), or with 24 g avocado oil (n=11) in humans. Values are means +/- SEM. Taken from (32).
Lutein, α-carotene, and β-carotene AUC were greater after salad consumption with 150 g avocado and 24 g avocado oil than after salad alone (P < 0.01, Tukey’s test). The AUCs for salad with 75 g avocado were significantly higher for α-carotene and β-carotene (P < 0.003, Tukey’s test).

In vitro studies by Huo et al. (33) have investigated the relationship between fatty acid chain length and fatty acid saturation level on carotenoid micellarization – an important step in carotenoid uptake. In this study, a mixed salad meal containing lutein, α-carotene, β-carotene, and lycopene was carried through the in vitro digestive procedure with triglycerides composed of fatty acids of various chain lengths, including butyric acid (c4:0), caprylic acid (c8:0), and stearic acid (c18:0), in addition to c18 fatty acids of various levels of saturation (Huo et al., 2007). A statistically significant increase in micellarization was observed for carotenes when the 18 carbon fatty acids were tested as compared to no fatty acids, 4, or 8 carbon fatty acids. However, the degree of unsaturation of the C18 fatty acids (c18:1, c18:2, c18:3) did not impact micellarization (33).

Similarly, various human studies have investigated the impact of fatty acid chain length on carotenoid uptake. A study by Borel et al. investigated the impact of medium chain triglycerides (c6-c16) and long chain fatty triglycerides (c14-c22) on β-carotene uptake (34). In this study, subjects were fed 120 mg of β-carotene with 40 g medium chain triglycerides, or 40 g long chain triglycerides, and chylomicron levels of β-carotene were monitored over 6 hours (34). Consumption of β-carotene with long chain triglycerides resulted in 4.6 fold greater β-carotene AUC levels as compared to β-
carotene consumed with medium chain triglycerides (34). More recently, another human study investigated the impact of the amount of fatty acids, fatty acid chain length, and fatty acid saturation on carotenoid uptake (35). In this study, human subjects were fed varying amounts (3, 8, and 20 g lipid) of different types of lipid (poly-unsaturated fatty acid rich soy oil, mono-unsaturated fatty acid rich canola oil, or short-chain fatty acid rich butter) with a mixed salad meal. The AUC of TRL carotenoids (α-carotene, β-carotene, lutein, zeaxanthin, and lycopene) was measured over 10 hours. For total carotenoids, there was a statistically significant increase in carotenoid absorbed when 20 g lipid was consumed as compared to 3 g lipid. While there was a trend for higher absorption of carotenoids at the 3 g lipid level of canola or soybean oil (long chain fatty acids) as compared to butter (short chain fatty acids), this was not statistically significant (35).

1.2.5 Conversion of Provitamin A to Vitamin A in the Enterocyte

The absorption of provitamin A and conversion to vitamin A in the enterocyte has been studied using a number of different methods. By measuring the provitamin A carotenoid and retinyl ester levels found in the TRL fraction, the amount of conversion which occurs in the enterocyte can be estimated.

Work by van Vliet et al. first demonstrated that the post-prandial TRL β-carotene and retinyl palmitate levels could be used as a measure of β-carotene absorption from the meal and subsequent conversion to vitamin A in the enterocyte, and estimated that 35%-71% of the β-carotene absorbed from a 15 mg dose fed with 50 g of oil was converted to vitamin A (36). Another study by Edwards et al. (2001), fed 6 mg of β-carotene with 20 g of lipid, and isolated TRL fractions post-prandially. It was determined that 4% of the
initial β-carotene dose was absorbed, of which 63% was converted into vitamin A in the enterocyte. A second study by Edwards at al, (2002), fed pureed cooked carrot (delivering 18.6 mg β-carotene and 7.5 mg α-carotene) or boiled mashed carrot (delivering 18.6 mg β-carotene and 7.2 mg α-carotene) to subjects, and again carotenes and retinyl esters were monitored in the TRL fraction of the blood. It was estimated that 5% of the carotene dose was absorbed from the pureed cooked carrot, and 3% of the carotene dose was absorbed from the boiled mashed carrot. Furthermore, of the absorbed carotene dose, the conversion efficiency of β-carotene to vitamin A was found to be 44% for the carrot puree meal, and 59 % for the boiled mashed carrot meal (Edwards et al., 2002). Likewise, in a study where red palm oil was fed (containing 2.4 mg β-carotene and 1.8 mg α-carotene), an average of 23% of the carotene dose was absorbed, of which 43% was converted into vitamin A in the enterocyte (You et al., 2002). Taken together, these studies (and numerous others) suggest that while a relatively low proportion of the carotene dose is absorbed from a carotene-rich meal, a high percentage of the carotene absorbed is converted into vitamin A in the enterocyte and secreted as retinyl esters in the chylomicron fraction when lipid is present.

1.2.6 Effects of Different Types and Levels of Lipid on Conversion of Provitamin A to Vitamin A

Much of the work of the effect of lipid level and lipid type on absorption and conversion of provitamin A to vitamin A has been done in animals. In one study with Mongolian gerbils, a diet containing carrot powder with 10% lipid (n=12) or carrot powder with 30% lipid (n=12) was fed for 2 weeks (37). Animals were sacrificed, and
hepatic carotenoid and vitamin A stores were measured. Animals in the 10% lipid group had significantly higher β-carotene and α-carotene and lower vitamin A levels in liver as compared to the 30% group (37). A similar study by Lakshman et al. (38) compared the effect of the consumption of β-carotene with 6%, 13.4%, or 23% lipid in ferrets. After 4 weeks of consumption of the various diets, hepatic β-carotene and retinyl esters levels were measured. The consumption of 13.4% or 23% resulted in hepatic β-carotene levels which were approximately double those of the 6% group. In contrast, a stepwise increase in dietary lipid was correlated with a stepwise increase in hepatic retinyl ester stores (38).

Taken together, these studies suggest that consuming a higher level of dietary lipid increased conversion of provitamin A to vitamin A. This increase in hepatic vitamin A stores and decrease in β-carotene stores may in fact be due to higher conversion of provitamin A to vitamin A in the intestine. Higher lipid consumption, as well as lipid type, has been shown to have an impact on BCO1 activity, the enzyme responsible for the conversion of provitamin A to vitamin A in rats. During et al. (39) fed four different groups of rats a standardized diet containing 2.5% polyunsaturated fat, 15% saturated fat, 15% monounsaturated fat, or 15% polyunsaturated fat for three weeks. Animals were sacrificed, the BCO1 protein fraction of the intestinal homogenate was incubated with β-carotene for 30 minutes, and the resulting retinal was quantitated. Animals in the 15% mono and poly unsaturated groups had 2.4 fold greater specific BCO1 activity (as measured by retinal formation) than the 2.5% polyunsaturated fat group. In contrast, there was no significant difference between the saturated fatty acid group and the 2.5% polyunsaturated fat group (39).
With regards to the absorption and conversion of provitamin A to vitamin A in humans, research has demonstrated that consumption of long-chain fatty acids increases both β-carotene and retinyl ester levels observed in chylomicrons as compared to medium chain fatty acids (34). The increase in β-carotene bioavailability with long chain fatty acids may be partially explained by an increase in chylomicron synthesis stimulated by long chain fatty acids (40) or an increase in bioaccessibility (amount of carotenoid which passes through the apical side of the enterocyte, Figure 3 (33). The increase in these retinyl ester levels may be may be due to an increase in β-carotene absorption, or due to increased intestinal BCO1 activity stimulated by long-chain fatty acids, as suggested by the rat study described above (39).

1.2.7 Effects of Other Carotenoids on Conversion of Provitamin A to Vitamin A

In a study by van den Berg et al. (41), the effect of various carotenoids on the absorption and conversion of β-carotene to retinyl palmitate was investigated. In this 3-way crossover study, healthy subjects consumed palm oil alone (delivering 15 mg of β-carotene and 4.7 mg β-carotene), palm oil with 15 mg lycopene, or palm oil with 15 mg lutein. The AUC of β-carotene and retinyl esters was monitored over 10 hours post meal consumption. The consumption of lycopene reduced β-carotene AUC by 27% and did not significantly affect retinyl palmitate AUC. The consumption of lutein reduced β-carotene AUC by 44% and retinyl palmitate AUC by 38%. For the lutein meal, the change in AUC β-carotene and retinyl palmitate levels was almost the same, suggesting that the rate of conversion of β-carotene to retinyl palmitate was maintained. Thus, these authors concluded that lutein interfered with β-carotene uptake (41).
1.3 Non-Provitamin A Carotenoids

1.3.1 Biological Effects of Non-Provitamin A Carotenoids

Other carotenoids have been associated with health-promoting properties as well. Lutein and zeaxanthin deposit in the retina of the eye, and studies are currently investigating the ability of these compounds to modulate the incidence and severity of macular degeneration (42,43). In addition, the consumption of carotenoid containing fruits and vegetables is associated with a reduced risk of developing cardiovascular disease and cancer (44,45). Below, we have chosen to highlight recent research from just a few studies which focus specifically on tomatoes or lycopene and disease risk.

Epidemiological studies have associated tomato consumption with a decreased risk of cardiovascular disease. A study by Sesso et al. (46) of 38,445 women found that higher levels of tomato-based product intake were associated with a reduced risk of cardiovascular disease (RR = 0.71, \( P_{\text{trend}} = 0.029 \)) and myocardial infarction (RR = 0.43, \( P_{\text{trend}} = 0.033 \)) between highest and lowest quintiles of intake. Increased plasma lycopene levels have been associated with reductions in CVD risk. A study in 1,028 Finnish men found an inverse association between serum lycopene concentrations and common carotid artery intima-media thickness, a measure of early atherosclerosis (47). In addition, a study using a subset of data (n=4,557) from the third National Health and Nutrition Survey (NHANES III) observed decreased serum levels of carotenoids (including lycopene) in individuals with higher levels of C-reactive protein, a marker of inflammation (48). Since the primary source of lycopene in the American diet comes from tomatoes (49), we could surmise that the associations observed in the studies...
measuring serum lycopene are due in large part to tomato and tomato product consumption.

Cancer is the second leading cause of death in the United States, with approximately 1.5 million new cases of cancer diagnosed in 2008 (50). The consumption of red tomatoes and tomato products has also been associated with a reduced incidence of a number of different types of cancers, most notably prostate, lung, and stomach (51,52). Some of the strongest epidemiological evidence to support an association between tomato product consumption and a reduced incidence of cancer has come from the Health Professionals Follow-Up Study (HFPS). Most recently, a prospective observational study by Giovannucci et al. (53) collected food frequency questionnaire (FFQ) data from the HPFS group of 47,365 men in 1986, 1990, and 1994. Intake of ≥ 2 servings of tomato sauce per week was associated with a reduced risk of prostate cancer (relative risk (RR) = 0.77 relative to < 1 serving of tomato sauce per month, P_trend < 0.001).

Researchers have presumed that lycopene, the most abundant carotenoid in tomatoes, is responsible for reducing cardiovascular disease and cancer incidence associated with tomato consumption. Lycopene has also been shown to act as an antioxidant in vitro. Research performed by Di Mascio et al. (54) demonstrated that lycopene was the most efficient carotenoid in quenching singlet oxygen. The rate of reaction between singlet oxygen and lycopene is twice as high as that of β-carotene and 100x as high as the rate of reaction with α-tocopherol (54). Researchers have suggested that health benefits observed from consuming tomatoes and tomato products may be due to the ability of carotenoids to quench free radicals in vivo, preventing the propagation of
free radicals and preventing damage to lipid membranes (55). However, this has never been proven. In fact, tomatoes contain a host of other phytochemicals, including lycopene precursors, \( \beta \)-carotene, vitamin C, flavonoids, folic acid, and tomatine, which may favorably impact health (56–59). More recently, researchers have also suggested that lycopene may be enzymatically metabolized into a bioactive compound (60,61).

1.3.2 Tomatoes, Tomato Products, and Lycopene

Lycopene is the predominant carotenoid in red tomatoes. Fresh red tomatoes contain 3.1 mg–7.74 mg lycopene/100 g wet wt., while processed products may contain significantly more (2). Tomato paste, the most concentrated product, has the highest amount of lycopene with approximately 30 mg/100 g wet wt. (2). Tomatoes also contain lycopene precursors. Whole processed tomatoes contain 1.86 mg phytoene, 0.82 mg phytofluene, 1.11 mg neurosporene, and 0.21 mg of \( \zeta \)-carotene per 100 g wet wt. (62). These precursors are found at even higher levels in mutant varieties of tomatoes such as the tangerine tomato. In this variety, the enzyme catalyzing the isomerization of tetra-cis lycopene to all-trans lycopene is impaired, causing a buildup of precursors in the carotenoid synthesis pathway (63). Consequently, the levels of phytoene and phytofluene are ~3-fold higher in this variety, while the levels of neurosporene and \( \zeta \)-carotene are ~25-fold higher (63).

Traditional processing methods do not have a significant effect on lycopene stability or cis/trans isomerization (22). In fact, greater than 90% of the lycopene found in commercially processed tomato products is in the all-trans conformation (64). In addition, the carotenoid precursors (phytoene, phytofluene, neurosporene, and \( \zeta \)-carotene)
appear to be resistant to heat treatment typically employed during commercial thermal processing, although few studies have been done (65,66).

1.3.3 Lycopene in Humans

Multiple studies have shown that lycopene from thermally processed tomato products is more bioavailable than lycopene from fresh tomatoes (67–69). This increase in bioavailability at least partially results from the disruption of cellular membranes during thermal processing, which allows lycopene to be released from the tissue matrix (64). *In vivo* studies demonstrate that the *cis*-isomers of lycopene are more bioavailable than the *all-trans* isomer (28,68). *In vitro* experiments suggest that increased bioavailability of lycopene *cis*-isomers is at least partially due to increased micellarization and increased uptake by the enterocyte relative to all-*trans* lycopene (70).

A study observed lycopene absorption after consumption of tomato juice (with constant % fat) containing a series of doses between 10 mg to 120 mg of lycopene (71). The range of lycopene absorbed, independent of dose, was between 1.8 mg – 14.3 mg, with an average of 4.7 mg. The amount of lycopene absorbed by the men consuming juice containing 120 mg lycopene was not significantly different from the men consuming juice containing 10 mg lycopene. However, since this study was not designed as a crossover, the fact that no difference was observed could be attributed to inter-individual variability between subjects (71).

In free living men and women of various ages, Yuem et al. (72) observed circulating levels of lycopene in blood between 0.44-0.64 μmol/L. Studies have shown that lycopene levels may be significantly impacted by diet. We have observed blood
lycopene levels as low as 0.27 μmol/L after a 7 day washout (73) and as high as 1.28 μmol/L after 4 weeks of tomato juice consumption delivering 21 mg lycopene/day (74). Levels of lycopene reported in human tissues vary. Levels as high as 21 nmol lycopene/g wet wt. tissue have been reported in human adrenal, testes, and liver of individuals (75,76). Adipose and kidney levels have been reported as high as 1.30 nmol/g wet wt. and 0.62 nmol/g wet wt., respectively (75,76). Prostate levels as high as 1.7 nmol/g wet weight have been reported (77). Skin levels of lycopene have been reported as high as 1.75 nmol/g wet wt. (78).

Studies suggest that approximately 40% of the lycopene found in blood and 20% of the lycopene found in human prostate tissue are in the all-trans form (73,74,79). Interestingly, the ratio of cis to trans lycopene in circulating blood plasma varies over time. In a study by (73), the ratio of 5-cis to all-trans lycopene at enrollment (before washout) was 0.877. After a 7 day washout where subjects abstained from consuming any lycopene containing foods, the ratio increased to 1.125 (i.e. 5-cis lycopene rose relative to all-trans lycopene). After 15 days of condensed tomato soup consumption (35 mg lycopene per day), the ratio of 5-cis to all-trans dropped to 0.943 (73). This study provides evidence to support the hypothesis that all-trans lycopene isomerizes after absorption in vivo. However, the significance of cis-isomers has yet to be elucidated.

1.3.4 Carotenoid Precursors in Humans

Levels of phytoene in plasma have been reported from 0.10 - 0.85 μmol/L (80,81), while levels of phytofluene range 0.27 – 0.72 μmol/L and levels of ζ-carotene range from 0.12 – 0.26 μmol/L (80). Phytoene, phytofluene, and ζ-carotene have also
been reported in human liver, lung, breast, cervix, prostate, and colon tissues at levels between 45-1275 ng/g tissue (82). A study by Hata et al. (83) of human abdominal skin found phytoene levels from 24 – 92 ng/g, phytofluene levels from 6 – 21 ng/g, and ζ-carotene levels from 4 – 25 ng/g.

1.3.5 Lycopene in Rodent Tissues

Lycopene can be absorbed and deposited into tissue in rodents, although the extent to which depends on the species and strain of animal. One study by Huang et al. (84) compared the levels of lycopene in tissues of male F344 rats, BALB/c mice, athymic nude mice, and Mongolian gerbils who had been supplemented with 20mg lycopene/kg BW every other day for 10 days. These researchers found lycopene in the highest concentrations in the liver, with lower levels found in kidney, and much smaller amounts in the lung, brain, and testes of all four animal models studied. Mongolian gerbils had the highest levels of lycopene in all tissues, followed by the nude mice, rats, and BALB/c mice. In the nude mice, liver and kidney levels were 524 nmol/g and 60 nmol/g, respectively, while the levels in the rats were ~15-fold lower and levels in BALB/c mice were ~100-fold lower (84). Nude mice had plasma lycopene levels of 0.22 μmol /L (84). Lindshield et al. (85) reported similar levels of lycopene in liver of C57BL/6 x 129/SvJ mice who had consumed diets supplemented with lycopene beadlets (providing 150 mg lycopene/kg diet) for 60 days (469 nmol/g liver tissue). However, the level of lycopene reported in the kidney was significantly lower (2.6 nmol/g), and the level found in blood serum was higher (0.59 μmol/L) (85). Studies in rats have shown that levels of lycopene
deposited in liver are generally lower than lycopene levels observed in mice liver (86–88), except for one report by Zhao et al. (89) where levels reached up to 244 nmol/g.

1.3.6 Lycopene and Plant Carotenoid Cleavage Enzymes

To date, nine carotenoid cleavage dioxygenase enzymes (CCD) have been identified in arabidopsis. Of these nine, five enzymes are involved in the synthesis of abscisic acid, an important hormone regulating plant growth. The activity of the other four CCD enzymes is still being characterized. CCD1 from maize and tomato has been shown to cleave lycopene at the 5’-6’ position and 9’-10’ position (90). While only the short, volatile products of these cleavages were determined by GC-MS (pseudo-ionone and 6-methyl-5-heptene-2-one), the complementary long-chain product of this enzymatic step would likely be apo-6’-lycopenal and apo-10’-lycopenal, respectively. In addition, carotenoid cleavage dioxygenase 1 (CCD1) in the annatto plant has been shown to cleave lycopene to produce bixin aldehyde, a precursor of the carotenoids bixin and norbixin (91). CCD7 appears to cleave the 9’-10’ double bond of lycopene, but the resulting product has only tentatively been identified with UV spectra data and retention time (92). It is not clear if CCD8 cleaves lycopene, and there is no literature discussing the ability of CCD4 to act on lycopene. It is also possible that enzymes demonstrated to be involved in the abscisic acid pathway may cleave lycopene. In addition, in vitro evidence suggests that lycopene could be non-specifically co-oxidized with lipid by lipoxygenase enzymes in the fruit (93).
1.3.7 Lycopene and Mammalian Carotenoid Cleavage Enzymes

The most widely characterized pathway of carotenoid metabolism is the formation of vitamin A by the cleavage of β-carotene (or other pro-vitamin A carotenoids) at the central double bond by β-carotene oxygenase 1 (BCO1) to form two molecules of retinal (6). However, research suggests that BCO1 does not metabolize lycopene (94). A second carotenoid cleaving enzyme, β-carotene oxygenase 2 (BCO2), has also been identified (95). BCO2 has been shown to eccentrically cleave β-carotene (95) and lycopene (95,96). Hu et al. (96) transfected COS-1 cells with ferret BCO2, and found that incubation with 5-cis-lycopene and 13-cis-lycopene (but not all-trans lycopene) produced apo-10'-lycopenal. Other researchers have incubated lycopene with various tissue homogenates ex vivo and produced oxidative products, but it is unclear whether these are products of a BCO reaction or whether they could be produced in vivo (93).

A few studies on knockout animals have investigated the direct effects of BCO1 and BCO2 in vivo. A study by Lindsheild et al. (85) fed a lycopene beadlet containing diet to wild type and BCO1 knockout mice for 60 days. Interestingly, hepatic liver stores of lycopene were 4.7 fold higher in the wild type mice as compared to the knockout mice, while serum levels remained the same. Since BCO1 has not been shown to metabolize lycopene (94), it would be expected that the knockout animals would accumulate higher levels of lycopene as compared to the wild type animals. These authors suggested that another enzyme may be responsible for metabolizing lycopene, or sequestering lycopene in tissues which were not tested, in the knockout animals (85). In another study by the same group, wild type and BCO2 knockout mice were fed an AIN-93G diet containing
10% tomato powder or a lycopene beadlet for 4 days or 30 days (97). After 4 days of feeding, liver lycopene was 6.3 fold higher in the BCO2 knockout mice as compared to the wild type mice for the lycopene beadlet group. Likewise, liver lycopene was 3 fold higher in the BCO2 knockout mice after 30 days (97). These results support the hypothesis that BCO2 may be responsible for metabolizing lycopene in vivo.

1.3.8 Lycopene and Phase I & Phase II Metabolism

In a study by Zaripheh et al. (98), rats were fed lycopene as part of their daily diet for 50 days, followed by a radioactive dose of $[^{14}\text{C}]$ labeled lycopene on day 51. Afterwards, radioactivity was observed in both the feces and the urine up to 168 hrs. post-dosing. While a majority of the excreted radioactivity was observed in feces, ~3% of the radioactivity observed was in urine (98). A similar study was recently conducted in humans, where $[^{14}\text{C}]$ labeled lycopene was fed to two healthy subjects and samples of blood plasma, urine, skin, and breath CO$_2$ were collected (14). Accelerator mass spectrometry (AMS) was used to measure the radioactive dose. It was determined that 17%-19% of the radioactivity was excreted in the urine over 6 days after the lycopene dose consumption (14). Since lycopene is particularly hydrophobic, it is reasonable to hypothesize that it would need to be metabolized to more polar compounds in order to be excreted via urine. Metabolism by carotenoid cleavage enzymes provide one possible route to a more water-soluble metabolite. However, an alternative route to creating such a product would be via metabolism by phase I and phase II enzymes. Ross et al. treated urine samples with β-glucuronidase and sulphatase to deconjugate potential phase II
metabolites, but specific compounds were not identified, so it is unclear at this time if phase II metabolites were present (14).

A study by Jewell & O’Brien (99) fed male Wistar rats a diet supplemented with lycopene (300 mg/kg diet) for 16 days. Afterwards, the activity of cytochrome P450 (CYP450) monooxygenase activity towards ethoxyresorufin (specific for P450 1A1), methoxyresorufin (specific for P450 1A2), pentoxyresorufin (specific for P450 2B1/2), benzyloxyresorufin (non-specific for P450 1A1/2, 2B1/2, and 3A) was determined in various tissues (liver, lung, kidney, small intestine). Glutathione-S-transferase activity and glutathione levels were also measured. There was no difference observed in the activity of all enzymes measured or in glutathione levels between the lycopene supplemented group and the negative control group (99). An almost identical study performed by a different research group determining activity of the same four CYP450 enzymes also found no change in enzyme activity after lycopene supplementation (100). However, this group found that lycopene reduced the activity of nitrosodimethylamine N-demethylase (100). Similarly, a study by Liu et al. (101) found no change in CYP 1A1 activity or NAD(P)H:quinone reductase 1 activity (NQO1) in male Copenhagen rats fed 10% tomato powder for 7 days. In contrast, a study by Breinholt et al. (102) observed dose dependent increases in CYP activity towards ethoxyresorufin (specific for P450 1A1) and benzyloxyresorufin (non-specific for P450 1A1/2, 2B1/2, and 3A) in female Wistar rats fed a lycopene/corn oil solution via gavage daily for 14 days. A dose response effect of lycopene on activity of glutathione-S-transferase was observed, and interestingly, a U-shaped dose response curve was observed for other phase II enzymes.
investigated in this study (NQO1, UDP-glucuronosyltransferase) (102). O’Brien and O’Connor (103) have suggested that these different results may be due to differences in gender, species strain, or differences in levels of lycopene achieved in vivo (which was not measured in most studies cited above).

One human study observed the difference in CYP1A2 activity between individuals with colorectal cancer (n=43) and healthy controls (n=47) in relation to plasma carotenoid levels (104). In this study, plasma lycopene was positively associated with CYP1A2 activity (p = 0.06).

1.3.9 Lycopene Oxidation Products in Plants and Animals

A handful of lycopenoids (a term used to describe oxidative metabolites of lycopene) have been identified in raw tomatoes and tomato products (Figure 1.6). Apo-6’-lycopenal and apo-8’-lycopenal, were reported in tomato paste (105). Later in 1973, Ben Aziz et al. (106) confirmed the presence of apo-6’-lycopenal and apo-8’-lycopenal in raw tomatoes, indicating that these products are present in the unprocessed fruit as well.
Figure 1.6 Structures of Various Lycopeneoids
Khachik et. al. first reported lycopene derivatives in human blood plasma (107), which were later identified as two diasteromeric forms of 2,6-cyclolycopene-1,5-diol (108). More recently, lycopenoids have been identified in the tissues of animals consuming lycopene. In one study, ferrets consumed a lycopene supplement (lycovit powder, 10% lycopene) daily for 9 weeks (96). Following sacrifice, apo-10’-lycopenol, and alcohol, was found in the lung tissue of these animals (96). These researchers went on to incubate ferret liver homogenate ex vivo with apo-10’-lycopenal (an aldehyde), and discovered that apo-10’-lycopenoic acid was produced when NAD+ was added as a cofactor (96). In a separate study by Zaripheh et al. (98), F344 rats were fed lycopene as part of their daily diet for 50 days, followed by a radioactive dose of lycopene on day 51. Various tissues were extracted and analyzed by high performance liquid chromatography (HPLC)-photo diode array (PDA). HPLC fractions were collected and further analyzed by scintillation counting (98). Polar products, defined as products containing radioactivity which eluted in the first 5 min. of the HPLC run, were found in multiple tissues. Polar products were observed in liver (20% of isotope label), seminal vesicle (38% of isotope label), and prostate (67% of isotope label). In contrast, it is noted that polar products in the kidney, adrenal glands, and feces did not change over time, but it is unclear what % of the total isotope label was attributed to polar products in these tissues (98). Upon further analysis, the rat livers from the Zaripheh et al. (98) study were pooled and extracted with non-polar solvent, and apo-8’-lycopenal and putative apo-12’-lycopenal were identified (109).
1.3.10 Chemical Oxidation of Lycopene

Effort has been put forth to oxidize lycopene to produce non-symmetric aldehyde metabolites which could be produced in vivo. In one study, lycopene dissolved in dichloromethane with methylene blue was oxidized by irradiation with a tungsten lamp combined with bubbling air (110). In this study, apo-5-lycopenone and apo-6’-lycopenal were the primary products identified. Kim et al. (111) produced an entire series of aldehyde cleavage products of lycopene using a variety of oxidative methods including ozonolysis, and incubation at 37°C for 72 hrs. with toluene, aqueous Tween 40, or liposomal suspension. Likewise, a similar series of aldehyde derivatives were generated by Caris-Veyrat et. al. (112) using potassium permanganate as an oxidant.

1.3.11 Biological Effects of Lycopenoids

Very few reports exist which investigate the biological effects of lycopenoids. King et al. (113) investigated the effectiveness of 2,6-cyclolycopene-1,5-diol in upregulating connexin 43 gene expression, a gap junction protein, in human HaCaT cells. After treating cells with lycopene and 2,6-cyclolycopene-1,5-diol in the $10^{-7}$ to $10^{-5}$ M range, a dose dependent increase in connexin 43 was observed (113).

Apo-10’-lycopenoic acid has been shown to be effective in the treatment of BEAS-2B human bronchial epithelial cells in vitro as measured by enhanced expression of Nrf2 (114). Nrf2, a nuclear receptor factor associated with the upregulation of phase II enzymes, increased intracellular glutathione, and oxidative stress responses in this model (115). Similarly, work by Linnewiel et al. (116) demonstrated that apo-10, 10’-lycopenaldehyde significantly increased electrophile response element/antioxidant
response element transcription system (EpRE/ARE) reporter activity in MCF-7 cells breast cancer cells and LNCaP prostate cancer cells. This dialdehyde also significantly inhibited MCF-7, LNCaP, and T47D (breast cancer cell) growth *in vitro*, a result which was correlated to EpRE/ARE transduction (116). The EpRE/ARE pathway is also activated via Nrf2 (116). The feeding of apo-10’-lycopenoic acid (0,10, 40, or 120 mg/kg diet) has also been shown to reduce lung tumor multiplicity in an A/J mouse model with lung tumorigenesis induced by 4-(N-methyl-N-nitrosamino)-1-(3-pyridal)-1-butanone (NNK) (114). Animals were fed the apo-10’-lycopenoic acid diet for 2 weeks before NNK administration, and then fed for 14 weeks after NNK injection. The effect of apo-10’-lycopenoic acid on final lung tumor burden was dose-dependent. Mice who had consumed no apo-10’-lycopenoic acid had 15 lung tumors on average, while mice consuming 120 mg/kg diet had 5 tumors on average (114). The feeding of apo-10’-lycopenoic acid (ALA) has also been shown to play a role in reducing liver steatosis in the *ob/ob* mouse model which is used to model obesity and non-alcoholic fatty liver disease (117). In this study, one group of mice (n=12) was fed a high-fat control diet with a daily level of apo-10’-lycopenoic acid that would be equivalent to ~15 mg/day of lycopene consumed by a 60 kg adult male (117), while another group of mice (n=12) was fed the control diet alone for 16 weeks. While body and liver weight was not significantly different between the two groups, a statistically significant lower distribution of liver steatosis (graded using hematoxylin and eosin staining) was observed in the ALA group as compared to the control group (117).
1.4 Carotenoids and Skin

1.4.1 Solar Ultraviolet Light and the Skin

The sun emits electromagnetic radiation in the ultraviolet (UV) range from 280nm-400nm. This range is further subdivided into three categories: UVC (<280nm), UVB (280-315nm), and UVA (315-400nm). UVC, the most damaging (i.e. highest energy) radiation, and some UVB radiation is completely filtered out by the ozone layer of the stratosphere which absorbs wavelengths up to 310 nm (118). However, some UVB and all UVA pass through to ground level and can cause damage to dermal tissue. UVB passes through the epidermis and can be absorbed directly by DNA and proteins causing damage (118). Cyclobutane pyrimidine dimmers (CPD), created by the reaction of neighboring DNA pyrimidine bases, are primarily formed by UVB (119). UVA rays penetrate further to the dermis and cause indirect damage through the generation of reactive oxygen species (ROS). ROS are formed when chromophores in the skin are excited by photons, and this energy is dissipated by transferring an electron to a substrate molecule or molecular oxygen (120). ROS may also be released by phagocytic cells during an acute inflammatory response like a sunburn (121). ROS can cause DNA damage. For example, the hydroxyl radical can oxidize the DNA base guanosine to produce 8-hydroxy-2’-deoxyguanosine (8-oxo-dG) (122). Over time, if these DNA adducts are not excised and repaired, this damage can lead to cancer (122).

1.4.2 Carotenoid Protection Against UV Light

The most important mechanism of photo-protection in the skin is the pigment melanin (123). Melanin absorbs light reducing the amount which passes through the
layers of skin and consequently reducing the subsequent damage (123). Carotenoids also have the ability to absorb light. In fact, researchers have suggested that one of the primary roles of plant carotenoids is to capture and assist in the dissipation of excess energy produced during photosynthesis (124). Research suggests that carotenoids may be able to protect the skin during sun exposure in humans as well. ß-carotene has been used therapeutically in individuals with erythropoetic protoporphyria (EPP). Individuals with EPP accumulate protoporphyrin in their tissues because of a defect in ferrochelatase, the enzyme which inserts iron into the protoporphyrin ring to make heme for red blood cells (125). Consequently, individuals with EPP are especially sensitive to light, a symptom which is ameliorated with oral ß-carotene supplementation (125).

Recent studies have suggested that the consumption of tomato products or lycopene may alter the UV sensitivity of healthy individuals. The ability of lycopene to affect UV induced erythema (mild sunburn) was investigated in 9 healthy humans consuming 40 g tomato paste (~16mg lycopene/day) with olive oil daily for 10 weeks (126). The control group (n=10) consumed olive oil alone. At week=0 and week=10, subjects were irradiated with a solar simulator, and the a-value (i.e. redness) of skin tone was measured by chromatometry. Individuals in the treatment group had a 32% reduction in a-values between week=0 and week=10. In addition, the a-values of the treatment group were 40% lower than controls, indicating that the tomato paste treatment was protective against UV induced sunburn (126). A separate clinical study was performed in 36 healthy adults where subjects consumed synthetic lycopene alone, a soft-gel encapsulated tomato extract, or a tomato drink for 12 weeks (127). Dorsal skin was
irradiated at weeks 0, 4, and 12 with artificial UV light at levels high enough to cause a mild sunburn. The subjects consuming the tomato extract and tomato drink had a 38% and 48% decrease, respectively, in solar simulator induced sunburn at week 12, compared to only a 25% decrease in the group treated with synthetic lycopene (127). These authors hypothesized that the phytoene and phytofluene found in the tomato extract capsule and tomato drink but not the synthetic lycopene capsule might be responsible for the greater decrease in erythema formation. Indeed, this hypothesis seems plausible since phytoene absorbs maximally at 285 nm in the middle of the UVB range, and phytofluene absorbs maximally at 350 nm in the middle of the UVA range (128). Interestingly, a study performed in the early 1980s using hairless mice observed that mice treated intraperitoneally with phytoene for 10 weeks before a single dose of tumor inducing UV radiation developed no tumors (129). This was in contrast to the control group of mice that developed tumors after receiving vehicle only (129).

1.5 Analysis of Carotenoids by Mass Spectrometry

Mass spectrometry data is often paired with UV-Visible spectra, NMR, or HPLC retention time for carotenoid identification. In addition, HPLC coupled with MS as a detector has been reported to be 100 times more sensitive than PDA for detection and quantification of some carotenoids (130,131). While mass spectrometry can be a powerful tool, it should be noted that the analysis of carotenoids (which are nonvolatile, thermally labile, and inherently unstable) presents a special challenge to the mass spectrometry analyst.
1.5.1 Ionization

There are many types of ionization methods that have been tested with carotenoids, which are discussed below. Because hydrocarbon carotenes do not contain functional groups which can easily gain or lose protons, it has been observed that multiple ionization methods produce a molecular anion $[M^-]$ in negative mode or molecular cation $[M^+]$ in positive mode as the predominant species of carotenes in-source. In these instances, an electron is either gained or lost, respectively, to produce a charged species. Since the molecular weight of an electron is negligible and most mass analyzers are not sensitive enough to detect such a small change, the mass of these ions is the same as the mass predicted by the chemical formula. Some compounds like peptides often produce multiply charges species in source, but carotenoids generally do not.

The background matrix often presents a challenge in carotenoid analysis by MS, especially with foods or biological samples in which a significant amount of lipid is present. These lipids (often not visible by PDA) can compete with the carotenoid for ionization in-source and increase background noise. If enough background is present, significant decreases in sensitivity and selectivity may be observed. It is often necessary to identify sample preparation steps (as discussed previously) which extract or selectively remove carotenoids from the lipophilic background without degrading carotenoids in the sample. Alternatively, the use of different HPLC methods (to chromatograph background differently) or use of stable isotopes as internal standards for quantification (132,133), can help to reduce or eliminate the problems posed by sample matrix.
Often a mass spectrometer is interfaced with an HPLC-PDA system. This technique is especially useful because isobaric species can be chromatographically separated before entering the MS. Interestingly, there are a large number of isobaric species in the field of carotenoids, such as lycopene, β-carotene, α-carotene, and γ-carotene which all have a parent mass of 536 mu, or β-cryptoxanthin, α-cryptoxanthin, zeinoxanthin, and rubixanthin which all have a parent mass of 552 mu.

Earlier methods of ionization applied to carotenoids, including electron impact (EI), chemical ionization (CI), a particle beam interface with EI or CI, and continuous-flow fast atom bombardment (CF-FAB), have been comprehensively reviewed elsewhere (134–136). These techniques have generally been replaced by “softer” ionization techniques like electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI), and more recently atmospheric pressure photoionization (APPI). It should be noted that ESI, APCI, and APPI can be used as ionization methods with a direct infusion of an analyte in solution (i.e. not interfaced with an HPLC system), or as the interface between the HPLC and the MS. In contrast, matrix-assisted laser desorption ionization (MALDI) cannot be used directly with HPLC.

ESI – Van Berkel first tested β-carotene with ESI positive in 1994 (137). In this study, a doubly charged molecular ion of β-carotene was observed as the primary species when trifluoroacetic acid was present in the solution. Van Breemen was the first to utilize ESI as an interface between HPLC and MS to analyze carotenoids (130). In this study, ESI operated in negative mode ionized xanthophylls (astaxanthin, β-cryptoxanthin, and lutein), but did not ionize hydrocarbon carotenes (lycopene and β-carotene). In contrast,
ESI positive produced only \([M^+]\) for all carotenoids in this study, and the addition of halogenated solvents to the post-column effluent greatly enhanced signal intensity (130). A later study by Guarantini et al. demonstrated the ability of ESI positive to produce both \([M^+]\) and \([M+H]^+\) for a number of xanthophylls, and these authors attributed the production of the two species to solvent system effects (138). In contrast, the only ion of β-carotene produced was \([M^+]\) (138), further supporting the results of van Breemen (130).

Novel methods of utilizing ESI have also been developed. LC-ESI-MS has been applied to the detection of silver adducts of tocopherols and carotenoids in various food products including tomato, carrot, infant formula, vegetable juices, and a vitamin drink (139). The addition of silver, a positively charged adduct, creates positively charged species which are easily detected. In this study, an AgClO₄ solution was added to effluent post-HPLC before entering the source. The Ag⁺-carotenoid adducts formed were then monitored, and \([Ag-M^+]\) was the primary ionization product for each of the carotenoids studied (139). Similarly, oxime derivatives of zeaxanthin metabolites (140) and the provitamin A carotenoid metabolite retinaldehyde (6) have been synthesized in biological samples and analyzed via ESI positive. The synthesized oxime derivatives are more stable than the unreacted metabolites, and more easily detected than the parent products.

In terms of quantitation, work by Wang et al. (2000) has demonstrated that the response of β-carotene, ionized by ESI positive and normalized to an internal standard, was non-linear over a range of concentrations (141). This study highlights the
importance of testing carotenoid linearity over the anticipated range of analysis on any instrument before quantitative analysis is attempted.

APCI – The most popular ionization technique used with carotenoids to date is APCI, which has been reviewed by Pajkovic and van Breemen (136). A comprehensive table listing studies utilizing APCI for carotenoid analysis can be found in a recent review by Rezanka et al. (142). In APCI negative, both carotenes and xanthophylls are ionized to produce [M•−] and [M-H]− species. In APCI positive, both carotenes and xanthophylls produce both [M•+] and [M+H]+ ions. However, the most abundant species for xanthophylls in APCI positive is often [M+H-18]+, corresponding to a loss of water (131,143,144). APCI techniques have been used to ionize carotenoids from plants and plant foods (143–145), blood plasma (146,147), and biological tissues (148).

APCI with in-source fragmentation has been utilized to determine the types and substitution locations of fatty acyl moieties of lutein esters (145,149,150). In a study of lutein esters from marigold, Breithaupt et al. (2002) observed that the most labile fatty acid or hydroxyl group in-source was covalently bound to the epsilon ring of lutein (149). These results were confirmed by Young and colleagues who synthesized various lutein esters, determined structures with NMR, and observed that the epsilon ring preferentially loses water or a fatty acid moiety before the β-ionone ring (150).

In contrast to ESI, APCI has been found to produce a linear response over a range of concentrations (i.e. has a large dynamic range) for β-carotene (141,151), lutein, zeaxanthin, β-cryptoxanthin (151) and a relatively linear response for lycopene (148).
**APPI** - In this method of ionization, the solvent is vaporized in a heated nebulizer and the gaseous analytes are then ionized with photons from a lamp (152). It has been observed that certain solvents, called “dopants,” enhance the ionization of analytes ionized via this technique. To date only one study has been published with carotenoids and APPI (152). APPI was compared to ESI and APCI as ionization techniques, and the authors observed that APPI positive produced approximately 2-4 fold greater total ion signal for lycopene and β-carotene as compared to APCI positive and ESI positive. In contrast, APCI positive outperformed APPI positive for a number of xanthophylls and phytoene and phytofluene.

**MALDI** - MALDI has been used to ionize multiple carotenoids and carotenoid ester standards (153) and carotenoids in a variety of plant tissue samples (154). Fraser et al. (154) found that the use of a nitrocellulose matrix produced the least variability in analyte detection. In addition, these authors observed that MALDI was able to detect large differences in carotenoid phenotypes, but not small differences in carotenoid levels. Likewise, MALDI was not able to differentiate between isobaric species (like β-carotene and lycopene, for example).

**1.5.2 Mass Separation**

*Time-of-Flight (TOF)* - The accurate mass capabilities of TOF are useful in determining the composition of new carotenoids or carotenoid metabolites which have not previously been identified (147,155,156). In addition, a TOF chamber (interfaced with MALDI or an ESI ion source) is often used with a targeted “metabolomics” approach, where
determining the exact mass of a mixture of components is important to successfully
differentiate carotenoids from a high level of background ions (154,157).

*Ion Mobility Spectrometry (IMS)* – IMS is a relatively new technique in which ions are
separated based on size and shape using an electric field. IMS was utilized by Dong et al.
(158) to separate all-*trans*-lycopene from *cis*-lycopene and all-*trans*-β-carotene from *cis*-
β-carotene. Unfortunately, the various *cis* isomers could not be separated from each
other using IMS alone. The authors provided evidence to suggest that *cis/trans*
isomerization of carotenoids occurs in-source (ESI positive mode was used in these
experiments). Because of this isomerization, it does not appear likely that IMS will
replace HPLC as a means of separating geometrical isomers of carotenoids in the near
future (158).
Figure 1.7 (A) LC-MS of lycopene, α-carotene, and β-carotene monitored at 536 m/z and (B) LC-MS-MS analysis of the same sample using selected reaction monitoring of m/z 536→467. Taken from (146)
Tandem mass spectrometry (MS/MS) - Tandem mass spectrometry has been used to identify key structural components of carotenoids, such as loss of a 69 mu terminal isoprene group from lycopene and γ-carotene (146) or the internal loss of a 92 mu toluene group observed for a variety of carotenes (159). Tandem mass spectrometry is also useful in identify substitutions like fatty acid esters of xanthophylls. In addition, tandem mass spectrometry (with triple quadrupole instruments) can be used for the quantitation of a compound by Selected Reaction Monitoring (SRM). For example, lycopene and γ-carotene can be distinguished from α-carotene and β-carotene by monitoring the selective daughter ion at 467 produced after CID (Figure 1.7). This approach has been used previously to quantify lycopene (146,147).

Accelerator Mass Spectrometry (AMS) – AMS is a special type of MS which has been used to determine various parameters of carotenoid absorption, distribution, and metabolism, and the basics of this technique have been reviewed by Buchholz et al. (160). A radio-labeled carotenoid (generally labeled with $^{14}$C) is fed to a subject, and biological samples or expired air are collected. Samples may be analyzed directly, or first extracted and analyzed by HPLC, where fractions containing the putative isotopically labeled parent carotenoid(s) and/or carotenoid metabolites are collected. The sample (biological sample or collected fraction) is oxidized to produce carbon dioxide and then converted to graphite (161). The sample is then ionized, and ions are accelerated to high levels of energy in the MS. The ratio of the rare isotope to the abundant isotope is determined ($^{14}$C/$^{12}$C for example). Because of the extreme sensitivity
of AMS (in the attomole range), only a small amount of radio-labeled carotenoid is necessary to study absorption and metabolism.

AMS has been used for many years to study the pharmacokinetics of β-carotene, as has been reviewed by van Lieshout et al. (162). Recent studies have been performed on the absorption of β-carotene and metabolism to vitamin A (163,164) and other metabolites (165) in humans. AMS has also been used to monitor the kinetics of lutein metabolism (166) and lycopene bioavailability and metabolism (167) in humans.

1.5.3 Use of Stable Isotopes

Stable isotopes of carotenoids (usually labeled with $^{13}$C or $^2$H), or of compounds which react with carotenoids, are often used with MS studies. We previously mentioned the use of stable isotopes as internal standards for carotenoid quantitation (especially important when matrix suppression of signal is observed), but there are many other applications as well.

Isotopes can be used to determine mechanism(s) of MS ionization and fragmentation. For example, deuterated chloroform was employed to determine the source of the proton for the molecular ion of β-carotene generated in APCI positive (131).

Stable isotopes can also be used to demonstrate the mechanisms of enzymatic cleavage. For example, isotopes were used to determine the mechanism of enzymatic cleavage and oxygenation of β-ionone by carotenoid cleavage dioxygenase 1 (CCD1), a plant enzyme (168). Likewise, stable isotopes have been used by many groups to follow a single dose of β-carotene and observe enzymatic conversion into vitamin A derivatives.
in humans (133,141,169). The feeding of an isotope allows the dose fed from a specific meal to be differentiated from circulating carotenoid from previous meals. In these studies, samples are extracted and separated by HPLC which is interfaced with a mass spectrometer. While this technique is not as sensitive as using radiolabeled isotopes with AMS, the ability to interface the HPLC with the MS eliminates the need for fraction collection and actually allows the specific compounds to be identified by MS. This also reduces HPLC analysis time, because as long as compounds are not isobaric, they do not need to be baseline separated to be accurately identified and quantified. In contrast, in AMS, fractions are collected for each peak, and any label detected is presumed to be derived from the peak collected.

Stable isotopes of retinal and β-carotene have also been employed to assess liver vitamin A stores in healthy individuals, the best measure of vitamin A status (170). The relative ratio of circulating isotopically labeled retinol is later assessed to estimate liver levels (141,171). Without this technique, liver biopsy would be necessary to estimate liver levels.

1.5.4 Analysis of Metabolites

Techniques used to identify new carotenoids are also employed to identify carotenoid metabolites in various photosynthetic organisms, as well as animals and humans. A new metabolite might be identified in a food or biological extract by HPLC-PDA with the observation of a new peak with a UV/Vis spectrum similar to a carotenoid, or which produces an MS fragment similar to other known carotenoids. Alternatively,
metabolism may be induced in vitro by creating ideal biological conditions for generating metabolites (with intestinal mucosa, for example (93)).

Once a new compound is observed, multiple steps are taken to properly identify the substance. If it is present in high enough quantities, the compound would be isolated and analyzed by NMR for conclusive identification. Alternatively, if the compound is present in small amounts (as is often the case for metabolites), putative “standards” are synthesized and classified by numerous analytical techniques (including UV/Vis, IR, NMR, HPLC, GC, and MS), and compared against the unknown compound. Standards might be synthesized by building the molecule from smaller intermediates (172), or by oxidizing a parent carotenoid to create smaller fragments (112).

These types of techniques have been employed to identify a number of carotenoids in multiple types of samples. Recently GC-MS and authentic standards were used identify volatile carotenoid metabolites from plant tissues (90). Numerous studies have identified β-carotene metabolites in animals and humans using a variety of analytical techniques (96,165). These techniques have also been used to identify lycopene metabolites in both foods and biological samples (91,147,173) and the metabolism of lutein, zeaxanthin, and β-cryptoxanthin (140,174,175).

It should be noted that extreme care should be taken when attempting to prepare and analyze samples for carotenoid metabolites. As mentioned previously, carotenoids are very labile, and putative “metabolites” can easily be created during sample handling and storage. With increasingly sensitive instrumentation, especially with MS, the ability to detect very small amounts of carotenoid degradation products is of concern. The
importance of working with pure solvents, avoiding harsh sample preparation procedures (like saponification), and reducing time from sample extraction to analysis cannot be overlooked.
1.6 Specific Aims

1.6.1 Aim 1: To determine if adding avocados to a carotene rich meal will promote the absorption of provitamin A carotenoids and enhance the delivery of greater quantities of vitamin A.

A definitive understanding of provitamin A carotenoid absorption and metabolism in humans, relative to the provitamin A content in foods, is still lacking. A more detailed knowledge of the bioavailability and metabolism of this carotenoid under a variety of meal conditions is needed. This objective will be accomplished by quantitation of the immediate post-prandial plasma concentrations of parent carotenoids and vitamin A metabolites after subjects consume a meal with or without avocado in combination with a serving of tomato sauce (containing nutritionally relevant amounts of β-carotene) or carrots (containing both α-carotene and β-carotene). I hypothesize that adding avocados (naturally rich in long-chain fatty acids) to a carotene-rich meal will promote the absorption of provitamin A carotenoids from the meal. In addition, I anticipate that greater levels of vitamin A will be observed after the consumption of avocado, an effect which is expected to be subject to inter-individual variability.
1.6.2 Aim 2: To determine whether oxidative products of lycopene are present in raw and processed tomatoes, in addition to blood plasma of humans who have consumed processed tomato juice.

An important step in determining if and how lycopene is metabolized is to identify the products of lycopene which may result from enzymatic or chemical oxidation. Through these studies, we hope to determine if oxidative metabolites are formed in vivo after the consumption of lycopene, what those products may be, and at what levels they are present. We will perform non-polar extraction of raw and processed foods containing lycopene. We will also extract plasma from a previous human clinical trial where tomato juice was consumed. We will seek to identify and quantify the products in the raw foods, food products, and blood plasma.

My hypothesis is that a number of lycopene oxidative products will be present in lycopene containing foods, as well as the blood plasma of individuals consuming a high tomato-juice diet.
1.6.3 Aim 3: To determine if the consumption of tomatoes containing lycopene, phytoene, and phytofluene

a. Results in accumulation of lycopene, phytoene, and phytofluene in murine skin tissue

b. Reduces the incidence or severity of acute UVB induced skin damage

We will feed a purified diet containing freeze-dried tomato powder to male and female SKH-1 hairless mice for ten weeks. We will then induce acute inflammation via UVB radiation. Mice will be sacrificed and plasma and dermal tissue will be analyzed for tomato carotenoids. Inflammatory parameters (i.e. edema, infiltration and activation of neutrophils) and alterations in UVB mediated DNA damage (i.e. CPD, p53) will also be measured.

My hypothesis for sub-aim A is that we will observe lycopene, phytoene, and phytofluene in murine skin. In addition, I expect that the levels of these carotenoids will be lower in animals exposed to ultraviolet light as compared to animals who were not exposed to ultraviolet light. My hypothesis for sub-aim B is that we will observe a reduction in inflammatory parameters and DNA damage in the dermal tissue of UVB exposed animals on the tomato diet, as compared to animals on the control diet.
CHAPTER 2

Comparison of HPLC-MS/MS and HPLC-PDA for the quantitation of carotenoids, retinyl esters, α-tocopherol, and phylloquinone in chylomicron-rich fractions of human plasma

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ABSTRACT

Bioavailability of essential lipophilic micronutrients and carotenoids is of utmost interest for human health, as the consumption of these compounds may help alleviate major nutritional deficiencies, cardiovascular disease, and cancer. High performance liquid chromatography-photo diode array (HPLC-PDA) and HPLC-tandem mass spectrometry (MS/MS) were compared for the quantitative analysis of α- and β-carotene, β-cryptoxanthin, lutein, lycopene, α-tocopherol, phylloquinone, and several retinyl esters from chylomicron-rich fractions of human plasma obtained from two clinical trials. After selecting an efficient extraction method for the analytes, both the HPLC-PDA and the HPLC-MS/MS methods were developed and several parameters validated using an HP 1200 series HPLC system interfaced with a QTrap 5500 (AB Sciex, Foster City, CA, USA) via an atmospheric pressure chemical ionization (APcI) probe operated in positive ion mode. For lycopene, α- and β-carotene, HPLC-MS/MS was up to 37 times more sensitive than HPLC-PDA. PDA detection was shown to be 8 times more sensitive for lutein standard as compared to MS/MS. In biological extracts, MS/MS signals were enhanced by matrix components for lutein. In contrast, matrix suppression was observed for retinyl palmitate, α-carotene, and β-carotene. Both detectors showed similar suitability for α-tocopherol and retinyl palmitate (representing ~73% of total retinyl esters). Exclusively MS/MS allowed the quantitation of minor retinyl esters, phylloquinone, and (Z)-lycopene isomers. HPLC-MS/MS was more sensitive than HPLC-PDA for 6 of the 8 analytes and represents a powerful tool for the analysis of chylomicron samples and potentially other biological samples of limited sample size. The
use of stable isotope internal standard is recommended when available. Employing MS/MS detection may reduce the necessary blood sample volume, which is particularly advantageous for minimizing risk and discomfort of human subjects during clinical studies.

INTRODUCTION

Fat soluble vitamins, including vitamin A, vitamin E, and vitamin K are essential nutrients provided in the human diet. Some carotenoids, including α-carotene, β-carotene, and β-cryptoxanthin, are vitamin A precursors, while carotenoids such as lutein and lycopene have been associated with other health benefits (46,53,176). Understanding the absorption of these compounds from the human diet is essential to study their bioavailability and to provide sound recommendations to eliminate nutritional deficiencies in developing countries. After consumption of a meal containing fat soluble nutrients, these compounds are absorbed by intestinal cells, packaged into chylomicrons, and released into the blood circulation (1). The compounds of interest can be isolated from the chylomicrons of the blood plasma by ultracentrifugation and extraction with lipophilic solvents. Subsequent instrumental analysis allows for the estimation of the bioavailability of a compound.

Carotenoid bioavailability has been studied widely by monitoring the levels of these compounds in chylomicron-rich fractions of human plasma by HPLC coupled to a photodiode array (PDA) detector and/or an electrochemical (EC) detector (31,177,178). For the simultaneous quantitation of carotenoid and tocopherol isomers, a rapid HPLC-
PDA method has been developed for vitamin supplemented (α-tocopherol and β-carotene, i.e. ATBC) drinks (179). However, more sensitive identification and quantitation methods for these compounds are necessary particularly for samples from human studies, since the analysis of minor components at low biological levels would require a large sample volume. Many strides have been made in recent years, particularly in mass spectrometry, offering distinct advantages over the other methods of detection (180). Higher sensitivity might facilitate the quantitation of minor components such as retinyl esters or carotenoid isomers. Moreover, the risk for patients during a clinical trial might be minimized due to the reduction of the necessary blood sample volume. Thus, we developed and partially validated an HPLC-MS/MS method to analyze fat soluble vitamins and carotenoids in one HPLC run. Our method involved an optimized extraction procedure and avoids saponification, which enables distinct measurements of esterified forms of retinol. The main focus of this study was the comparison of analytical parameters of the HPLC-MS/MS and the HPLC-PDA method, investigating extracts of chylomicron-rich fractions from two clinical trials, which contained challenging lipid matrix components.

**MATERIALS AND METHODS**

*Reagents*: HPLC grade methyl tert-butyl ether (MTBE), toluene, acetone, and ethanol, and Optima grade hexane, water, and methanol, and ACS grade sodium chloride were purchased from Fisher Scientific (Pittsburg, PA, USA). High purity formic acid (≥ 95%) and polyvinyl alcohol (PVA) with average molecular weight from 70,000-100,000 was
purchased from Sigma-Aldrich (St. Louis, MO, USA). Ammonium acetate was obtained from J.T. Baker (Phillipsburg, NJ, USA). Lutein and α-carotene were acquired from Chromadex (Irvine, CA, USA). Unlabelled β-carotene, retinyl palmitate, β-cryptoxanthin, phylloquinone, and α-tocopherol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Lycopene was purified and crystallized as described previously (147). d8-β-carotene was purchased from Cambridge Isotopes (Andover, MA, USA).

Clinical Trials: Two human clinical trials were performed, the details of which will be published elsewhere. Both studies were approved by the Institutional Review Board of the Ohio State University. In trial 1, fasted healthy adult subjects were fed raw carrots containing 28 mg β-carotene, 17 mg α-carotene, 0.5 mg lutein, 32 µg of phylloquinone, and 3 mg α-tocopherol with a breakfast meal containing 24 g lipid from avocado. Blood samples were taken at 0, 2, 3, 4, 5, 6, 8, 10, and 12 hours. During trial 2, fasted healthy adult subjects received fresh papaya (Carica papaya L. cv. ‘Pococi’) slices containing ~1.3 mg β-carotene, 2.1 mg β-cryptoxanthin, and 13.0 mg lycopene (181). Additionally, subjects were fed 150 g of a low-fat yogurt enriched with soy oil to a lipid content of 10% (w/w). Blood samples were drawn at 0, 2, 3, 4, 5, 6, 8, and 9.5 hours after test meal consumption.

Chylomicron Isolation: The following procedure and the subsequent carotenoid extractions were carried out under subdued light. For trial 1, venous blood was drawn via needle and syringe from the lower forearm into EDTA vacutainer tubes (Becton, Dickinson and Co., Franklin Lakes, NJ, USA). Plasma was immediately separated from red blood cells by centrifuging at 1000 x g at 4°C for 10 min. Blood plasma was then
placed into cryovials and stored at 4°C before chylomicron isolation. Polyallomer tubes (13 mm x 51 mm) were washed with a 5% PVA solution (w/v) overnight, rinsed three times, and allowed to dry. Plasma (2.5 mL) was transferred into a polyallomer tube and overlaid with 1 mL of NaCl solution having a density of 1.006 kg/L (36). Samples were subjected to ultracentrifugation for 33 minutes at 40,700 rpm in a SW 50.1 swinging bucket rotor of a Beckman L8-M ultracentrifuge to separate the triglyceride rich lipoprotein (TRL) fraction containing the chylomicrons. The lower plasma fraction was drained from the tube and the TRL-containing supernatant (top 0.5 mL) was removed and set aside. The tube was rinsed twice with 250 µL of saline solution which was combined with the TRL fraction. The TRL fraction was frozen at -80 °C until extraction. All TRL fractions were isolated from fresh (i.e. previously unfrozen) plasma.

For trial 2, the TRL isolation protocol was carried out as described above with the following modifications: For plasma separation, the blood samples were centrifuged at 1700 x g at 4 °C for 10 min. Separation of TRL fractions from plasma was performed as described above, except that 0.8 g potassium bromide were added to the plasma samples prior to overlaying with NaCl solution and ultracentrifugation (WX Ultra 80, Thermo Fisher Scientific, Asheville, NC, USA) following a modified method of Terpstra (182). After removing the TRL fraction, the polyallomer tube was rinsed once with 250 µL of saline solution, and the fractions were stored at -80 °C. Prior to carotenoid extraction, the TRL samples were made up to 1.0 mL with saline solution.

*Extraction:* First, a 0.5 mL aliquot of the TRL fraction was mixed with 0.5 mL ethanol. After vortexing, 2 mL of the extraction solvent were added. The sample was probe-
sonicated for 8 seconds thrice and then centrifuged for 5 min at 300 x g. The upper non-polar layer was removed and the remaining aqueous plasma mixture was re-extracted as described above, unless otherwise specified. The two non-polar extracts were combined in a glass vial and dried under nitrogen gas. The dried extract was stored at -80°C until HPLC-MS/MS analysis. Various extraction solvents were tested, including hexane alone, hexane/acetone (1:1, v/v), and hexane/ethanol/acetone/toluene (10:6:7:7, v/v/v/v). Overspike recovery experiments were performed by adding 100 µL of a standard mixture containing all analytes of interest at levels expected to be observed at 5 hours into 5 hour TRL samples.

**HPLC-PDA-MS/MS Analysis**: TRL extracts were re-dissolved in 100 µL MTBE containing the d8-β-carotene internal standard. Then, the samples were sonicated in a water bath, followed by the addition of 100 µL of MeOH and centrifugation (1500 rpm, 2 min, model 5424, Eppendorf, Hamburg, Germany) to pellet any solid matrix remaining in the sample. The compounds were separated using reverse phase chromatography with a YMC C30 column (Waters, Milford, MA, USA), 4.6 mm x 150 mm, with a 5 µm particle size. Samples were separated on an HP 1200 series system HPLC with an HP 1200 series diode array detector (Agilent Technologies, Santa Clara, CA, USA). The column was operated at 40 °C and the injection volume was 20 µL. Total run time was 19 min at a flow rate of 1.8 mL/min. The following gradient was employed to separate the samples: 0% B with a linear gradient to 100% B over 16 minutes, holding at 100% B for 1 minute, and then returning to 0% B and holding for 2 minutes. Solvent A = 80:20 MeOH/water (v/v) and solvent B = 78:20:2 MTBE/MeOH/water (v/v/v). Besides the described solvent
composition without further additives, two additional solvent modifications were tested. Ammonium acetate was added at 0.4 g/L solvent. Furthermore, the water proportion of solvent A and B was replaced by 0.1% aqueous formic acid. After several optimization experiments, the solvent combination containing ammonium acetate proved to most effectively enhance chromatographic separation and MS signal intensity of parent ions for all carotenoids tested, but particularly for the carotenes α-carotene, β-carotene, and lycopene (Figure 1). The LC solvents were therefore enriched with 0.4 g ammonium acetate per liter of solvent throughout the method development.

The eluent from the HPLC was interfaced with a QTrap 5500 mass spectrometer (AB Sciex, Foster City, CA, USA) via an atmospheric pressure chemical ionization (APcI) probe operated in positive ion mode. After various optimization experiments, the following parameters were chosen to analyze the final samples: curtain gas = 30 psi, source temperature = 450 °C, ion source gas 1 (GS1) = 45 psi, declustering potential (DP) = 100 V, entrance potential (EP) = 10 V, collision cell exit potential (CXP) = 11 V. Various parent>daughter ion combinations for each analyte were identified in preliminary experiments. Only those parent>daughter ion combinations which were selective for the compounds of interest and for which background interference was minimized or eliminated were chosen for the final method (Table 2.1 and 2.2). Final data acquisition was carried out using MRM windows with dwell times of 50 msec for each channel, and 5 msec pauses between channels. All data were acquired and peak areas integrated using Analyst 1.5.1 software (AB Sciex, Foster City, CA, USA).
Figure 2.1 Influence of MS source temperature and LC solvent additives on MS signal intensity for parent ions using SIR in APcI positive mode. AA: ammonium acetate, FA: formic acid, H$_2$O: no additive. AA at 450 °C provided optimal signal for all analytes tested. For more details, see Materials & Methods section.
Quantitation: Carotenoids and other micronutrients were quantitated using calibration curves generated with authentic standards at five different dilution levels. For PDA quantitation, the peak area at the UV/Vis absorption maximum of each analyte was used. For MS/MS quantitation, the specific parent>daughter fragment denoted in Table 2.1 was chosen for each compound. However, all parent-daughter fragments listed in Table 2.1 were monitored during the experiment as sufficient duty cycle was available for each parent>daughter pair, providing additional confirmation of compound identity.
Table 2.1 UV-Vis Spectra and MS Data of the Investigated Analytes.

<table>
<thead>
<tr>
<th>No.</th>
<th>Retention time (min)</th>
<th>Compound Identity</th>
<th>HPLC-PDA spectrum $\lambda_{\text{max}}$ (nm)</th>
<th>HPLC-APCI(+)–MS/MS experiment $m/z$ parent ion &gt; $m/z$ daughter ion</th>
<th>Collision energy for MS/MS experiment(#)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.4</td>
<td>retinol</td>
<td>340</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>2</td>
<td>8.7</td>
<td>(all-E)-lutein</td>
<td>430/446/472</td>
<td>551.5*&gt;533.0, 551.5*&gt;429.5, 551.5*&gt;345.3(#)</td>
<td>20, 20, 20</td>
</tr>
<tr>
<td>3</td>
<td>8.9</td>
<td>$\alpha$-tocopherol</td>
<td>294</td>
<td>431.4&gt;205.1($), 431.4&gt;165.1</td>
<td>30, 30</td>
</tr>
<tr>
<td>4</td>
<td>9.8</td>
<td>phylloquinone</td>
<td>n.d.</td>
<td>451.4&gt;227.2, 451.4&gt;197.0, 451.4&gt;187.2($)</td>
<td>40, 35</td>
</tr>
<tr>
<td>5</td>
<td>11.1</td>
<td>(all-E)-β-cryptoxanthin</td>
<td>430/454/478</td>
<td>553.3&gt;473.5, 553.3&gt;429.5($)</td>
<td>20, 25</td>
</tr>
<tr>
<td>6</td>
<td>11.1</td>
<td>retinyl ester 1</td>
<td>n.d.</td>
<td>269.2*&gt;239.2, 269.2*&gt;213.2($)</td>
<td>20, 25</td>
</tr>
<tr>
<td>7</td>
<td>11.6</td>
<td>retinyl ester 2</td>
<td>n.d.</td>
<td>269.2*&gt;239.2, 269.2*&gt;213.2($)</td>
<td>20, 25</td>
</tr>
<tr>
<td>8</td>
<td>11.8</td>
<td>retinyl ester 3</td>
<td>n.d.</td>
<td>269.2*&gt;239.2, 269.2*&gt;213.2($)</td>
<td>20, 25</td>
</tr>
<tr>
<td>9</td>
<td>12.0</td>
<td>retinyl palmitate</td>
<td>330</td>
<td>269.2*&gt;239.2, 269.2*&gt;213.2($)</td>
<td>20, 35</td>
</tr>
<tr>
<td>10</td>
<td>12.9</td>
<td>(all-E)-α-carotene</td>
<td>430/446/472</td>
<td>537.4&gt;415.4, 537.4&gt;413.4($)</td>
<td>30, 30</td>
</tr>
<tr>
<td>11a</td>
<td>13.1</td>
<td>(all-E)-β-carotene</td>
<td>430/454/478</td>
<td>537.4&gt;415.4, 537.4&gt;413.4($)</td>
<td>30, 30</td>
</tr>
<tr>
<td>11b</td>
<td>13.1</td>
<td>(all-E)-d8-β-carotene(\¥)</td>
<td>430/454/478</td>
<td>545.4&gt;423.4(\¥), 545.4&gt;421.4(\¥)</td>
<td>30, 30</td>
</tr>
<tr>
<td>12</td>
<td>14.3</td>
<td>(Z)-lycopene isomer 1</td>
<td>n.d.</td>
<td>537.4&gt;455.6, 537.4&gt;427.4, 537.4&gt;413.4($)</td>
<td>25, 20, 25</td>
</tr>
<tr>
<td>13</td>
<td>14.5</td>
<td>(Z)-lycopene isomer 2</td>
<td>n.d.</td>
<td>537.4&gt;455.6, 537.4&gt;427.4, 537.4&gt;413.4($)</td>
<td>25, 20, 25</td>
</tr>
<tr>
<td>14</td>
<td>15.2</td>
<td>(Z)-lycopene isomer 3</td>
<td>n.d.</td>
<td>537.4&gt;455.6, 537.4&gt;427.4, 537.4&gt;413.4($)</td>
<td>25, 20, 25</td>
</tr>
<tr>
<td>15</td>
<td>16.0</td>
<td>(all-E)-lycopene + 5-(Z)-lycopene</td>
<td>446/472/502</td>
<td>537.4&gt;455.6, 537.4&gt;427.4, 537.4&gt;413.4($)</td>
<td>25, 20, 25</td>
</tr>
</tbody>
</table>

*: in source-fragment as parent ion.
*: Collision energy used for the respective MS/MS is displayed in the same order as the parent ion/daughter ions (MRM).
\$: Parent-daughter ion pair selected for quantitation
\¥: Internal standard
n.a.: not applicable, n.d.: not determined
## Table 2.2 Analytical Parameters for HPLC-PDA and HPLC-MS/MS Analysis of Standards.

<table>
<thead>
<tr>
<th>Compound name</th>
<th>Detector type</th>
<th>Wavelength [nm] and MS/MS transition</th>
<th>Extraction Recovery(^\text{a}) [%] (n=5)</th>
<th>Calibration linearity (n=4)</th>
<th>LOD [pmol on column]</th>
<th>LOQ [pmol on column]</th>
<th>Accuracy (n = 7)</th>
<th>Inter-day Precision (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(all-E)-lutein</td>
<td>PDA</td>
<td>446</td>
<td>99.3 ± 5.8</td>
<td>0.9976</td>
<td>0.042</td>
<td>0.139</td>
<td>98%</td>
<td>6.0%</td>
</tr>
<tr>
<td></td>
<td>MS/MS</td>
<td>551.5&gt;345.3</td>
<td>-</td>
<td>0.9983</td>
<td>0.323</td>
<td>1.077</td>
<td>94%</td>
<td>12%</td>
</tr>
<tr>
<td>(all-E)-β-cryptoxanthin</td>
<td>PDA</td>
<td>452</td>
<td>101.0 ± 3.3</td>
<td>0.9984</td>
<td>0.381</td>
<td>1.270</td>
<td>97%</td>
<td>5.6%</td>
</tr>
<tr>
<td></td>
<td>MS/MS</td>
<td>553.3&gt;429.5</td>
<td>-</td>
<td>0.9983</td>
<td>0.148</td>
<td>0.493</td>
<td>97%</td>
<td>15%</td>
</tr>
<tr>
<td>(all-E)-α-carotene</td>
<td>PDA</td>
<td>446</td>
<td>100.9 ± 5.3</td>
<td>0.9987</td>
<td>0.473</td>
<td>1.575</td>
<td>100%</td>
<td>4.7%</td>
</tr>
<tr>
<td></td>
<td>MS/MS</td>
<td>537.4&gt;413.4</td>
<td>-</td>
<td>0.9984</td>
<td>0.133</td>
<td>0.443</td>
<td>93%</td>
<td>14%</td>
</tr>
<tr>
<td>(all-E)-β-carotene</td>
<td>PDA</td>
<td>452</td>
<td>99.5 ± 0.7</td>
<td>0.9963</td>
<td>1.184</td>
<td>3.945</td>
<td>97%</td>
<td>6.2%</td>
</tr>
<tr>
<td></td>
<td>MS/MS</td>
<td>537.4&gt;413.4</td>
<td>-</td>
<td>0.9997</td>
<td>0.304</td>
<td>1.014</td>
<td>102%</td>
<td>4.5%</td>
</tr>
<tr>
<td>(all-E)-lycopene</td>
<td>PDA</td>
<td>472</td>
<td>100.2 ± 2.5</td>
<td>0.9989</td>
<td>0.899</td>
<td>2.995</td>
<td>93%</td>
<td>8.3%</td>
</tr>
<tr>
<td></td>
<td>MS/MS</td>
<td>537.4&gt;413.4</td>
<td>-</td>
<td>0.9997</td>
<td>0.025</td>
<td>0.082</td>
<td>96%</td>
<td>11%</td>
</tr>
<tr>
<td>retinyl palmitate</td>
<td>PDA</td>
<td>330</td>
<td>95.9 ± 9.7</td>
<td>0.9959</td>
<td>0.613</td>
<td>2.042</td>
<td>99%</td>
<td>3.7%</td>
</tr>
<tr>
<td></td>
<td>MS/MS</td>
<td>269.2&gt;213.2</td>
<td>-</td>
<td>0.9999</td>
<td>0.574</td>
<td>1.912</td>
<td>107%</td>
<td>7.2%</td>
</tr>
<tr>
<td>phyloquinone</td>
<td>PDA</td>
<td>248</td>
<td>100.5 ± 4.3</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>MS/MS</td>
<td>451.4&gt;187.2</td>
<td>-</td>
<td>0.9981</td>
<td>0.003</td>
<td>0.009</td>
<td>97%</td>
<td>13%</td>
</tr>
<tr>
<td>α-tocopherol</td>
<td>PDA</td>
<td>295</td>
<td>100.0 ± 4.3</td>
<td>0.9965</td>
<td>0.003</td>
<td>0.011</td>
<td>100%</td>
<td>10%</td>
</tr>
<tr>
<td></td>
<td>MS/MS</td>
<td>431.4&gt;205.1</td>
<td>-</td>
<td>1.0000</td>
<td>0.004</td>
<td>0.014</td>
<td>79%</td>
<td>41%</td>
</tr>
</tbody>
</table>

N/A: not available.
\(^{a}\): Values are reported as mean ± standard deviation.
Limit of detection (LOD) and limit of quantitation (LOQ) were determined by using the least squares regression method of the residuals of the standards at each dilution level. According to the method of Vial et al. (183), the y-intercept was used to calculate LOD (x 3) and LOQ (x 10). To determine precision and accuracy of the HPLC-PDA-MS/MS method, quality control (QC) standards were run on multiple days. The standard mixture was created in solvent, dried under nitrogen gas, and stored dry at -80 °C until use. Each QC injection contained 6 pmol of α-carotene, β-carotene, β-cryptoxanthin, lutein, lycopene, and retinyl palmitate and 0.03 pmol of α-tocopherol and phylloquinone.

RESULTS & DISCUSSION

**Extraction Recovery:** A variety of extraction solvents and solvent combinations have been described in the literature for removing lipophilic analytes like carotenoids from TRL fractions (32,36,74,184). As a first step in plasma sample preparation for HPLC, most methods use an organic solvent such as methanol, ethanol, or acetonitrile to precipitate proteins (185). Adding ethanol and vigorously vortexing the TRL fraction before the addition of the extraction solvent was vital for an acceptable extraction recovery of the analytes. Multiple extraction solvents were tested during a series of overspike recovery experiments, maintaining the ratio of 1:4 (v/v) TRL fraction to extraction solvent (see Materials & Methods). Since all extraction solvents provided comparable recovery results, a repeated extraction with hexane was chosen and the results are summarized in Table 2.2.
**Validation Results:** Parent-daughter transitions unique to each compound were chosen for each analyte. The instrument speed (minimum dwell of 2 msec, delay of 3 msec) allowed us to monitor multiple parent-daughter transitions without sacrificing sensitivity. Thus, several transitions for each compound were monitored to increase confidence in identification, and the relative ratio of multiple parent-daughter transitions for each compound was monitored to ensure that we were indeed detecting the analyte of interest. In our samples, we observed that higher daughter masses were more selective to a particular analyte, but less sensitive, and thus for most analytes the lower mass daughter ions were chosen for quantitation (Table 2.1).

The LOD and LOQ for all analytes investigated are provided as pmol injected onto the HPLC column in Table 2.2. LC-MS/MS was more sensitive for most analytes except for lutein, where PDA was more sensitive than LC-MS/MS by 7 fold. No clear sensitivity advantage was observed for α-tocopherol between the two detection methods. In contrast, LC-MS/MS was 3.6-fold more sensitive for α-carotene, 3.9-fold more sensitive for β-carotene, and a remarkable 36-fold more sensitive for lycopene as compared to PDA. For phylloquinone, which is a comparatively poor absorber of light in the UV-visible range, LC-MS/MS was the only viable method of detection, particularly when UV-absorbing background compounds from the TRL fractions were present.

Our LOQ values for the LC-MS/MS analysis of lutein, β-cryptoxanthin and β-carotene are comparable to those values reported by Hao et al. (151) and our LOQ values for lycopene by LC-MS/MS are similar to those reported by Fang et al. (146).
In contrast, our LC-MS/MS LOD value for α-tocopherol (4.2 fmol) is 121-fold lower than that reported by Hao et al. (151) and 5.7-fold lower than that reported by Andreoli et al. (186). Although the MS/MS detection limit was quite low, precision and accuracy values were not within an acceptable range, and thus PDA is the favorable detection method for quantitation of α-tocopherol using our method.

Sensitivity parameters have also been reported for selected ion monitoring (SIM, LC-MS) by various groups. Our LOQ values (LC-MS/MS) for retinyl palmitate (1.91 pmol) and α-carotene (0.44 pmol) were very similar to those reported by van Breemen et al.(187) and Matsumoto et al.(188), respectively, for the LC-MS analysis of selected ions (SIM). Likewise, we observed significantly higher sensitivity for phylloquinone (LOQ = 8.8 fmol) than Martinis et al. (189), who reported an LOQ value of 0.89 pmol for the parent ion as compared to 0.009 pmol in our method. Loss in analyte signal during fragmentation was expected with our tandem mass spectrometry as compared to the SIM methods of van Breemen et al. (187), Matsumoto et al.(188) and Martinis et al. (189). However, this was not observed for the above-mentioned analytes.

Some studies have employed electrochemical (EC) detection to quantitate carotenoids at low levels typically observed in biological samples. In our study, our LC-MS/MS LOD for β-carotene was 304 fmol, as compared to 10 fmol reported earlier by EC detection (177). In contrast, we report a LOD of 24 fmol for lycopene by MS/MS, while 50 fmol has been reported by EC for lycopene. Likewise, the LOD for α-tocopherol by LC-MS/MS was 4 fmol, while EC LOD has been reported as 150 fmol (190). While EC appears to be more sensitive for some carotenoids, MS/MS analysis provides
confirmation of the compound via parent and daughter ions consistent with standards. EC provides less selectivity as the measured dominant oxidation potentials are not specifically unique to individual compounds. In addition, unknown interfering compounds may be present.

As shown in Table 2.2, detection accuracy was >90% for all analytes except α-tocopherol by MS/MS analysis. Except for β-carotene, the precision for all analytes was better for the PDA measurement as compared to MS/MS measurement, suggesting that the PDA response is more stable than that of MS/MS. The higher precision for β-carotene by MS/MS analysis was likely due to the use of the stable isotope internal standard, which can account for instrumental variability. The precision for α-tocopherol was comparatively high regardless of whether the 205 daughter ion (Table 2) or the 165 daughter (data not shown) was chosen. Overall, precision for most analytes was acceptable. Thus, it should be noted that Figures 2.2-2.6 are meant to illustrate MS/MS vs. PDA response differences, and standard error of the mean (SEM) reflects inter-individual subject differences and not differences in response.

The developed method using HPLC-PDA-MS/MS for the determination of carotenoids, retinyl esters, α-tocopherol, and phylloquinone was applied to TRL fractions acquired during two clinical trials in 2011 (Trial 1 and 2). Figure 2.2 illustrates the chromatographic separation, as monitored by PDA and corresponding MS/MS signals of a representative TRL sample (t = 6 h, Trial 2).
Figure 2.2 HPLC separation of lipophilic micronutrients from a human TRL sample at hour 6 after meal consumption, showing maximum levels of analytes of interest monitored at (A) 295 nm, (B) 450 nm, and (C) 325 nm. Dotted lines assign UV-Vis peaks to the corresponding MS/MS traces. For peak assignments, see Table 1.
Figure 2.3 shows β-carotene and α-carotene absorption curves for the average of 2 subjects from Trial 1 corrected to the baseline (i.e. t = 0h) level of these compounds. All time plots showed a typical peak in concentration at 3 hours after meal consumption, and a second peak for α-carotene was observed at 5 hours after meal consumption (Figure 2.3). Clearly, for both α- and β-carotene, the raw MS/MS signal delivered lower values as compared to the PDA signal (Figure 2.3). The difference between both signals was substantially higher at carotenoid peak levels as compared to other data points. Since other lipophilic compounds show similar absorption behavior after the consumption of a meal, the signal suppression observed when the internal standard normalization was not used may be related to the co-elution of such lipids present in the sample matrix (36). Mechanisms of ion suppression are discussed elsewhere (191,192).
Figure 2.3 Baseline-corrected plasma TRL concentrations of (A) \( \beta \)-carotene and (B) \( \alpha \)-carotene as measured by HPLC-PDA (solid line) and HPLC-MS/MS (dotted lines) during the 12 h after consumption of the test meal from trial 1. Normalization of the MS/MS signal by the internal standard (d\(_8\)-\( \beta \)-carotene) was demonstrated for \( \beta \)-carotene only. Values are means \( \pm \) SEM from two subjects.
The use of a stable isotope internal standard (d8-β-carotene) allowed the MS/MS signal of native β-carotene to be corrected for matrix suppression. This adjustment was successful as the normalized MS/MS signal directly overlaid the PDA signal (Figure 3.3A). Although co-elution could be an issue for PDA detectors in some cases, the concurrence of the PDA and the raw MS/MS signals allowed mutual cross-validation. The AUC for β-carotene measured by the non-normalized MS/MS (180 nmol*h/L) was only 43-45% of the AUC determined by PDA (404 nmol*h/L) and normalized MS/MS (414 nmol*h/L). Hence, the use of an internal standard represents a prerequisite for accurate measurements when using MS/MS for the determination of β-carotene in TRL samples with our instrument. α-carotene elutes just 0.2 min prior to β-carotene in the method. Due to their close proximity and the assumed similarity in matrix suppression, we attempted to use the β-carotene IS to compensate for α-carotene matrix suppression. Unfortunately, the β-carotene IS over-compensated for the matrix suppression of α-carotene and, thus, was not useful (data not shown).

Various retinyl esters (compounds 6,7,8,9) as well as free retinol (compound 1) in small amounts were observed in the TRL samples (Figure 2.2). Particularly at earlier time points (0, 2, 3, 4 h), retinyl palmitate was the only retinyl ester quantifiable by PDA, whereas the MS/MS signal also delivered acceptable S/N ratios for other, minor retinyl esters. Therefore, the MS/MS signal for total retinyl esters delivered a higher absorption curve (AUCMS/MS = 222 nmol*h/L) as compared to PDA (AUCPDA = 184 nmol*h/L) in the samples studied (Figure 2.4A). The PDA signal in Figure 2.4A represents retinyl
palmitate only, which was the most abundant retinyl ester (73% of total retinyl esters determined by MS/MS). AUC\textsubscript{PDA} for retinyl palmitate only (184 nmol*h/L) was higher than AUC\textsubscript{MS/MS} for retinyl palmitate only (162.9 nmol*h/L), suggesting the application of an internal standard due to a minor amount of ion suppression.

The free retinol (compound 1) detected in the TRL samples might represent a baseline remnant, which was not derived from the test meal. After feeding isotopically labeled d8-β-carotene, our group in collaboration with others showed that the TRL fraction only contained labeled d4-retinyl esters, whereas labeled free d4-retinol was not detected (184). At the same time, unlabeled free retinol was found in the TRL fractions, although no unlabeled β-carotene was fed. Hence, the presence of unlabeled free retinol was related to native circulating species (184).

By analogy to retinyl esters, MS/MS analysis of lycopene allowed the quantitation of further (Z)-lycopene isomers (compounds 12, 13, 14). At some time points, these isomers were not quantifiable by PDA (S/N < 10). Thus, as shown in Figure 2.4B, the AUC\textsubscript{PDA} (109 nmol*h/L) for total lycopene was slightly lower than the respective AUC\textsubscript{MS/MS} (138 nmol*h/L) for total lycopene. For estimating the matrix effect, only (all-E)-lycopene and lycopene isomer 4 (both eluting in peak 15) were considered, since the determination of both compounds was feasible by MS/MS and PDA. The AUC\textsubscript{PDA} and AUC\textsubscript{MS/MS} was 109 and 116 nmol*h/L, respectively, indicating an apparently small matrix effect on average. It should be noted that fluctuations of the MS/MS signal were observed at single data points, and, if available, an isotopically labeled internal standard would reduce this noise.
Figure 2.4 Baseline-corrected plasma TRL concentrations of (A) retinyl esters and (B) lycopene during the 9.5 h after consumption of the test meal from trial 2. In figure 4A, HPLC-PDA of retinyl palmitate only (solid line), HPLC-MS/MS of retinyl palmitate only (dashed line), and HPLC-MS/MS of total esters (dotted line) are shown. In figure B, HPLC-PDA of (all-E)-lycopene + (Z)-lycopene isomer 4 (solid line), HPLC-MS/MS of (all-E)-lycopene + (Z)-lycopene isomer 4 (dashed line), and HPLC-MS/MS of total lycopene (dotted line) are shown. Analysis by MS/MS for both retinyl esters and lycopene delivered higher results as compared to PDA, since quantitation of minor retinyl esters and lycopene isomers was only feasible by MS/MS. Values are means ± SEM from two subjects.
β cryptoxanthin and lutein absorption curves are shown in Figure 2.5. Using the PDA signal as reference, the MS/MS signal delivered artificially high results for the β-cryptoxanthin ion pair at m/z 553>473 and for all lutein ion pairs, respectively. Regarding β-cryptoxanthin, the inconsistent signal enhancement for different ion pairs indicated that a co-eluting matrix compound might show the same transition at m/z 553>473, but not at m/z 553>429. The apparent signal enhancement was more consistent for lutein and might therefore be caused by enhancement of ionization in the source. Furthermore, a matrix-related cause for the observed ion enhancement of lutein is probable, since the differences between PDA and MS/MS are larger at data points with higher expected levels of matrix (3 - 6 hours). Regarding lutein, a more than 2.2-fold matrix enhancement was revealed when comparing the AUC_{MS/MS} of the ion pairs at m/z 551>345 (120 nmol*h/L) and m/z 551>429 (115 nmol*h/L) with the reference AUC_{PDA} (52 nmol*h/L). For β-cryptoxanthin, the MS/MS ion pair at m/z 553>473 showed a much stronger enhancement (AUC_{MS/MS} = 180% of AUC_{PDA}) as compared to the ion pair at m/z 553>429 of β cryptoxanthin (AUC_{MS/MS} = 111% of AUC_{PDA}). Although the matrix enhancement on the latter ion pair is rather low, the use of an internal standard would be ideal in order to compensate for the observed matrix enhancement.
Figure 2.5 Baseline-corrected plasma TRL concentrations of (A) β-cryptoxanthin from trial 2 and (B) lutein from trial 1 as measured by HPLC-PDA (solid line) and HPLC-MS/MS (dotted lines) during the 9.5 h and 12 h after consumption of the test meals, respectively. MS/MS delivered higher results as compared to PDA, presumably due to matrix enhancement. Values are means ± SEM from two subjects.
Phylloquinone and α-tocopherol absorption is shown in Figure 2.6. Unlike the other analytes, the PDA signal was unavailable for phylloquinone in the real TRL samples. However, in preliminary experiments, TRL test samples were spiked with phylloquinone at high enough levels that PDA response could be compared to MS/MS response, and no matrix effect was observed (data not shown). The MS/MS daughter ions studied showed a very consistent behavior in all samples (Figure 6). For α-tocopherol, the AUCs determined by PDA (5.8 nmol*h/L) were slightly higher as compared to the MS/MS response (5.2 nmol/L), possibly due to a low matrix-related ion suppression.
Figure 2.6 Baseline-corrected plasma TRL concentrations of (A) phylloquinone and (B) α-tocopherol as measured by HPLC-PDA and HPLC-MS/MS during the 12 h after consumption of the test meal from trial 1. Values are means ± SEM from two subjects.
CONCLUSIONS

In conclusion, an IS was found to be necessary for the proper quantitation of β-carotene in TRL samples by MS/MS. A stable isotope IS is strongly recommended for quantitation of lutein, β cryptoxanthin, and α-carotene for quantitation by MS/MS of these compounds in lipid-rich TRL fractions due to matrix enhancement and suppression. In contrast, an IS does not appear to be necessary for retinyl palmitate and lycopene for MS/MS quantitation. In addition, MS/MS detection was necessary for quantitation of retinyl esters and lycopene (Z)-isomers present at low levels and for phylloquinone at all levels. While the inherent absence of ion suppression/enhancement made HPLC-PDA a better choice for quantitation of some carotenoids and α-tocopherol, in-line MS/MS detection clearly provides compound identification confirmation, which could not be obtained by PDA or EC at these low levels.
CHAPTER 3

Provitamin A carotenoid absorption and conversion from a novel high β-carotene tomato and from carrot is enhanced with avocado

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ABSTRACT

Provitamin A carotenoids serve as precursors of vitamin A, an essential nutrient, in the human diet. Dietary triglycerides have been shown to increase bioavailability of provitamin A carotenoids from a single meal, but the effects of dietary lipid on conversion to vitamin A during digestion and absorption are largely unknown. Based on results from previous animal studies, we hypothesized that feeding provitamin A carotenoids with dietary lipid in humans would enhance conversion to vitamin A during the digestive process, as compared to feeding provitamin A carotenoids alone. 12 healthy men and women were recruited for each of two randomized, two-way crossover studies in which one meal was served with lipid in the form of avocado fruit (delivering 23 g lipid) and a second meal was served without lipid. In study 1, the source of provitamin A carotenoids was a tomato sauce made from a novel, high β-carotene variety of tomatoes (delivering 33.7 mg β-carotene). In study 2, the source of provitamin A carotenoids was carrots (delivering 18.7 mg α-carotene and 27.3 mg β-carotene). Subjects consumed a low vitamin A and provitamin A diet for two weeks before each test meal was served. Test meals were fed, post-prandial blood samples were taken over 12 hours, and provitamin A carotenoids and vitamin A (as retinyl esters) were quantitated in triglyceride rich lipoprotein fractions to determine baseline-corrected area under the concentration-versus-time curve (AUC). The feeding of lipid-rich avocado enhanced the absorption of β-carotene from study 1 by an average of 2.3 fold (P < 0.001), and the absorption of α-carotene and β-carotene by an average of 4.9 and 5.8 fold, respectively, in study 2 (P < 0.001 for both). In addition, feeding lipid-rich avocado enhanced the
conversion to vitamin A (as measured by retinyl esters) by an average of 4.5 fold in study 1 and an average of 7.2 fold in study 2.

In conclusion, consumption of provitamin A carotenoids with dietary lipid enhances absorption and increases the absolute amount of vitamin A released into the blood. In addition, subjects who initially exhibited a lower level of intestinal conversion of \( \beta \)-carotene to vitamin A when consuming the sauce meal demonstrated enhanced efficacy of conversion when co-consuming the sauce meal with lipid-rich avocado.

**INTRODUCTION**

Vitamin A deficiency is a global problem. It is the leading cause of blindness in children, and is also associated with increased burden of infectious disease, xerophthalmia (dry eye syndrome), night blindness, and increased risk of mortality (14). Vitamin A is an essential nutrient in humans which must be obtained from dietary sources (1). Vitamin A is derived from the diet in two forms: preformed vitamin A (from animal products such as milk and meat) and provitamin A (from lipophilic plant compounds called carotenoids) (1). In the United States, it is estimated that approximately 30\% of the vitamin A consumed in the typical American diet is derived from provitamin A carotenoids found in fruits and vegetables (1). Carotenoids that contain at least one unsubstituted \( \beta \)-ionone ring have provitamin A activity. These carotenoids, which include \( \beta \)-carotene and \( \alpha \)-carotene, can be enzymatically cleaved to produce vitamin A in mammalian systems by \( \beta \)-carotene oxygenase 1 (BCO1) (6).
Bioavailability of a carotenoid is defined as the amount of the carotenoid which is absorbed, metabolized, packaged into chylomicrons (as parent carotenoid or as a vitamin A metabolite), and released into circulation after consumption of a carotenoid-containing meal. Thus, bioavailability of carotenoids can be monitored by measuring levels in the triglyceride rich lipoprotein (TRL) fraction of plasma which contains chylomicrons. Bioavailability can be affected by a number of factors. Studies by our group and others have demonstrated that the consumption of a carotenoid containing meal with lipid or a lipid rich fruit like avocado dramatically enhances carotenoid bioavailability as compared to a meal with little or no lipid (31,32,35,193). However, the effects of lipid on provitamin A conversion to vitamin A have yet to be investigated in humans. In addition, a definitive understanding of provitamin A carotenoid absorption and metabolism in humans, relative to the provitamin A content in foods, is still lacking. A more detailed knowledge of the bioavailability and metabolism of this carotenoid under a variety of meal conditions is needed in order to optimize vitamin A status and prevent deficiency.

Our primary objective was to determine if adding lipid to a carotene-rich meal, in the form of lipid-rich avocado fruit, would promote the absorption of provitamin A carotenoids and enhance conversion to vitamin A. We accomplished this by monitoring the immediate post-prandial plasma concentrations of parent carotenoids and retinyl esters after subjects consume a meal with or without avocado in combination with a serving of a novel high β-carotene tomato sauce (containing nutritionally relevant amounts of β-carotene) for study 1, or carrots (containing β-carotene and α-carotene) for
study 2. We also monitored the absorption of other carotenoids and vitamins from the avocado fruit (i.e. lutein, α-tocopherol, and phylloquinone).

**SUBJECTS AND METHODS**

*Subjects:* Healthy adult volunteers were recruited for these studies (sauce study n=12, carrot study n=12). Subjects were non-pregnant, non-smoking, normocholesterolemic (<200 mg/dL total cholesterol), normolipidemic, body mass index (BMI) of 17-30, without history of cancer, gastrointestinal diseases, diabetes, or use of medication affecting lipid uptake or transport. The study was approved by the Institutional Review Board of the Ohio State University, Protocol # 2011H0159, and the Clinical Research Center (CRC) of the Ohio State University, CCTS ID #987. The study was registered on ClinicalTrials.gov. Written, informed consent was obtained from all subjects before beginning the study, and all clinical procedures were carried out at the CRC.

*Study Instruments:* Subjects were asked to fill out a health and lifestyle questionnaire during their initial visit to the CRC. The primary purpose of this questionnaire was to identify individuals who met exclusion criteria and could not participate in the study. Subjects were given a list of foods and supplements to avoid. Throughout the 4 week duration of the study, subjects were asked daily to review a diet compliance checklist, and to document any deviations from the dietary restrictions. Dietary restrictions were determined based upon the U.S.D.A. Carotenoid Database for U.S. Foods 1998 and National Nutrient Database for Standard Reference Release 23, and included no consumption of foods or supplements containing > 1 mg of β-carotene or α-carotene per
100 g serving, > 0.5 mg lutein per 100 g serving, or foods containing high levels or fortified with preformed vitamin A (ready-to-eat cereals, dairy or dairy replacement products, liver, fish oil). This diet compliance checklist was used to determine whether subjects were consuming any restricted foods.

*Experimental Design:* For each study, an equal number of men and women were randomly assigned to one of two groups. One group consumed the test meal containing avocado on the first daylong visit, the other group consumed the test meal without avocado on the first daylong visit. Subjects were asked to abstain from consuming foods rich in provitamin A and vitamin A for 2 weeks prior to their first daylong clinic visit. After an overnight (12 h) fast, subjects arrived at the clinic in the morning and had an angiocatheter inserted. Hour 0 blood was drawn, and then subjects immediately consumed the test meal. Subjects were given 20 minutes to eat the sauce meal, and 30 minutes to eat the carrot meal. Subjects were allowed to consume water ad libitum throughout the course of the first daylong visit. Blood samples were then taken at hours 2, 3, 4, 5, 6, 8, 10, and 12 hours after the meal was consumed. A lunch very low in carotenoids, provitamin A, and lipid was served at 4.5 hours.

Subjects returned home, continued the low provitamin A and vitamin A diet for two additional weeks, and then again returned to the clinic for the second daylong visit. Subjects crossed over to the test meal they had not yet consumed on the second daylong visit. *Blood Lipids:* Blood lipids were tested at all three clinic visits, and are shown in Table 3.1.
**Test Foods:** For study 1, a novel variety of tomato (*Solanum lycopersicum* L.) rich in β-carotene (variety 97L97) was grown at the OSU North Central Agricultural Research Station near Fremont, Ohio. This variety has been used by our group previously (6). Tomatoes were harvested and processed into tomato juice using a hot-fill process in a pilot plant of the Food Industries Center of the Ohio State University. Later, the tomato juice was concentrated in a steam-jacketed kettle to a brix value of 15° to make tomato sauce. The tomato sauce was hot filled into #300 cans, and sealed the cans to create a shelf stable product. For study 2, petite baby carrots were purchased from the local grocery store. Avocados were provided by the Hass Avocado Board.

**Meals:** Test foods were served with the breakfast meal. For study 1, 300 g of processed sauce was weighed and served at room temperature. On the day subjects consumed the test meal with avocado, 150 g of avocado was sliced fresh and placed atop the sauce. For study 2, 300 g of raw petite baby carrots were weighed into a bowl. On the avocado days, the carrots were served with guacamole consisting of 150 g of mashed avocado, 1 tsp. lemon juice, 1/8 tsp. garlic powder, and 1/8 tsp. salt. Subjects were also given one English muffin to completely clean and consume the sauce from the bowl for the sauce study, or to clean and consume the guacamole from the bowl for the carrot study. In addition, cooked egg whites (from 2 eggs), a medium banana, and a cup of coffee were served with breakfast. The breakfast with tomato sauce alone provided 406 calories, with 17 g of protein, 2 g of lipid, and 80 g of carbohydrate. The breakfast with carrot alone provided 390 calories, with 15 g of protein, 2 g of lipid, and 78 g of carbohydrate. When
the breakfast meal was consumed with avocado or guacamole, an extra 275 calories were consumed, with 3 g protein, 23 g total lipid, and 14 g carbohydrate.

*Carotenoid Extraction from Food*: The raw carrots were blended in a food processor to prepare a fine pulp. Approximately 2 g of carrot pulp, sauce, or mashed avocado were weighed into 12 mL glass tubes. 5 mL of methanol was added, and the mixture was probe sonicated. The sample was centrifuged at 2000 x g for 10 min. The methanol was decanted into a clean glass vial, and 5 mL of hexane/acetone (1:1) was added to the remaining food material. The sample was again sonicated and centrifuged, and the hexane/acetone extract was removed and combined with the methanol. The hexane/acetone extraction was repeated twice more. To the pooled extracts, 10 mL of water and 1 mL of saturated aqueous NaCl solution was added to induce phase separation. The extract was shaken, and the upper phase was transferred to a 25 mL volumetric flask. An aliquot was removed and dried under nitrogen gas, and stored at -20 °C before analysis.

*Methods of Analysis*: The blood preparation, TRL isolation, carotenoid extraction, and quantitation methods are detailed in Chapter 2.

Conversion Efficiency: To estimate the amount of vitamin A formed in the enterocyte from the β-carotene dose in study 1, we used the following equation from Fleshman et al., (184):

**Equation 1**: 
\[
\frac{(\text{Total AUC}_{\text{retinyl esters}}/2)}{(\text{Total AUC}_{\beta\text{-carotene}} + (\text{Total AUC}_{\text{retinyl esters}}/2))}.
\]
To estimate the amount of vitamin A formed in the enterocyte from the β-carotene and α-carotene doses in study 2 with carrots, the equations of Edwards et al., (20) were used with slight modifications. Briefly, the contribution of each carotene to the TRL vitamin A pool was calculated by taking into account the relative ratio of β-carotene and α-carotene in the test meal as follows:

**Equation 2:** 
\[
\frac{(\text{Total AUC}_{\text{retinyl esters}} \times (27.4 \text{ mg } \beta\text{-carotene/46.2 mg total carotenes})/2) + (\text{Total AUC}_{\text{retinyl esters}} \times (18.8 \text{ mg } \alpha\text{-carotene/46.2 mg total carotenes}))}{\text{X}}
\]

This value was then divided by the sum of the estimated total carotenes derived from the meal, using the following equation:

**Equation 3:** 
\[
\frac{\text{X}}{\text{Total AUC}_{\beta\text{-carotene}} + \text{Total AUC}_{\alpha\text{-carotene}} + \text{X}} \times 100\%
\]

**Statistical Analysis:** Each compound is expressed as the baseline-corrected AUC value in the TRL fraction for the 12 hours after meal consumption. AUC values were determined by using trapezoidal approximation. Statistical analyses were performed using Minitab version 15.1. Raw AUC values for all compounds were right-skewed and did not satisfy normality parameters. Thus, AUC median values, and 25th and 75th percentiles of each intervention group are reported in Table 3.3. The raw AUC data was log transformed before additional calculations and analyses were performed, and normality assumptions of the log transformed data were verified by the Kolmogorov-Smirnov test and Q-Q plots.
for residuals. Fold differences were calculated for each subject by the following equation: \((\text{test meal with avocado} - \text{test meal alone})/\text{test meal alone}\). A student’s t-test was performed to determine whether the average fold difference in AUC was greater than 0. Analysis of variance (ANOVA) was used to determine if gender or meal order significantly impacted average fold AUC difference. A P of < 0.05 was considered statistically significant.

**RESULTS**

*Subjects:* Table 3.1 provides the baseline characteristics of study subjects at their initial visit to the clinic. Twelve subjects completed study 1, and twelve subjects completed study 2. After reviewing the data, one female subject in study 1, who had indicated that she typically followed a “paleo-diet” in the health questionnaire, had a large negative AUC value for \(\beta\)-carotene and retinyl esters in the sauce meal alone, and no \(\beta\)-carotene and retinyl ester change from baseline for the sauce meal with avocado. It is not clear if the adaptation of a paleo-diet affected carotenoid absorption and metabolism, or whether this subject was a so called “non-responder” or “poor absorber”. This subject’s data was dropped from the final data set.
### Table 3.1 Subject Characteristics at Initial Screening Visit

<table>
<thead>
<tr>
<th>Study</th>
<th>Gender</th>
<th>Age (y)</th>
<th>BMI (kg/m²)</th>
<th>Plasma Total Cholesterol level (mg/dL)</th>
<th>Plasma Triacylglycerol level (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tomato Sauce Study</td>
<td>Female (n=5)</td>
<td>24.6 ± 4.6</td>
<td>22.4 ± 3.3</td>
<td>167.2 ± 24.7</td>
<td>79.2 ± 42.9</td>
</tr>
<tr>
<td></td>
<td>Male (n=6)</td>
<td>26.7 ± 5.0</td>
<td>25.8 ± 2.2</td>
<td>151.2 ± 26.9</td>
<td>107.2 ± 65.1</td>
</tr>
<tr>
<td>Carrot Study</td>
<td>Female (n=6)</td>
<td>28.5 ± 5.0</td>
<td>23.1 ± 2.7</td>
<td>172.0 ± 14.8</td>
<td>59.2 ± 29.8</td>
</tr>
<tr>
<td></td>
<td>Male (n=6)</td>
<td>27.2 ± 4.0</td>
<td>25.3 ± 2.4</td>
<td>165.8 ± 31.2</td>
<td>88.3 ± 87.6</td>
</tr>
</tbody>
</table>

*Values are means ± standard deviation.*

Note that characteristics between genders within each study are not statistically different from one another using a two-tailed unpaired Student’s t-test (*P* < 0.05)
Absorption of Carotenoids: Table 3.2 provides information on the level of fat soluble carotenoids and vitamins fed during each test meal. Median AUC values for nutrients of interest are provided in Table 3.3.
<table>
<thead>
<tr>
<th></th>
<th>β-carotene (mg)</th>
<th>α-carotene (mg)</th>
<th>lutein (mg)</th>
<th>lycopene (mg)</th>
<th>α-tocopherol (mg)</th>
<th>phylloquinone (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tomato Sauce</td>
<td>33.7 ± 0.21</td>
<td>N.D.</td>
<td>N.D.</td>
<td>2.34 ± 0.01</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Sauce with Avocado</td>
<td>33.7 ± 0.21</td>
<td>0.014 ± 0.007</td>
<td>0.12 ± 0.03</td>
<td>2.34 ± 0.01</td>
<td>2.80 ± 0.29</td>
<td>26.2 ± 9.8</td>
</tr>
<tr>
<td>Carrots</td>
<td>27.3 ± 7.7</td>
<td>18.7 ± 5.5</td>
<td>0.40 ± 0.11</td>
<td>0.04 ± 0.01</td>
<td>N.D.</td>
<td>19.8 ± 8.6</td>
</tr>
<tr>
<td>Carrots with Avocado</td>
<td>27.4 ± 7.9</td>
<td>18.8 ± 5.5</td>
<td>0.50 ± 0.13</td>
<td>0.04 ± 0.01</td>
<td>2.80 ± 0.29</td>
<td>46.6 ± 19.9</td>
</tr>
</tbody>
</table>

*Values are means ± standard deviation.
N.D. = not determined
Table 3.3  AUC levels (nmol.hr/L) of the Carotenoids and Fat Soluble Vitamins After Test Meal Consumption

<table>
<thead>
<tr>
<th></th>
<th>β-carotene</th>
<th>α-carotene</th>
<th>lutein</th>
<th>α-tocopherol</th>
<th>phylloquinone</th>
<th>retinyl esters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tomato Sauce</td>
<td>202 (111, 273)\textsuperscript{a}</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>127 (25, 327)\textsuperscript{a}</td>
</tr>
<tr>
<td>Sauce with Avocado</td>
<td>437 (269, 730)\textsuperscript{b}</td>
<td>N.D.</td>
<td>15 (6.5, 74)</td>
<td>4.4 (1.0, 7.4)</td>
<td>7.9 (7.0, 16.3)</td>
<td>367 (237, 802)\textsuperscript{b}</td>
</tr>
<tr>
<td>Carrots</td>
<td>88 (24, 125)\textsuperscript{a}</td>
<td>70 (31, 97)\textsuperscript{a}</td>
<td>34 (3.5, 63)</td>
<td>N.D.</td>
<td>0.5 (0.0, 0.7)\textsuperscript{a}</td>
<td>51 (22, 97)\textsuperscript{a}</td>
</tr>
<tr>
<td>Carrots with Avocado</td>
<td>366 (276, 460)\textsuperscript{b}</td>
<td>260 (170, 313)\textsuperscript{b}</td>
<td>39 (7.4, 70)</td>
<td>1.6 (1.0, 2.8)</td>
<td>4.6 (3.9, 10)\textsuperscript{b}</td>
<td>327 (234, 490)\textsuperscript{b}</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Values are medians (25\textsuperscript{th}, 75\textsuperscript{th} percentiles). Within a study, medians in a column without a common letter indicate a significant difference between the two meals, P < 0.001

N.D. = not determined
In study 1, the consumption of the sauce meal with avocado lead to a 2.3 fold increase in AUC β-carotene (P < 0.001), as shown in Figure 3.1A. Likewise, consumption of the sauce meal with avocado lead to a 4.5 fold increase in AUC retinyl esters as compared to the sauce meal alone (P < 0.001), as shown in Figure 3.1B. Gender and meal order did not significantly impact the AUC fold difference. The mean fractional absorption of β-carotene from the sauce meal alone was 35%, while the mean fractional absorption of β-carotene from the sauce meal when consumed with avocado was 86%.
Figure 3.1 Baseline-corrected plasma TRL concentrations of β-carotene (A) and retinyl esters (B) over 12 hours after consumption of the high β-carotene tomato sauce with avocado (▲) and high β-carotene tomato sauce alone (♦). Values are means ± standard error of the mean.
In study 2, the consumption of the carrots with guacamole led to a 5.8 fold AUC increase in β-carotene and a 4.9 fold AUC increase in α-carotene (P < 0.001) as compared to carrots alone, which are depicted in Figure 3.2A and 3.2B, respectively. A 7.2 fold increase in AUC of retinyl esters was observed when subjects consumed carrots with guacamole as compared to carrots alone (P < 0.001), shown in Figure 2C. Similarly, a 4.8 fold increase in phylloquinone AUC was observed when subjects consumed carrot with avocado as compared to carrot alone (P < 0.001). In contrast, no fold difference was observed for AUC lutein. Gender and meal order did not significantly impact the AUC fold difference for any of the analytes.
Figure 3.2 Baseline-corrected plasma TRL concentrations of β-carotene (A), α-carotene (B), and retinyl esters (C) over 12 hours after consumption of the carrot with avocado (▲) and carrot alone (♦). Values are means ± standard error of the mean.
Conversion Efficiency:  Figure 3.3 plots the percent conversion of provitamin A to vitamin A for each individual subject when the tomato sauce meal was consumed alone as compared to the sauce meal with avocado. The range of β-carotene conversion to vitamin A for the sauce and avocado meal in study 1 was 25%-48%. A strong linear relationship between conversion efficiency of the two meals was observed. Subjects with low conversion efficiency when consuming the sauce meal alone had notably improved conversion when the meal was consumed with avocado. Subjects with high conversion efficiency with sauce alone had less improvement when the meal was consumed with avocado.
Figure 3.3  A plot of the percent conversion of provitamin A into vitamin A, following Equation 1. Each data point represents a single subject in the study, and plots their conversion efficiency from the sauce meal alone against the sauce meal when consumed with avocado. The line through the origin represents an equivalent conversion for both meals. The linear equation and $R^2$ value describe the relationship between the data points.
While a similar trend was observed with the carrot study, there was much wider variation in percent conversion efficiency, and the linear relationship was weaker ($R^2 = 0.30$), Figure 3.4. The ratio of $\alpha$-carotene to $\beta$-carotene in the carrot meal (approx. 1:1.4 $\alpha$-carotene/$\beta$-carotene) was largely maintained in the blood plasma of subjects when they consumed the carrot meal with avocado, but this ratio was not maintained when subjects consumed the carrot alone (data not shown). The range of $\beta$-carotene and $\alpha$-carotene conversion to vitamin A from the carrot with avocado meal was 8%-69%, demonstrating a very large range and interindividual variation.
Figure 3.4 A plot of the percentage conversion of provitamin A into vitamin A, following Equations 2 and 3. Each data point represents a single subject in the study, and plots their conversion efficiency from the carrot meal alone against the carrot meal when consumed with avocado. The line through the origin represents an equivalent conversion for both meals. The linear equation and $R^2$ value describe the relationship between the data points.
DISCUSSION

In this study, a higher absorption of β-carotene was observed in study 1 and β-carotene and α-carotene in study 2 when the test meal was consumed with lipid-rich avocado fruit as compared to no avocado. Likewise, previous work from our group and others has demonstrated that carotenoids are more readily absorbed from a meal containing lipid as compared to no lipid or lower levels of lipid (31,32,35). A previous study from our group demonstrated that the absorption of β-carotene (11.5 mg) and α-carotene (6.6 mg) from a mixed salad meal was enhanced 15.3 fold and 7.2 fold respectively, when the meal was consumed with 150 g of lipid-rich avocado fruit as compared to no avocado fruit (32). In the current studies, a smaller magnitude of increase of AUC carotenoid was observed when the meals were consumed with avocado. It is hypothesized that this difference may be attributed to the larger dose of carotenoid delivered from the sauce in study 1 (33.7 mg β-carotene) and from the carrots in study 2 (27.4 mg β-carotene and 18.8 mg α-carotene) as compared to the previous work (32).

Interestingly, our work here demonstrates that the feeding of the avocado with the test meals lead to a higher absolute level of AUC retinyl esters (i.e. vitamin A) in the TRL fraction. In study 1, we observed a 4.5 fold increase in retinyl esters and in study 2 we observed a 7.2 fold increase in retinyl esters when the test meal was consumed with lipid. While increased absorption of provitamin A carotenoids provides more provitamin A to be converted, lipid may also impact other variables which affect conversion. Higher lipid consumption has been shown to have an impact on BCO1 specific activity, as demonstrated in a rodent study. During et al. (39) fed four different groups of rats a
standardized diet containing 2.5% polyunsaturated fat, 15% saturated fat, 15% monounsaturated fat, or 15% polyunsaturated fat for three weeks. Animals were sacrificed, the BCO1 protein fraction of the intestinal homogenate was incubated with β-carotene for 30 minutes, and the resulting retinal was quantitated. Animals in the 15% mono and poly unsaturated groups had 2.4 fold greater specific BCO1 activity (as measured by retinal formation) than the 2.5% polyunsaturated fat group (39). Recent work by Lobo et al. (197) has demonstrated that PPAR-γ activation in adipocytes regulates BCO1 transcription. Thus, it is plausible that dietary lipid could lead to increased synthesis of BCO1 in the enterocyte, resulting in increased formation of vitamin A. In addition, other researchers have demonstrated that dietary lipid is necessary for chylomicron synthesis in the enterocyte (40). Thus, it is possible that increased levels of retinyl esters in the chylomicron fraction are a product of increased synthesis and release of chylomicrons containing retinyl esters. Regardless of the mechanism(s) involved, increasing vitamin A formation and delivery to the circulatory system by consuming lipid-rich may have practical implications for populations where vitamin A deficiency is prevalent.

No statistically significant difference in lutein AUC values was observed for study 2. This result is not surprising as there was only a 0.1 mg difference in the lutein content of the two test meals. In addition, AUC values of α-tocopherol (vitamin E) and phylloquinone (vitamin K) were quite low. In other studies where statistically significant AUC changes were observed for α-tocopherol, a labeled dose ranging from 15 mg – 150 mg was fed (198,199) as compared to 2.8 mg fed in our studies when the avocado fruit
was consumed. Likewise, studies demonstrating significant AUC changes for unlabeled phylloquinone have been observed fed ~0.4 mg - 1 mg of this nutrient (200–202) as compared to up to 0.047 mg fed in our study. Subjects were not required to abstain from consuming α-tocopherol and phylloquinone during the washout, which may have also impacted the uptake of these nutrients from the test meal.

The range of β-carotene conversion to vitamin A for the sauce and avocado meal was 25%-48%, and the range of total carotene conversion to vitamin A from the carrot and avocado meal was 8%-69%. These values are within range of what has been reported by other groups. For example, van den Berg observed a 69%-71% conversion rate for β-carotene when it was fed in palm oil (41). A study by van Vliet et al. (36) fed 15 mg of β-carotene in oil and observed a range of 33%-71% conversion. Similar ratios have been reported for β-carotene in foods. Edwards et al. (20) found a mean conversion efficiency of 44% for carrot puree, 59% for boiled-mashed carrots, and 63% for raw chopped carrots for an 18.6 mg dose of β-carotene when healthy subjects were fed with 9 mg safflower oil. A striking linear relationship is displayed in Figure 3.3 for provitamin A conversion to vitamin A when sauce was consumed alone, plotted against the conversion to vitamin A when the sauce was consumed with avocado lipid. Furthermore, the effect of avocado lipid consumption on conversion efficiency is not equal among subjects. Those with lower conversion efficiency have a more appreciable increase in conversion with avocado lipid than those with higher levels of conversion. It is unclear why this phenomena was observed. As suggested before, differences in percent conversion between subjects could be related to specific polymorphisms in BCO1 (203).
or potentially PPAR-γ, or any number of other proteins involved in carotenoid uptake, transport, and/or metabolism in the enterocyte, as recently reviewed by Borel (196).

When subjects consumed the carrot meal with avocado, the ratio of α-carotene to β-carotene in the carrot was maintained in the blood TRL’s. However, the ratio of α-carotene to β-carotene is not maintained in blood TRL’s when subjects consumed the carrot meal alone. We hypothesize that the weaker relationship of conversion efficiency with and without lipid observed in the carrot study (shown in Figure 3.4) as compared to the sauce study may be attributed to multiple factors. These factors include food matrix effects (cooked sauce vs. raw carrot), the contribution of both β-carotene and α-carotene to vitamin A in the carrot study as compared to β-carotene alone in the sauce study, etc.

One subject in study 1 appeared to be a “non-responder” to the carotenoid feeding treatment, as the subject consumed both meals in the clinic, and no obvious deviations from the study protocols were noted. Non-responders (sometimes referred to as poor absorbers) have been previously reported for carotenoid absorption (194,195) although this seems to be a small percentage of the population (34). This phenomenon may be attributed to polymorphisms in enzymes involved in carotenoid uptake, storage, and conversion (196).

In conclusion, consuming provitamin A carotenoids with lipid-rich avocado enhances carotenoid absorption in healthy humans. Importantly, a higher level of vitamin A, as measured by retinyl esters in the TRL fraction, is observed when the meal is fed with lipid-rich avocado as compared to no avocado. This observation highlights the importance of feeding provitamin A carotenoids with lipid in the meal in vitamin A
deficient populations, where maximum delivery of active vitamin A is desired. Furthermore, consuming lipid-rich avocado with provitamin A from a high β-carotene tomato sauce leads to a higher efficiency of conversion to vitamin A in subjects with low conversion efficiency. Novel varieties of tomatoes, including those used in this study, represent a potential food source of provitamin A which could be utilized in developing nations where tomatoes are consumed and vitamin A deficiency is prevalent.

Acknowledgments: We would like to acknowledge Dr. Dennis Pearl for his advice regarding the statistical analysis of the data.
CHAPTER 4

Identification and quantification of apo-lycopenalns in fruits, vegetables, and human plasma

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Published in 2010 in the Journal of Agricultural and Food Chemistry. 58(6):3290-3296
ABSTRACT

Research has suggested that lycopene may be metabolized by eccentric cleavage, catalyzed by β-carotene oxygenase 2 (BCO2), resulting in the generation of apo-lycopenals. Apo-6'-lycopenal and apo-8'-lycopenal have been reported previously in raw tomato. We now show that several other apo-lycopenals are also present in raw and processed foods, as well as in human plasma. Apo-lycopenal standards were prepared by in vitro oxidation of lycopene, and a high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) method using atmospheric pressure chemical ionization in negative mode was developed to separate and detect the apo-6', 8', 10', 12', 14', and 15'-lycopenal products formed in the reaction. Hexane/acetone extracts of raw tomato, red grapefruit, watermelon, and processed tomato products were analyzed, as well as plasma of individuals who had consumed tomato juice for eight weeks. Apo-6', 8', 10', 12', and 14'-lycopenals were detected and quantified in all food products tested, as well as plasma. The sum of apo-lycopenals was 6.5 µg/100 g ‘Roma’ tomato, 73.4 µg/100 g tomato paste, and 1.9 nmol/L of plasma. We conclude that several apo-lycopenals, in addition to apo-6'- and 8'-lycopenal, are present in lycopene containing foods. In addition, the presence of apo-lycopenals in plasma may derive from the absorption of apo-lycopenals directly from food and/or human metabolism.

INTRODUCTION

Epidemiological studies have repeatedly correlated increased consumption of tomato and tomato products with reduction in the incidence of a number of cancers and
other chronic diseases (46,52,53,204,205). Many researchers have suggested that lycopene, the most abundant carotenoid in red tomatoes, may be responsible for this effect (206–208). It has also been suggested that metabolites of lycopene may be bioactive (60,61). Khachik et al. (107) first reported lycopene derivatives in human blood plasma, which were later identified as two diastereomeric forms of 2,6-cyclolycopene-1,5-diol (108). These researchers proposed that these two structures were either absorbed from tomato products in the diet, or were metabolic products of 2,6-cyclolycopene-1,5-epoxide, a compound which has been identified in raw tomatoes, tomato juice, and tomato paste (209).

Lycopene may be metabolized in humans by a mechanism analogous to that of β-carotene, another C40 hydrocarbon carotenoid. The most widely characterized pathway of β-carotene metabolism is the formation of two molecules of retinal by the cleavage of β-carotene at the central double bond by β-carotene oxygenase 1 (BCO1) (6). A second enzyme, β-carotene oxygenase 2 (BCO2), has been shown to cleave β-carotene eccentrically (210). Interestingly, a central cleavage product of lycopene has never been identified in a mammalian system. Thus, a focus upon BCO2 cleavage has stimulated efforts to oxidize lycopene to produce aldehydes which could result from BCO2 cleavage. Kim and colleagues (111) produced a series of aldehyde cleavage products of lycopene using a variety of oxidative methods. Likewise, a similar series of aldehyde derivatives were generated by Caris-Veyrat et al. (112) using potassium permanganate as an oxidant.
We have limited knowledge of lycopene metabolites in mammals. Apo-lycopenals have been reported in laboratory animals consuming high lycopene diets. Apo-10′-lycopenol was found in the lung tissue of ferrets consuming a high lycopene diet for 9 weeks (96). In a separate study, apo-8′-lycopenal, and putatively apo-12′-lycopenal were observed in the hepatic tissue of rats consuming a high lycopene diet for 30 days (109). Interestingly, these apo-lycopenal products are not limited to mammalian systems. Apo-6′-lycopenal and apo-8′-lycopenal were previously identified in extracts of tomato paste (105) and raw tomatoes (106).

Given the highly unsaturated structure of lycopene, containing 11 conjugated double bonds available for oxidative cleavage, we hypothesized that an array of apo-lycopenals, in addition to apo-6′- and apo-8′-lycopenal, might be present in tomatoes or tomato products, and possibly as mammalian metabolites. We chose to focus our search on the long chain non-volatile products (> 20 carbons, i.e. acycloretinal or longer) that would result from a double bond cleavage and apply highly sensitive and selective tandem HPLC-MS/MS techniques to detect and identify apo-lycopenals by comparison to synthesized compounds and authentic standards. We also sought to identify and quantify apo-lycopenals in other lycopene-containing foods (red grapefruit, watermelon). Additionally, we identified and quantified apo-lycopenals in the plasma of humans consuming a daily serving of tomato juice.
MATERIALS AND METHODS

Chemicals: Apo-6′-lycopenal, apo-8′-lycopenal, apo-12′-lycopenal, and β-apo-12′-carotenal were purchased from Carotenature (Lupsingen, Switzerland). Pure lycopene was isolated and crystallized from tomato paste following a procedure outlined below. Acetone, acetonitrile, butylated hydroxytoluene (BHT), calcium carbonate, chloroform, dichloromethane, hexane, methanol, methyl tert.-butyl ether, potassium hydroxide, tetrahydrofuran, and toluene were purchased from Fisher Scientific (Pittsburgh, PA). Tetrahydrofuran was stabilized before use by passing the solvent through a column of alumina. Formic acid, ethylenediaminetetraacetic acid (EDTA), diatomaceous earth, β-apo-8′-carotenal, and β-carotene were purchased from Sigma Aldrich (St. Louis, MO).

Isolation of lycopene from tomato paste: We found it necessary to isolate pure lycopene from tomato paste due to our observation that analytical grade lycopene standard purchased from two commercial sources contained detectable levels of apo-lycopenals. Sixty grams of tomato paste was blended in an Oster blender with 12 g CaCO₃, and 250 mL of MeOH to dehydrate the tissue. The homogenate was filtered through a Whatman #1 filter using a Buchner funnel. The MeOH rinse was discarded, and the filtrant was then mixed with 150 mL of acetone and filtered again. The acetone rinse was also discarded. The filtrant was then mixed with 150 mL of hexane/acetone (1:1) and filtered again. Next, the hexane/acetone filtrate (containing carotenoids and lipids) was placed into an Erlenmeyer flask and 75 mL of 30% KOH in MeOH solution was added in addition to a stir bar. The Erlenmeyer flask was placed into a large beaker containing ice, and the mixture was stirred on a stir plate for 2 h to saponify the components. The
mixture was then transferred to a 2 L separatory funnel and washed three times with water. The aqueous phase was drained and discarded, and the hexane was placed in a 500 mL round bottom flask and dried under reduced pressure in a 40 °C water bath.

The dried extract was removed from the round bottom flask, transferred to a vial, and 1:1 chloroform/MeOH solution was added. The vial was placed into a 55 °C water bath, and the mixture was stirred until all of the dried extract dissolved. The water bath containing the dissolved lycopene was removed from the heat source and allowed to slowly cool to room temperature to induce lycopene crystallization. The sample was then transferred to the -20 °C freezer for two hours to facilitate further crystallization. Afterwards, the supernatant was filtered off and the crystals were washed four times with ice cold MeOH. The crystals were dried under vacuum and analyzed for apo-lycopenals using the LC-MS/MS method detailed below. Purity was determined by HPLC-photodiode array (PDA).

*Synthesis of apo-lycopenals for identification:* A series of apo-lycopenals was generated from lycopene using ozone as an oxidant. The lycopene used for this oxidation was isolated as above with modifications. That is, the initial blending of 360 g of tomato paste with MeOH (600 mL) and CaCO₃ (70 g) also included 100 g of diatomaceous earth. Further, once the combined acetone/hexane 1:1 filtrate was prepared, it was washed with 3 x 100 mL portions of saturated NaCl solution and the organic layer was evaporated. The resulting crude tomato oleo resin was transferred to a LiChroprep RP-18, 40-63 μM, reverse phase column (EM Separations, Gibbstown, NJ) which was washed with 150 mL of MeOH/acetonitrile/CH₂Cl₂ 45:45:10 (211) and then pure CH₂Cl₂ to elute 188 mg of
lycopene which as used as obtained. For ozonolysis, according to the procedure of Schwartz et al. (212), 4 mg (0.01 mmol) of this lycopene and 4-methylmorpholine-N-oxide (40 mg; 0.34 mmol) were dissolved in 8mL of CH$_2$Cl$_2$ and the solution divided into 4 x 2 mL aliquots. Phosphate buffer (2 mL of 25 mM, pH 6) was also added to each aliquot. Ozone was generated by a model T-816 laboratory ozonator (Welsbach, Philadelphia, PA) with oxygen pressure of 8 psi, dielectric voltage of 105 V, and ozone was bubbled into CH$_2$Cl$_2$ for 30 s. Different aliquots (0.5, 1, 1.5 and 2 mL) of this saturated ozone solution were added to the four lycopene containing vials which were immediately shaken to mix with the buffer and quench the reaction. The CH$_2$Cl$_2$ layers were then separated, concentrated, and the residues subjected to HPLC-MS/MS analysis as described below.

**HPLC-MS/MS Method for separating apo-lycopenals in the oxidized lycopene mixture:**

An HPLC method was developed to resolve long-chain apo-lycopenals produced in the mixture of lycopene oxidation products. The mixture was separated by a model 2996 HPLC (Waters) connected to a model 2996 PDA (Waters) using a 150 mm x 4.96 mm i.d., 5 µm, YMC C30 column (Waters Corp., Milford, MA). Composition of solvents was as follows: A= 88:5:5:2 MeOH/water/methyl tert.-butyl ether/0.1% aq. formic acid solution; B = 78:20:2 methyl tert.-butyl ether/MeOH/0.1% aq. formic acid solution. A gradient of A and B was used to separate the synthesized compounds at a flow rate of 1.3 mL/min, column temperature was 30 °C. The gradient was as follows: isocratic at 0% B for 1 min, ramp to 4.5% B over 0.1 min, linear gradient to 90% B over 19.9 min, linear gradient to 100% B over 6 min, and 4 min to return to 0% B. The HPLC was interfaced
with a Q-Tof Premier quadrupole time-of-flight hybrid mass spectrometer (Micromass UK Ltd., Manchester, UK) interfaced using an atmospheric pressure chemical ionization (APCI) source operated in negative ion mode. Additional settings were as follows: corona current = 30 μA, cone = 35 V, desolvation temp. = 400 °C, desolvation gas flow = 300 L/h, and gas for collisionally induced dissociation (CID) was argon at 4 x 10^{-3} bar.

**Extraction of foods:** Foods used for analysis were purchased from a local supermarket (Columbus, OH). Three different brands of each processed tomato product (catsup, spaghetti sauce, pizza sauce, tomato soup, tomato paste, tomato juice) were tested. Raw red vine ripened tomatoes, cherry tomatoes, ‘Roma’ tomatoes, ruby red grapefruit, and watermelon were also tested. Raw fruits were purchased, diced, and mixed in an Oster blender to produce a puree before extraction.

Foods were extracted following the method developed previously (177). All extractions were performed under red light. Hexane extracts were dried immediately under argon gas and stored at -80 °C for not more than one week before analysis. Samples were reconstituted in 1:1 methyl tert.-butyl ether /MeOH before being injected into the UPLC for analysis. Each extraction of food products was performed in duplicate.

**UPLC-MS/MS Method for Food:** A method was developed to quantify the apolyycopenals present in lycopene-containing foods. Separation was performed on a Waters Acquity UPLC (Waters Corp., Milford, MA). The column used was a 50 mm x 2.1 mm i.d., 1.7 μm particle size, BEH C18 (Waters Corp., Milford, MA). Solvent A = 80:20 MeOH/0.1% aqueous formic acid solution. Solvent B = 85:15 MeOH/tetrahydrofuran. Flow rate was 0.7 mL/min, column temperature = 40 °C. Gradient was as follows: 0% B
to 90% B over 1 min, to 95% B over 0.7 min, to 100% B over 0.8 min which was held for 0.5 min, then quickly back to 0% B for 2 min. Quantitation of compounds was performed on a Quattro Ultima triple quadrupole mass spectrometer (Micromass UK Ltd., Manchester, UK) interfaced with the UPLC via an APcI source operated in negative ion mode. The mass spectrometer was operated in selected reaction monitoring (SRM) mode transmitting the radical ion form of the parent \([M^-]\) in the first quadrupole, fragmenting it by collision induced dissociation (argon-filled chamber at \(3 \times 10^{-3}\) mBar) and selecting the corresponding \([M-69]^-\) daughter ion with the last quadrupole to deliver to the detector. Mass transitions were as follows: 284>215, 310>241, 350>281, 376>307, 416>347, 442>373, 536>467 for acycloretinal, apo-14’-12’, -10’, -8’, -6’-lycopenal and lycopene, respectively. Instrumental parameters included: corona current = 30 μA, cone =35 V, desolvation temp. = 400 °C, desolvation gas flow = 700 L/h.

**Plasma analysis:** Seven human plasma samples from a previous dietary intervention study were analyzed for apo-lycopenals. In this study, healthy men and women consumed a diet supplemented with soy-fortified tomato juice for 8 weeks (delivering 21.9 mg of lycopene/day). Blood was sampled after 8 weeks of juice consumption. Samples were collected into 10 mL EDTA tubes, centrifuged immediately to remove red blood cells, and stored at -80 °C until extraction. Written informed consent was obtained from all study participants, and this study was approved by the Institutional Review Board of the Ohio State University, protocol # 2004H009.

Plasma (2 mL) was extracted using HEAT (hexane/ethanol/ acetone/toluene; 10:6:7:7, v/v/v/v) as the extraction solvent (213). Briefly, plasma was mixed with an
equal volume of ethanol containing 0.1% butylated hydroxytoluene, 0.5 mL of a saturated NaCl solution, and 10 mL of HEAT. The mixture was vortexed and then centrifuged for 5 min at 300 x g. The upper non-polar layer was removed with a glass pipette and the remaining aqueous plasma mixture was extracted two more times. The non-polar layers were combined and dried under argon. The dried extract was stored at -80 °C until analysis by HPLC-MS/MS.

**HPLC-MS/MS method for human plasma:** The C30 HPLC method used to separate the lycopene oxidation mixture was also used to separate apo-lycopenals present in human plasma extracts with slight modifications (solvent A for plasma analysis was 80:20 MeOH/0.1% aqueous formic acid solution). Both the C30 and the C18 methods were tested with plasma extracts, and the C30 method provided twice the sensitivity of the C18 method. This superior sensitivity was likely due to matrix components which were diluted or resolved from analytes in the longer C30 method. Since levels of apo-lycopenals are low in plasma, the more sensitive C30 method was chosen for quantitation of these compounds in plasma.

The C30 method was run on a UPLC instrument, which is capable of operating under the same parameters as a traditional HPLC system. A Waters Acquity UPLC (autosampler, pump, and PDA detector) was interfaced with the Quattro Ultima mass spectrometer (operated using the same settings as for food analysis) to quantify apo-lycopenals in the plasma extracts.

**Recovery Experiments:** Recovery experiments help validate that analytes are not being lost during extraction. Since aldehydes (i.e. apo-lycopenals) have the ability to react with
nucleophiles, we hypothesized that the aldehydes might covalently bind to protein in the food or blood plasma matrix and be underestimated. To determine whether such reactions were occurring during our extraction process, we performed the lipophilic extraction of both the plasma and the food in the presence of excess formaldehyde.

Data Analysis: Quantification of compounds in plasma and food samples is based on external calibration curves of apo-6′-, 8′-, 12′-lycopenal from Carotenature, and all-trans-lycopene purified by crystallization. Peak areas of the [M-69] daughter fragment (see UPLC-MS/MS Method for Food) were used to generate the calibration curves. Concentrations of lycopene and apo-lycopenals in the samples were calculated by comparing their peak areas to the respective calibration curve. In lieu of authentic apo-10′-lycopenal standard, apo-10′-lycopenal levels were estimated using the average response of apo-8′-lycopenal and apo-12′-lycopenal standards. In lieu of authentic apo-14′-lycopenal standard, levels of apo-14′-lycopenal were estimated using the response of apo-12′-lycopenal standard.

Values for apo-lycopenals in raw fruits and vegetables are the average of duplicate extractions. Apo-lycopenal levels in processed products are the average from the measurement of three different brands of product extracted in duplicate.

RESULTS AND DISCUSSION

Synthesis of apo-lycopenals: Figure 4.1 displays the structures of all-trans lycopene and the long chain apo-lycopenals observed after oxidation of lycopene with ozone.
Oxidation occurs at each double bond in the chain, leading to the series of products detected.
Figure 4.1 Series of apo-lycopenals produced after ozone treatment of lycopene.
**Figure 4.2** shows an HPLC-MS chromatogram of the separated mixture of apo-lycopenals and lycopene after lycopene ozonolysis. All apo-lycopenals in the series (apo-6’-, 8’-, 10’-, 12’-, 14’-, and 15’-lycopenal) display the characteristic isoprenyl loss upon CID in the mass spectrometer (M-69) (130). The m/z of the synthesized apo-lycopenals were within 2 ppm mass accuracy of the respective theoretical m/z values and elute in order of increasing molecular weight as expected. Identity of apo-6’-, 8’-, and 12’-lycopenal were confirmed by retention time (RT) and ultraviolet (UV) spectra coincident with commercial standards. MS/MS chromatograms for the m/z values consistent with apo-10’-,14’-, and 15’-lycopenal were observed in the oxidation mixture and tentatively identified based on anticipated RT (relative to the RT of the apo-6’-, 8’-, and 12’-lycopenal standards), UV spectra, and characteristic M-69 MS/MS transition.
Figure 4.2  An HPLC-MS chromatogram of the products resulting from the oxidation of lycopene with ozone acquired on a quadropole-time-of-flight mass spectrometer (QTof Premier, Micromass, Beverley, MA). The chromatogram displays the sum of ion intensities for the radical anions of lycopene and apo-lycopenals in APcI negative (note: HPLC gradient slightly modified from that used for blood plasma analysis – see methods). 1 = all-trans-lycopene (536.44), 1’ = cis-lycopene isomers (536.44), 2 = apo-6’-lycopenal (442.32), 3 = apo-8’-lycopenal (416.31), 4 = apo-10’-lycopenal (376.28), 5 = apo-12’-lycopenal (350.26), 6 = apo-14’-lycopenal (310.23), and 7 = apo-15’-lycopenal (284.21).
Since many of the foods and blood plasma samples that we analyze contain a significant amount of β-carotene in addition to lycopene, it is necessary to develop HPLC methods that separate apo-lycopenals from potential apo-carotenals which are isobaric, e.g. apo-8′-lycopenal and β-apo-8′-carotenal. In addition, it is desirable to distinguish these species according to their MS/MS behavior. β-carotene was oxidized with ozone and examined using the HPLC-MS/MS method detailed above. The β-apo-carotenals observed were found to have different retention times than their corresponding apo-lycopenals (data not shown). Furthermore, HPLC-MS/MS analysis revealed that the β-apo-carotenals do not produce the M-69 fragment due to cyclization of the terminal isoprenyl group into an ionone ring. Commercial standards of β-apo-8′-carotenal and β-apo-12′-carotenal were further used to verify the RT and UV spectra of the compounds produced in the β-carotene oxidation mixture.

Apo-lycopenals in food extracts: Initial tests showed that the concentrations of apo-lycopenals in food extracts were far below the PDA detection limit necessitating the selectivity and sensitivity of mass spectrometry. Consequently, apo-lycopenals were analyzed using HPLC-MS/MS and identified by coincident behavior with the apo-lycopenal standards according to retention time, accurate mass, and MS/MS fragmentation patterns. Figure 4.3 displays the standard curves generated to quantitate the apo-lycopenals. Lycopene, apo-6′-, 8′-, 10′-, 12′-, and 14′-lycopenal were observed in all food tested. Interestingly, apo-15′-lycopenal was not detected in any of the food
products although it was generated in the lycopene oxidation mixture. Quantities of food apo-lycopenals are listed in Table 4.1.
Figure 4.3 Standard calibration curves for the quantification of lycopene (⊙), apo-6′-lycopenal (●), apo-8′-lycopenal (□), and apo-12′-lycopenal (■)
Table 4.1 Apo-lycopenals in Commonly Consumed Lycopene Containing Foods

<table>
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<tr>
<th>Food</th>
<th>lycopene</th>
<th>apo-6’-lycopenal</th>
<th>apo-8’-lycopenal</th>
<th>apo-10’-lycopenal</th>
<th>apo-12’-lycopenal</th>
<th>apo-14’-lycopenal</th>
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<td>4.3</td>
<td>2.0</td>
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<td>0.027</td>
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<td>nd</td>
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<td>Watermelon</td>
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<td>0.45</td>
<td>0.80</td>
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</tr>
<tr>
<td>Catsup&lt;sup&gt;c&lt;/sup&gt;</td>
<td>11,000 ± 1,600</td>
<td>4.9 ± 1.3</td>
<td>9.3 ± 1.6</td>
<td>1.2 ± 0.3</td>
<td>5.5 ± 0.2</td>
<td>0.27 ± 0.02</td>
</tr>
<tr>
<td>Spaghetti Sauce&lt;sup&gt;c&lt;/sup&gt;</td>
<td>11,000 ± 2,700</td>
<td>6.3 ± 1.5</td>
<td>10 ± 2</td>
<td>1.2 ± 0.1</td>
<td>5.3 ± 0.8</td>
<td>0.31 ± 0.05</td>
</tr>
<tr>
<td>Pizza Sauce&lt;sup&gt;c&lt;/sup&gt;</td>
<td>15,000 ± 4,100</td>
<td>10 ± 4</td>
<td>16 ± 7</td>
<td>1.7 ± 0.6</td>
<td>7.1 ± 2.2</td>
<td>0.43 ± 0.11</td>
</tr>
<tr>
<td>Tomato Soup&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9,600 ± 2,400</td>
<td>6.9 ± 3.7</td>
<td>11 ± 4</td>
<td>1.7 ± 0.8</td>
<td>6.1 ± 2.3</td>
<td>0.54 ± 0.27</td>
</tr>
<tr>
<td>Tomato Paste&lt;sup&gt;c&lt;/sup&gt;</td>
<td>34,000 ± 4,100</td>
<td>19 ± 5</td>
<td>34 ± 3</td>
<td>3.7 ± 1.2</td>
<td>16 ± 4</td>
<td>0.69 ± 0.13</td>
</tr>
<tr>
<td>Tomato Juice&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8,400 ± 1,800</td>
<td>1.8 ± 0.5</td>
<td>5.0 ± 1.3</td>
<td>0.56 ± 0.13</td>
<td>2.8 ± 0.3</td>
<td>0.11 ± 0.02</td>
</tr>
</tbody>
</table>

nd = not detected

<sup>a</sup>Level was estimated by averaging apo-8’-lycopenal and apo-12’-lycopenal slopes.

<sup>b</sup>Level was estimated by using apo-12’-lycopenal equivalents.

<sup>c</sup>Standard deviation reflects variation between three different commercial manufacturers of the same food product.
The mass spectrometric method was very sensitive to, and selective for, apolyycopenals. However, there were mass spectrometer performance limitations in terms of day-to-day and run-to-run variability. To mitigate this instability, standards were run daily. In addition, we investigated a number of possible internal standards (etretinate, astaxanthin, $^{13}$C$_{40}$ β-carotene) yet none of these candidates improved run-to-run relative standard deviation (RSD). The best solution is to use stable isotopes of the apolyycopenals as internal standards, and efforts are being made to synthesize these for future studies.

Lycopene was present at levels roughly ~1000 times higher than each apolyycopenal observed. Apo-8′-lyycopenal was the predominant apolyycopenal found in all foods, followed by apo-6′-lyycopenal and apo-12′-lyycopenal. In contrast, apo-10′- and 14′-lyycopenal were present in smaller quantities. The levels of apolyycopenals varied across three different commercial manufacturers of a given product, as reflected in higher standard deviations in certain products.

*Apo-lyycopenals in human plasma:* Apo-6′-,8′-,10′-,12′-, and 14′-lyycopenal were observed in the plasma of all seven individuals tested who consumed tomato juice daily for eight weeks. **Figure 4.4** shows a chromatogram of lycopene and apolyycopenals observed in one subject, representative of all plasma samples analyzed.
Figure 4.4 HPLC-MS/MS analysis of lycopene and apo-lycopenals in blood plasma extract of one representative individual. (A) MS chromatogram of the collection of SRM channels for all-trans lycopene (536>467), apo-6′- (442>373), -8′- (416>347), and -12′-lycopenal (350>281) standards and (B-G) lycopene and apo-lycopenals in blood plasma. Parent radical anion m/z values are shown in the upper right of each trace, with the respective transition to M-69 daughter ions that were monitored.
Table 4.2 provides the average concentration and the standard deviation of apo-lycopenals observed over seven individuals. Apo-15'-lycopenal was not detected in the plasma of any of the seven subjects. As shown in Table 4.2, apo-8'- and 12'-lycopenal were the predominant apo-lycopenals observed in blood. Relative to lycopene, apo-lycopenals were present at levels roughly 1,000 times lower, similar to their relative abundance in food.
Table 4.2 Apo-lycopenals in Blood Plasma of Seven Subjects Consuming a High Tomato Juice Diet for 8 Weeks.

<table>
<thead>
<tr>
<th></th>
<th>average nmol/L ± standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>lycopene</td>
</tr>
<tr>
<td>blood plasma</td>
<td>1,089 ± 380</td>
</tr>
</tbody>
</table>

*a* Level was estimated by averaging apo-8′-lycopenal and apo-12′-lycopenal slopes.  
*b* Level was estimated by using apo-12′-lycopenal equivalents.
Lycopene preservation during extraction: Lycopene is extremely sensitive to degradation by heat, light, and oxygen; therefore, the presence of apo-lycopenals in such small quantities stimulated our concern that they may form as degradation products during sample processing and analysis. During extraction, we maintained standard precautions to assure chemical stability, including minimizing handling time, extracting under red light, drying samples under nitrogen, and storing immediately at \(-80\) °C until analysis. We also performed several experiments to determine if specific technical procedures impacted apo-lycopenal levels. First, we extracted raw tomato with 2% BHT in the extraction solvent as BHT is commonly used as an antioxidant in the extraction of compounds sensitive to oxidation. The presence of BHT did not change the amount of apo-lycopenals observed in the extract. We also performed the extraction of raw tomato with EDTA at various concentrations to determine if the apo-lycopenal formation was catalyzed by free metals or metal-dependent enzymes (e.g. oxidases) during extraction. There was no change in the amount of apo-lycopenals observed between the no EDTA condition and as a function of EDTA concentration. In fact, neither BHT nor EDTA appear to protect against such generation. In summary, apo-lycopenals were likely not generated artifactually during extraction through free radical or metal-catalyzed reactions. However, we were able to increase the level of apo-lycopenals if we kept a closed vial containing lycopene dissolved in tetrahydrofuran or CH\(_2\)Cl\(_2\) at room temperature overnight.
Another possible route to the formation of apo-lycopenals is by metal independent enzymes native to the native food sample matrices which may have the capacity to generate apo-lycopenals during extraction. To test whether samples had this capacity, we added exogenous lycopene (prepared in-house) and performed the extraction. We observed slight changes in apo-lycopenal levels. We also performed the same experiment using blood plasma samples by spiking with a 200-fold excess of lycopene over unspiked plasma samples. We observed no change in the amount of apo-lycopenals as compared to unspiked plasma samples.

*Recovery Experiments:* No significant improvement in recovery was observed (data not shown). Thus, it appears we are not losing apo-lycopenals due to aldehyde reactivity with the sample matrices during extraction.

Lycopene metabolism in mammals is poorly understood, and the role of metabolites as biomarkers of metabolic degradation pathways or potentially bioactive metabolites remains speculative. Researchers have suggested that lycopene may be metabolized into aldehyde derivatives, and previous reports have identified a few apo-lycopenal products in tomatoes (106), tomato paste (105), and in tissues of rodents fed lycopene (96,109). We now document that several apo-lycopenals are present in a variety of raw and processed foods containing lycopene. In addition, we report for the first time the presence of a series of apo-lycopenals in human plasma.

The hypothesis that oxidative metabolites of lycopene have biological activity is supported by a few *in vitro* studies. The products of lycopene oxidized with hydrogen peroxide were shown to increase gap junction communication in rat liver epithelial cells.
In a separate study, the lycopene derivative 2,6-cyclolycopene-1,5-diol was shown to increase connexin 43 levels in vitro in murine and human cells to a greater extent than the parent lycopene molecule. Apo-10'-lycenoic acid was also shown to inhibit growth of human bronchial cells in vitro and to reduce the number of lung tumors in mice injected with the cancer inducing chemical 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK). As pure compounds become available, additional studies of bioactivity in experimental models will be a high priority.

The apo-lycopenals we observed in raw and processed fruits could be the result of enzyme-catalyzed metabolism of lycopene. In fact, nine carotenoid cleavage dioxygenase enzymes (CCD) have been identified in the genome of Arabidopsis. Of these nine, five enzymes are reportedly involved in the synthesis of abscisic acid, an important hormone regulating multiple plant functions. The other four CCD enzymes are being more fully characterized. CCD1 from maize has been shown to cleave lycopene at the 5'-6' position and 9'-10' position. While only the short, volatile products of these cleavages were determined by GC-MS (pseudo-ionone and 6-methyl-5-heptene-2-one), the complementary long-chain products of these enzymatic cleavages were likely apo-6'-lycopenal and apo-10'-lycopenal, respectively. CCD7 appears to cleave the 9'-10' double bond of lycopene, but the resulting product has only tentatively been identified with UV spectroscopic data and retention time. It is not clear if CCD8 cleaves lycopene, and there is no literature discussing the ability of CCD4 to act on lycopene. It is also possible that the apo-lycopenals observed in these foods might be formed by enzymes involved in the abscisic acid pathway. In contrast to
specific biosynthesis, these aldehydes could be the result of a non-specific lipoxygenase cleavage (93,217), or produced from photo-oxidation in the fruit (110).

The presence of apo-lycopenals in plasma could be due to enzymatic cleavage of lycopene. It has been demonstrated that BCO1 cleaves β-carotene at the 15-15′ double bond to produce two molecules of retinal in vertebrates (6). In addition, a second carotenoid oxygenase (BCO2), has been shown to eccentrically cleave β-carotene to produce β-apo-10′-carotenal (210). In vitro, BCO2 from ferret has also been shown to cleave both β-carotene and lycopene at the 9′-10′ double bond (96). However, a single cleavage site would not explain the array of aldehydes we observed in plasma.

The feeding of lycopene (as Lycovit 10%) to ferrets resulted in the detection of apo-10′-lycopenol in lung tissue (96). This alcohol was presumed to be a metabolite of the aldehyde (apo-10′-lycopenal) formed enzymatically in vivo. In addition, the liver of rats consuming a lycopene containing diet for 30 days produced apo-8′-lycopenal and putative apo-12′-lycopenal in this tissue (98,109). These products were also hypothesized to be the result of enzymatic cleavage in the animal.

In summary, we have observed a full series of apo-lycopenals in foods, processed food products, and analytical grade lycopene standard. In addition, we have documented the presence of multiple apo-lycopenals in the plasma of humans consuming tomato juice. This evidence suggests that these products may in fact be absorbed from the food and not solely a product of metabolism in vivo. The presence of apo-lycopenals in the plasma, either from the diet or as metabolic products, supports a hypothesis that these compounds may have bioactivity and potentially mediate some of the health promoting
beneficial effects proposed for tomato products. In addition, the development of analytical methods for the measurement of apo-lycopenals in mammalian systems provides a tool for the investigation of lycopene metabolism. The continued improvement of analytical approaches provides investigators with new approaches that will help to determine the importance of apo-lycopenals from the diet or as a product of enzymatic cleavage in vivo.
CHAPTER 5

Consumption of a tomato carotenoid containing diet reduces UV-induced inflammation and DNA damage in a SKH-1 hairless mouse model.

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\end{itemize}
ABSTRACT

Ultraviolet (UV) light exposure is a primary risk factor in the development of skin cancer. Human studies have shown that the consumption of a tomato containing diet for \( \geq 10 \) weeks reduces the intensity of a UVB-induced erythema more than a lycopene containing diet alone. We hypothesize that the tomato carotenoids phytoene and phytofluene might be responsible for this increase in effectiveness. For this study, four week old SKH-1 hairless mice (40 females, 40 males) were divided into two feeding groups (0 % or 10 % tomato powder) and two UV exposure groups (with or without UV exposure). For the 10% tomato powder diet, we chose to use tangerine tomatoes which contain higher levels of the UV absorbing carotenoids phytoene and phytofluene. Animals were maintained on their respective diets for 10 weeks and at week 11, the UV group was exposed to a single dose of UVB radiation, and sacrificed 48 hours post-UV exposure. Backskin samples were taken to assess inflammation and DNA damage. Blood and backskin samples were taken for carotenoid analysis by high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) analysis. Dermal myeloperoxidase levels were lower in males but not females consuming the 10% tomato diet as compared to the control diet. Cyclopyrimidine dimer levels were lower in both females and males consuming the 10% tomato diet as compared to the control diet. Females had significantly higher levels of carotenoids in skin and blood as compared to males, while UV exposure significantly reduced skin carotenoid levels in females but not in males. UV exposure did not effect carotenoid blood levels. In conclusion, the consumption of carotenoid containing tomatoes leads to carotenoid deposition in the skin.
of SKH-1 mice. Furthermore, the consumption of tomato carotenoids reduced inflammation and DNA damage resulting from acute UVB-exposure.

**INTRODUCTION**

Skin cancer is the most common type of cancer in the United States, with more individuals being diagnosed with some type of skin cancer than all other cancers combined\(^1\). Solar ultraviolet-B (UVB) light, with radiation emitted from 280 nm - 315 nm, is considered to be the major cause of sunburn and is the primary cause of sun induced damage to DNA and proteins in skin tissue (118). Furthermore, UVB exposure has been implicated in the development of both basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), collectively known as non-melanoma skin cancers (NMSC). While UVB exposure can be reduced, it will never be eliminated.

Consumption of dietary compounds that may inhibit UVB-induced skin damage presents a novel approach to reducing skin cancer risk. The carotenoids phytoene and phytofluene are found in tomatoes and tomato products, and are strong absorbers of UV light. Phytoene absorbs maximally at 286 nm, while phytofluene absorbs maximally at 350 nm. A study performed in the early 1980’s using hairless mice observed that mice treated intraperitoneally with purified phytoene for 10 weeks before a single dose of tumor inducing UV radiation developed no skin tumors (129). This was in contrast to the control group of mice that developed tumors after receiving the vehicle only. More recently, a clinical study was performed in humans where subjects consumed synthetic lycopene alone, a soft-gel encapsulated tomato extract, or a tomato drink for 12 weeks.
The subjects consuming the tomato extract and tomato drink had a 38% and 48% decrease, respectively, in erythema formation at week 12, compared to only a 25% decrease in the group treated with synthetic lycopene (127). These authors suggest that phytoene and phytofluene found in the tomato extract capsule and tomato drink (but not present in the synthetic lycopene capsule) may be responsible for the greater decrease in erythema formation. However, this hypothesis has never been tested (218).

Red tomatoes contain all-trans lycopene, which confers the red color. In contrast, tangerine tomatoes are a unique varietal of tomato which contain tetra-cis lycopene, a geometrical isomer of all-trans lycopene, which imparts a “tangerine” hue. Tangerine tomatoes also contain levels of phytoene and phytofluene which are 2 ½ to 3 times higher than red tomatoes (63).

The objective of this study was to determine if the consumption of tangerine tomatoes by SKH-1 hairless, immunocompetent mice reduces UVB-induced skin damage. We hypothesized that mice consuming phytoene and phytofluene-rich tangerine tomatoes would have a reduction in skin inflammation and DNA damage as compared to control animals. We also hypothesized that animals exposed to UVB light would have lower levels of skin carotenoids as compared to animals not exposed to UVB light.

**MATERIALS AND METHODS**

*Materials* - HPLC grade methyl tert-butyl ether (MTBE), dichloromethane, ethanol, and Optima grade hexane, water, and methanol were purchased from Fisher Scientific (Pittsburg, PA, USA). High purity formic acid (≥95%) was purchased from Sigma-
Aldrich (St. Louis, MO, USA). Ammonium acetate was obtained from J.T. Baker (Phillipsburg, NJ, USA). Lycopene was purified and crystallized as described previously (147). Phytoene and phytofluene were isolated from a tangerine tomato extract using a C18 preparatory HPLC method, adapted from an analytical HPLC method by Isaacson et al., (63). The isolated carotenoids were individually tested for purity by HPLC-PDA and HPLC-MS/MS, and molar extinction coefficients were used to quantitate the level of these carotenoids. The levels of ζ-carotene, and neurosporene are provided by estimating the relative response by UV-VIS of ζ-carotene, and neurosporene as compared to lycopene standard, and then estimating MS/MS response.

Tomatoes - Tangerine tomato variety FG04-169 was grown and harvested by the Francis Lab at the OSU North Central Agricultural Research Station in Fremont, Ohio. Following harvest, the tomatoes were diced into cubes and frozen. Frozen samples were then lyophilized in a freeze dryer in the Food Industries Center at the Ohio State University, and then powdered in a food processor (Cuisinart, East Windsor, NJ). The powder was stored frozen until it was sent by express delivery to Research Diets, Inc. (New Brunswick, NJ) to be incorporated into a AIN-93G based diet at a 10% level and pelleted. The breakdown of the control diet and the 10% tangerine tomato powder diet is detailed in Table 5.1. The diets were stored at 4°C throughout the duration of the study. Carotenoids were extracted from the finished 10% tomato diet pellets using the method previously published (219), and analyzed using the HPLC-MS/MS method described below.
**Table 5.1** Composition of Diets (in g per kg diet)

<table>
<thead>
<tr>
<th>Component</th>
<th>Control Diet</th>
<th>10 % Tomato Powder Diet&lt;sup&gt;†&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>200</td>
<td>186</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Corn Starch</td>
<td>397</td>
<td>348</td>
</tr>
<tr>
<td>Maltodextrin</td>
<td>102</td>
<td>88</td>
</tr>
<tr>
<td>Sucrose</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50</td>
<td>32</td>
</tr>
<tr>
<td>Soybean Oil</td>
<td>100</td>
<td>95</td>
</tr>
<tr>
<td>t-butylhydroquinone</td>
<td>0.014</td>
<td>0.014</td>
</tr>
<tr>
<td>Mineral Mix</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Vitamin Mix</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Choline Bitartrate</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Tomato Powder</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

*Each kg of diet contains 7.6 mg lycopene, 12.1 mg ζ-carotene, 6.5 mg neurosporene, 15.3 mg phytofluene, and 27.0 mg phytoene.*
Animals – Animals were housed in a facility approved by the American Association for the Accreditation of Laboratory Animal Care, and the Ohio State University Animal Care Advisory Committee approved all procedures. SKH-1 hairless mice (n = 40 females, 40 males) were purchased from Charles River Laboratories (Wilmington, MA) and were received at 4 weeks of age. Animals of the same gender were randomized into different cages, with 5 animals housed per cage. Each cage was assigned to a specific feeding group, and each group was maintained on their respective diet (either control diet or 10% tomato powder diet) for 10 weeks which they were allowed to consume both food and water ad libidum. 10 weeks of feeding was chosen as previous studies in humans have suggested that at least 10 weeks of daily carotenoid consumption is required to reduce erythema induced by UVB exposure (127,218). Within a gender, 20 animals received the control diet, and 20 animals received the 10% tomato powder diet. Animals were weighted weekly. The feed was weighted in and out of the cage, and changed every 2 days during the week and 3 days over the weekend to minimize carotenoid degradation.

At week 10, a subset of each gender-feeding group (n=15) was irradiated with 2240 J/m² of UVB light. The irradiation was delivered by Phillips FS40 UVB bulbs (American Ultraviolet Company, Murray Hill, NJ) with Kodacel filters (Eastman Kodak, Rochester, NY), and UVB exposure was determined by a UVR meter equipped with a UVB sensor (UVP Inc., Upland, CA). The remaining 5 animals in each gender-feeding group received no UVB exposure. 48 hrs. afterward, skinfold thickness was determined using metric calipers with submillimeter measurement capacity (Bel-Art Products, Pequannock, NJ), and animals were sacrificed by lethal inhalation of carbon dioxide.
Blood samples were taken via butterfly needle and placed into a microcentrifuge tube. The tubes were stored on ice, and then centrifuged at 11,000 rcf at 4 °C for 20 minutes. The supernatant was collected and centrifuged for 10 additional minutes, to ensure removal of all red blood cells. The upper serum layer was collected and stored at -80 °C for analysis of carotenoids. Dorsal skin samples for p53 analysis were fixed in 10% neutral buffered formalin for histochemical analysis following previously developed methods of Wilgus et al., (220). Likewise, dorsal skin samples for the myeloperoxidase, cyclopyrimidine dimer assays were collected and analyzed following previously developed methods (220,221). All remaining dorsal skin was stored at -80°C for carotenoid analysis.

Carotenoid Extraction and Analysis – Carotenoids were extracted from blood serum following the method previously published (222) scaled to a 300 µL sample. Carotenoids from backskin were extracted by first grinding the frozen skin tissue into a powder with liquid nitrogen in a mortar and pestle. Next, 400 mg of tissue was weighted into a glass vial and 1 mL of ethanol containing 0.1% BHT (w/v), 1 mL of water, and 5 mL of 5:1 hexane/dichloromethane (the solvent system used by (223) was added to the vial. The sample was probe sonicated for 30 seconds using a medium intensity setting. Next, the sample was centrifuged at 300 x g for 2 minutes to induce phase separation. The upper layer of solvent was removed with a glass pipette, and placed into a clean glass vial. The extraction procedure with hexane/dichloromethane was repeated, and the extracts were pooled together. Extracts were dried under nitrogen gas, and stored at -80°C until analysis.
**HPLC-MS/MS Analysis of Carotenoids** – Extracts were reconstituted in 100 µL of 1:1 MTBE/MeOH and a 10 µL injection volume was used. Carotenoids in the sample were separated using reverse phase chromatography with a Zorbax C18 column, 4.6 mm x 50 mm, 1.8 µm particle size (Agilent Technologies, Santa Clara, CA, USA) on a 1200 series system HPLC with an HP 1200 series diode array detector (Agilent Technologies, Santa Clara, CA, USA). The column heater was set at 30 °C. Total run time was 8.5 min at a flow rate of 2.33 mL/min. The following gradient was employed to separate the compounds in the sample: 0% B with a linear gradient to 25% B over 3 minutes, holding at 25% B for 2 minute, increasing to 100% B over one minute and holding for 0.5 minutes, then returning to 0% B over 0.1 minutes and holding for 1.9 minutes. Solvent A = 20:80 water/MeOH (v/v) and solvent B = 2:20:78 water/MeOH/MTBE (v/v/v).

The eluent from the HPLC was interfaced with a QTrap 5500 (AB Sciex, Foster City, CA, USA) via an atmospheric pressure chemical ionization (APCI) probe operated in positive ion mode. Additional mass spectrometer parameters were as follows: curtain gas = 10 psi, source temperature = 550 °C, ion source gas 1 (GS1) = 60 psi, declustering potential (DP) = 100 V, entrance potential (EP) = 10 V, collision cell exit potential (CXP) = 7 V. Parent > daughter ions for each analyte which were selective for the compounds of interest and for which background interference was minimized were chosen for quantitation of the samples (Table 5.2). Final data acquisition was carried out using MRM windows with dwell times of 15 msec for each channel, and 3 msec pauses between channels. Analyst 1.5.1 software (AB Sciex, Foster City, CA, USA) was used for data acquisition and analysis.
Table 5.2 Analyte-Specific Parameters for HPLC-MS/MS Analysis of Standards

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Parent &gt; Daughter Transition</th>
<th>Collision Energy</th>
</tr>
</thead>
<tbody>
<tr>
<td>phytoene</td>
<td>545.5 &gt; 463.6, 421.6, 395.6, 327.4</td>
<td>22, 22, 29, 25</td>
</tr>
<tr>
<td>phytofluene</td>
<td>543.5 &gt; 393.6, 325.4</td>
<td>29, 22</td>
</tr>
<tr>
<td>ζ-carotene</td>
<td>541.5 &gt; 457.6, 349.4, 271.3</td>
<td>22, 22, 20</td>
</tr>
<tr>
<td>neurosporene</td>
<td>539.5 &gt; 457.6, 415.4, 389.6, 269.3</td>
<td>22, 29, 29, 20</td>
</tr>
<tr>
<td>lycopene</td>
<td>537.5 &gt; 455.6, 269.3</td>
<td>22, 20</td>
</tr>
</tbody>
</table>
Statistical Analysis - Statistical analysis was performed using SAS (SAS Institute, Inc., Cary, NC, USA). Final body weights for each diet were assessed by gender using a 2-sample t-test, and $P < 0.05$ was considered statistically significant. Differences in carotenoid levels were determined using a mixed model (PROC MIXED). Gender, feed, and UV exposure were used as fixed effects. The interactions of ‘gender x feed’, ‘gender x UV exposure’, ‘feed x UV exposure’, and ‘gender x feed x UV exposure’ were also considered.

RESULTS

Animals – Animals consumed both diets well, and normal weight gain was observed. Final body weights are displayed in Table 5.3. There was no significant difference between final body weights for female mice on the control diet as compared to the tangerine tomato diet. In contrast, the average body weight of male mice consuming the tangerine tomato diet was 2.75 g greater than male mice consuming the control diet ($P = 0.001$).
Table 5.3 Final Animal Body Weights

<table>
<thead>
<tr>
<th>Gender</th>
<th>Diet</th>
<th>Average Body Weight in grams (± Std. Dev.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>Control</td>
<td>24.0 ± 1.95</td>
</tr>
<tr>
<td></td>
<td>10% Tomato</td>
<td>25.1 ± 2.02</td>
</tr>
<tr>
<td>Male</td>
<td>Control</td>
<td>30.7 ± 2.23&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>10% Tomato</td>
<td>33.4 ± 2.15&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Within a gender, a different superscript letter indicates a statistically significant difference ($P < 0.05$)
Skinfold Thickness – In the UVB exposure groups within each gender, no significant difference in skinfold thickness was observed between the animals who consumed to
tomato diet as compared to animals who consumed the control diet.

Myeloperoxidase (MPO) Assay – Results of the myeloperoxidase assay are shown in
Figure 5.1, with panel A displaying results for females, and panel B displaying results
for males. The extent of skin inflammation was determined by the measurement of
myeloperoxidase, an enzyme produced by dermal infiltrating neutrophils. UVB
irradiation induced a significant increase in MPO activity compared to unexposed skin in
both sexes (*p<0.05). Feeding male mice a 10% tomato diet significantly decreased the
MPO level (*p<0.00004). In contrast, the 10% tomato diet had no statistically significant
effect on MPO levels in female skin (p<0.5).
Figure 5.1 Mean units of myeloperoxidase activity in females (A) and males (B).

Values displayed are means ± standard deviation.
p53 — At 48 hours-post UVB irradiation, no significant difference was observed the number of p53 positive cells which were present in UVB irradiated animals which had consumed the tomato diet as compared to the control diet (data not shown).

Cyclopyrimidine Dimer Assay — Results of the CPD assay are shown in Figure 5.2. As expected, exposure to UVB light significantly increases CPD levels. Panel A, depicting females, reveals that UVB exposed animals consuming the 10% tomato feed had a lower CPD ratio than UVB exposed animals consuming the control diet. Likewise, as shown in panel B, male animals consuming the 10% tomato feed exposed to UVB also had a lower CPD ratio than males consuming the control diet exposed to UVB.
Figure 5.2 Ratio of cyclopyrimidine dimer to 4',6-diamidino-2-phenylindole (DAPI) stain in females (A) and males (B). Values displayed are means ± standard deviation.
Blood and Skin Carotenoid Levels – Blood serum levels of carotenoids in mice consuming the 10% tomato diet are shown in Table 5.4. Within gender, no significant difference in any blood carotenoids was observed when comparing the UVB exposed animals to the No UVB exposed animals. Comparing between genders, females had approximately 3.7 x higher levels of circulating phytoene, and 1.5 higher circulating level of phytofluene, ζ-carotene, neurosporene, and lycopene as compared to males.
Table 5.4  Blood Serum Levels of Carotenoids in nmol/L (± standard deviation) in Mice Consuming the 10% Tomato Diet

<table>
<thead>
<tr>
<th>Gender</th>
<th>Diet</th>
<th>Phytoene</th>
<th>Phytofluene</th>
<th>ζ-Carotene</th>
<th>Neurosporene</th>
<th>Lycopene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>No UVB</td>
<td>133 ± 33</td>
<td>108 ± 30</td>
<td>255 ± 57</td>
<td>153 ± 40</td>
<td>204 ± 60</td>
</tr>
<tr>
<td></td>
<td>UVB</td>
<td>136 ± 21</td>
<td>103 ± 18</td>
<td>233 ± 48</td>
<td>139 ± 28</td>
<td>191 ± 42</td>
</tr>
<tr>
<td>Male</td>
<td>No UVB</td>
<td>37 ± 6</td>
<td>66 ± 19</td>
<td>166 ± 34</td>
<td>104 ± 32</td>
<td>151 ± 48</td>
</tr>
<tr>
<td></td>
<td>UVB</td>
<td>35 ± 16</td>
<td>68 ± 38</td>
<td>146 ± 84</td>
<td>97 ± 70</td>
<td>122 ± 84</td>
</tr>
</tbody>
</table>
**Figure 5.3** is a representative HPLC-MS/MS chromatogram of carotenoids extracted from dorsal skin. All carotenoids observed in the 10% tomato feed were also present in the murine dorsal skin. It should be noted that a significant number of geometrical isomers of each carotenoid were observed in the feed, thus the 10% tomato feed was used to assist in identification of all carotenoids present in the skin sample.
Figure 5.3 Chromatogram of Carotenoids in Murine Dermal Tissue. (*) in each trace denotes the carotenoids of interest. The carotenoids, with corresponding (parent > daughter) ions displayed are as follows: A = phytoene (545.5 > 327.4), B = phytofluene
(543.5 > 325.4), C = ζ-carotene (541.5 > 349.4), D = neurosporene (539.5 > 389.6), E = lycopene (537.5 > 455.6).

**Figure 5.4** displays the dorsal skin level of all of the carotenoids in the 10% tomato animals. Comparing between genders in the non-irradiated animals, females had higher skin levels of carotenoid as compared to males, with the most striking difference for phytoene and lycopene, where females had 10 x higher levels than males. Comparing exposure to UVB radiation within a gender, UVB exposure reduced the levels of carotenoid down to 20 % of the of the no-UVB animals in females and down to 50 % of the no-UVB animals in males.
Figure 5.4 Mean level of carotenoid in dorsal skin tissue of animals consuming the 10% tomato diet, expressed as nmol carotenoid/g tissue. Female No UVB (solid bar), Female
UVB (striped bar), Male No UVB (gray bar), and Male UVB (open bar). Error bars represent standard deviation.

Finally, comparing blood levels of circulating carotenoids to dorsal skin levels of the same carotenoid reveals some interesting observations. For example, skin lycopene levels are approximately 10x higher than skin phytoene levels. In contrast, blood levels of lycopene are only 1.5x higher than blood phytoene levels.

**DISCUSSION**

After UVB exposure, the human inflammatory response activates neutrophils which release MPO. In this study, MPO activity was observed as a surrogate measure of neutrophil activation. The feeding of a 10% tangerine tomato diet in male mice resulted in a decrease in MPO activity after UVB exposure as compared to the control diet. This effect was not observed in female mice.

Within a gender, no significant difference in p53 positive cells was observed in animals which had consumed the tomato diet as compared to the control diet. Previous work by our group has observed statistically significant differences in p53 levels at 24 hours (220), but these differences appear to be no longer visible at 48 hours.

CPD levels have been shown to be lower when sunscreen is used to protect against UV-induced damage (224). Thus, the significant decreases in the CPD levels in UVB irradiated animals who had consumed the 10% tomato diet, in conjunction with the decreased level of carotenoids in the skin of UVB irradiated animals, suggest that the
carotenoids deposited in the skin of these animals may be conferring “sunscreen” like protection against UVB damage.

Human studies (73,74) and rodent studies (85,97,225) have previously reported blood lycopene levels in the 0.2 to 2 µmol/L range. As shown in Table 4, blood levels of lycopene in this study fell within this range, but on the lower end, from 0.1 to 0.2 µmol/L. Human plasma levels of phytoene have been reported from 0.10 - 0.85 µmol/L (80,81), while levels of phytofluene range 0.27 – 0.72 µmol/L and levels of ζ-carotene range from 0.12 – 0.26 µmol/L (80). Surprisingly, blood levels of phytoene, phytofluene, and ζ-carotene reported in Mongolian gerbils were 0.0003, 0.0002, and 0.0006 µmol/L, respectively (226). In contrast, blood levels of phytoene, phytofluene, and ζ-carotene reported in Fisher 344 rats were 0.5, 0.4, and 0.2 µmol/L, respectively (225). The levels of phytoene, phytofluene, and ζ-carotene we observed in the SKH-1 mice in our study fall within the same range as what has been previously reported in humans and in Fisher 344 rats. To the best of our knowledge, blood levels of neurosporene have not previously been reported in rodents or in humans, likely because the level of this carotenoid in widely consumed fruits and vegetables is quite low.

We observed significantly higher levels of blood carotenoids in females as compared to males. A few reports have indicated that human carotenoid levels may be higher in females as compared to males. A study by Nierenberg et al. fed a 50 mg β-carotene supplement to 582 subjects for 1 year, and observed median blood levels of β-carotene that were approximately 35% higher in women as compared to men (227).
Likewise, blood levels of lycopene and β-carotene were higher in women before and after a 3 weeks of dietary supplementation with watermelon juice (228).

Skin levels of various carotenoids in humans have been previously reported. Ribaya-Mercado et al., (78) reported lycopene levels of up to 1.75 nmol/g in human skin, while a more recently report found 0.04-0.70 nmol lycopene/g human skin tissue (229). In contrast, a recent study by Conlon et al. fed Mongolian gerbils a 10% tomato powder diet with different types of lipid and measured lycopene levels from 0.08-0.10 nmol lycopene/g skin tissue (226). We measured 0.03-3.9 nmol lycopene/g skin tissue, with higher levels found in the female animals. We hypothesize that the higher levels of lycopene observed in the skin of animals in our study as compared to human (229) and Mongolian gerbil (226) studies may be due to the fact that we fed a tangerine tomato containing high levels of tetra-cis and other-cis levels of lycopene which have been suggested to be more bioavailable than the all-trans lycopene found in red tomato products (28). It is presumed that the lycopene consumed by subjects in the Mayne et al. (229) article was primarily all-trans lycopene, and the tomato diet in the Conlon et al. (226) study was reported to be approximately 77% all-trans lycopene, 23% other-cis lycopene.

There are limited reports of skin levels of the other carotenoids investigated in this study. While phytoene, phytofluene, and ζ-carotene were detected in the skin of the Mongolian gerbils, they were below the PDA limit of detection and thus not quantitated (226). A human study by Hata et al. (83) of free-living individuals found phytoene levels from 0.04-0.16 nmol/g, phytofluene levels from 0.01-0.03 nmol/g, and ζ-carotene levels
from 0.01-0.05 nmol/g. In contrast, we measured phytoene, phytofluene, and ζ-carotene levels which were approximately 10-fold higher. Again, the 10-week feeding of a tangerine tomato which contains higher levels of these carotenoids, combined with anticipated species differences, may be responsible for this effect.

In conclusion, our data suggests that tangerine tomatoes mediate acute UVB-induced skin damage via different mechanisms, an affect which seems to be gender specific. Tangerine tomatoes reduce CPD levels in both males and females, and MPO activity in males. In addition, the feeding of tangerine tomatoes to SKH-1 mice over 10 weeks leads to circulating blood levels of these carotenoids, and deposition of these carotenoids into skin. Circulating levels and skin levels of these carotenoids are higher in female animals, and skin levels of these carotenoids are lower in animals irradiated with UVB light. To the best of our knowledge, we are the first group to report the skin levels of various carotenoids in the SKH-1 mouse model. This mouse model is routinely used for acute and chronic UVB exposure studies, and a better understanding of the circulating blood levels and skin deposition of these carotenoids is an important first step. Importantly, our data suggest that relative levels of circulating blood carotenoids may not directly reflect the distribution of carotenoids in skin. Future work will focus on the feeding of novel tomatoes with chronic UVB exposure, and the resulting differences in the development and progression of skin tumors.
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